BIOLOGY AND ECOLOGY OF ROOT-FEEDING BEETLES AND

OPHIOSTOMATOID FUNGI IN SANDHILLS LONGLEAF PINE STANDS

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BIOLOGY AND ECOLOGY OF ROOT-FEEDING BEETLES AND OPHIOSTOMATOID FUNGI IN SANDHILLS LONGLEAF PINE STANDS

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BIOLOGY AND ECOLOGY OF ROOT-FEEDING BEETLES AND

OPHIOSTOMATOID FUNGI IN SANDHILLS LONGLEAF PINE STANDS

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VITA

James Warren Zanzot was born on 9 May 1972 in North Kingstown, Rhode Island, the son of Douglas H. and Diana M. Zanzot. He spent most of his youth in Sudbury, Massachusetts. He attended Reed College in Portland, Oregon, graduating with a Bachelor of Arts degree in Biology in 1994. At Reed, his curriculum focused on plant biology and ecology and his senior thesis addressed the non-mycorrhizal status of the Brassicaceae. Upon graduation, he was invited to serve in the Peace Corps in Niger, West Africa. As an agroforestry extension agent, he encouraged subsistence farmers to plant trees to combat desertification and supplement their income. It was in Niger that he was given the Zarma name "Djibo". He earned his Master of Science in Botany and Plant Pathology at Oregon State University in 2004, writing a thesis on the potential for the sudden oak death pathogen, *Phytophthora ramorum*, to infect plant species of conservation concern and tanoak-associated species in Oregon. After completing his MS, he worked as a forest pathology technician in the laboratory of Dr. David Rizzo at the University of California, Davis. From there he came to the Forest Health Dynamics Laboratory in the School of Forestry and Wildlife Sciences at Auburn University. He is married to Jocelyn (Eisenberg) Zanzot, with whom he has two daughters, Veronica and Violet.

DISSERTATION ABSTRACT

BIOLOGY AND ECOLOGY OF ROOT-FEEDING BEETLES AND OPHIOSTOMATOID FUNGI IN SANDHILLS LONGLEAF PINE STANDS

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Ophiostomatoid fungi, especially those of the genus *Grosmannia* Goid. and its *Leptographium* Lagerb.& Melin anamorphs, are vectored by beetles and have been implicated as factors in decline of loblolly pine (*P. taeda* L.) and longleaf pine (*P. palustris* Mill.). In this dissertation the biology and ecology of ophiostomatoid fungi and their vectors were studied in longleaf pine stands at Fort Benning and a loblolly pine

decline risk model tested for its ability to predict areas of symptom development in longleaf pine. Longleaf pine data included tree height and diameter, increment growth, crown characteristics, and standing resin reserves. Longleaf pine roots were excavated and assayed for *Leptographium* infection. Root-feeding insects were collected using pitfall traps and assayed for *Leptographium* infestation.

Differences in growth parameters were observed only in the 20-40 year age class between predicted symptomatic and predicted asymptomatic plots. Trees in all age classes were found to be infected with *Leptographium procerum* (Kendr.) Wingfield and *L. terebrantis* Barras & Perry, but infection had no effects on growth or crown variables. *Leptographium* distribution in longleaf pine roots was not as predicted by the loblolly pine decline risk model. *Grosmannia huntii* (Robinson-Jeffrey & Grinchenko) Zipfel, de Beer & Wingfield was isolated from a single symptomatic longleaf pine tree.

Peak numbers of beetle captures and fungal infestation varied by insect species and by collection season. *Grosmannia huntii*, *L. procerum*, and *L. terebrantis* were the predominant fungi on *Hylastes* spp. Erichson.

The presence of *G. huntii* and *L. serpens* (Goid.) Siemaszko in the southeastern United States was confirmed by DNA sequence analysis, and molecular and morphological data are presented to aid in differentiation. An *Ophiostoma* sp. Syd & P. Syd isolated from declining loblolly pine roots and root-feeding beetles in this dissertation is described as a novel species, *O. sparsiannulatum* Zanzot, de Beer & Wingfield.

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CHAPTER I –LITERATURE REVIEW

1.1. Forest Decline

Declines may be contrasted with other types of forest diseases in that declines are typically protracted, and their etiologies are either difficult to detect or attribute to a single agent. Often, potential causal agents are ascribed to the condition when the decline is recognized, but these claims are often disputed as those causal agents may be acting secondarily, either singly or in combination with other biotic agents and/or abiotic factors that may precede or coincide with the causal agent put forth as responsible.

The etiology of forest declines are typically a complex of biotic and abiotic agents, which either exacerbate or mitigate the extent of tree growth reduction and tree mortality differentially. The decline model developed by Manion, (1981) suggested that declines begin with predisposing factors related to host genotype, site or other typically abiotic factors which may be permanent or long term. Soil nutrients, air pollutants, and slope are examples of predisposing factors. Root diseases caused by soil fungi such as *Armillaria* spp. (Fr.) Staude, and *Phaeolus schweinitzii* (Fr.) Pat. can also act as predisposing factors (Paine and Baker, 1993). Inciting factors are short term stressors such as insect defoliation, a frost event, drought or mechanical injury that may cause injury to the tree, which under ideal conditions would not lead to tree death.

Predisposing factors reduce the defenses against contributing factors, which are typically biotic agents such as fungi, viruses, or insects that normally do not affect healthy trees, but may infect or infest trees that have been compromised by the predisposing and inciting factors (Manion, 1981). Forest declines have been observed in many conifer and hardwood species around the world including; oaks (*Quercus* spp. L.) in Europe (Jung *et al.*, 2000) and the eastern U.S. (Oak *et al.*, 1996), sugar maple (*Acer saccharum* Marsh) in the northeastern U.S. and eastern Canada (Bauce and Allen, 1991), Alaska yellow-cedar (*Callitropsis nootkaensis* [D. Don] Örsted) in Alaska and British Columbia (Hennon *et al.*, 1990), and Cordilleran cypress (*Austrocedrus chilensis* [D.Don] Florin & Boutleje) in Chile (Filip and Rosso, 1999).

1.2. Pine declines

Decline of eastern white pine (*Pinus strobus* L.) has been observed in plantations in several northeastern states (Dochinger, 1967; Houston, 1969; Towers, 1977; Lackner and Alexander, 1981; Meyer *et al.*, 1983; Horner *et al.*, 1987). Houston (1969) reported a basal canker disease of eastern white pine. The symptomatology described by Houston is similar to other pine decline scenarios; needle chlorosis signaling the onset of disease, followed by reddening and loss of needles, a reduction in height and radial growth, and tree mortality (Dochinger, 1967; Houston, 1969). A study in Christmas tree plantations demonstrated that *Leptographium procerum* (Kendrick) Wingfield (Ascomycota, Ophiostomataceae) was common in symptomatic trees (Lackner and Alexander, 1984).

Subsequent inoculation trials on eastern white pine showed that another *Leptographium* Lagerberg & Melin species, *L. terebrantis* Barras & Perry, produces larger lesions than *L. procerum* (Wingfield, 1986).

Red pine (*Pinus resinosa* Soland), planted extensively in the Great Lakes region, has also experienced decline episodes (Raffa and Hall, 1988; Klepzig *et al.*, 1991; Klepzig *et al.*, 1995). In red pine decline, disease centers or "pockets" have been observed, with symptomatic trees radiating outward from foci of mortality. In the red pine decline situation, a suite of bark beetles and weevils associated with *Leptographium* spp., (especially *L. procerum* and *L. terebrantis*) were shown to be consistently present within and at the edges of pockets, but were absent from asymptomatic areas beyond the margins of pockets (Klepzig *et al.*, 1991).

Western white pine (*Pinus monticola* Douglas ex D. Don) decline was observed in the mid 20th century. A phenomenon called pole blight due to the size class of affected trees, 40-100 years of age (Buchanan *et al.*, 1951). While biotic agents were explored, including *Leptographium* spp., symptoms consistent with field observations were not reproducible in inoculation trials (Leaphart, 1956; Leaphart and Gill, 1959). The effects of water deficit were shown to be an important part of the decline, as increased incidence was correlated with shallow, droughty soils and a prolonged drought correlated with decreases in increment growth (Leaphart and Stage, 1971; Leaphart and Johnson, 1973).

1.2.1. *Pine decline in the southeastern United States*

In the southeastern U.S., decline has been reported in loblolly (*Pinus taeda* L.) (Williams and Tainter, 1971; Nevill *et al.*, 1995; Eckhardt *et al.*, 2004b; Eckhardt *et al.*, 2007), shortleaf pine (*P. echinata* Mill.) (Campbell and Copeland, 1954; Zarnoch *et al.*, 1994), and longleaf pine (*P. palustris* Mill.) (Otrosina *et al.*, 1999). Reports of pine decline date to the 1950s, in the phenomenon called littleleaf disease, which affects shortleaf pine predominantly (Campbell and Copeland, 1954). Loblolly pine was affected by littleleaf disease, but to a lesser degree than shortleaf pine, and most often in stands where infected shortleaf pines were also present (Campbell and Copeland, 1954). As the name suggests, one salient symptom of the disease is shortened needles, as well as thinning crowns, chlorosis, and in the final stages, an abundance of cones, reduced in size and with few viable seeds (Campbell and Copeland, 1954). Longleaf pine is rarely affected by littleleaf disease (Campbell and Copeland, 1954). *Phytophthora cinnamomi* Rand (Chromista: Oomycota, Pythiaceae) was noted as a potential contributing agent, a finding supported in subsequent inoculation trials (Zak and Campbell, 1958), but the role of this oomycete *in situ* is not clear (Eckhardt, 2003).

Reports of loblolly pine die-off or decline also date to the 1950's (Roth and Peacher, 1971). Loblolly die-off was observed on the Oakmulgee Ranger District, Talladega National Forest (Roth and Peacher, 1971), and also Anniston Army Depot (Ciesla, 1970). Symptoms on these sites also included chlorotic, shortened needles, poor needle retention, stress crops of cones, and ultimately mortality (Figure 1.1). However,

decline of loblolly pine has also been observed on droughty soils that are not typical littleleaf sites, and where there are few or no shortleaf pines (Eckhardt *et al.*, 2007). While the symptomatology of loblolly pine decline is similar to littleleaf disease, the biotic agents involved and site factors are quite different (Eckhardt, 2003). As with littleleaf, the crown symptoms suggest root damage, but in loblolly pine decline, the roots are commonly infected with *Leptographium* spp., which are vectored by root-feeding bark beetles and pine regeneration weevils (Coleoptera: Curculionidae, subfamilies Scolytinae and Molytinae, respectively) (Eckhardt *et al.*, 2007).

1.2.2. *Longleaf pine decline*

In addition to shortleaf and loblolly pine, longleaf pine (*P. palustris*) decline has also been observed in the southeastern U.S. Numerous predisposing and inciting factors have been investigated as effectors of longleaf pine growth and survival, particularly in juvenile (grass-stage) longleaf pine due to historical problems with natural regeneration of longleaf pine (Boyer, 1979). These include fire effects, interspecific competition, and drought stress. These factors are interrelated and are briefly reviewed here.

Figure 1.1. Declining loblolly pine at Fort Benning, GA. Visible symptoms include thinning, shortened foliage, dieback at the top of the crown.

Fire is an integral part of longleaf pine ecosystems (see Section 1.5.1). Historically, longleaf pine savannahs were maintained by frequent fire caused by lightning strikes or human application. While fire is essential to maintenance of these ecosystems, longleaf pine restoration ecologists have found fire application to be highly variable in effects. Fire applied during summer months negatively affects mature longleaf pine root growth more than fall or winter burns, but drought can exacerbate negative effects of dormant season burns also (Sayer and Haywood, 2006). The effects of fire on nutrient availability are also important, as burning releases stored carbon into the atmosphere, and temporarily increases plant available nitrogen (at a cost of nitrogen also being released into the atmosphere) (Carter and Foster, 2004).

Another benefit of fire to young longleaf pine is the control of competing vegetation, as longleaf pine is shade-intolerant (Wahlenberg, 1946). On sites with high water availability, failure to control competing woody growth by herbicide or mechanical means led to poor seedling growth (Knapp *et al.*, 2008), and the effects of weed control measures on growth has been observed in sapling longleaf pine as well (Haywood, 2005).

Compared to other southern pines, longleaf pine seedlings are less sensitive to drought stress (Sayer *et al.*, 2005), although water stress may inhibit longleaf growth to a greater extent than low nutrient or light availability (Jose *et al.*, 2003) and drought can also have synergistic effects with pathogenic fungi (Matusick *et al.*, 2008). Historic effects of drought on mature trees have also been demonstrated in dendrochronological analyses (Henderson and Grissino-Mayer, 2009).

An investigation into the contribution of root-infecting fungi to longleaf pine decline was previously published by Otrosina *et al.* (1999). They noted typical decline symptoms at the Savannah River Site in South Carolina; thinning crowns were followed by needle chlorosis, occasionally followed by wilting and cone stress crops before death. Symptom development followed prescribed burning of a 35-year old longleaf pine stand, though longleaf pine is dependent on fire to maintain its dominance. In addition to *Leptographium* spp., they also found the root decay fungus *Heterobasidion annosum* (Fr.) Bref. (Basidiomycota, Bonderzewiaceae), but the latter was found more frequently in trees where decline symptoms were more advanced (Otrosina *et al.*, 1999).

As in loblolly pine decline, the agents involved in longleaf pine decline have been debated. Although fire is essential to longleaf pine, the return of fire to long unburned sites has been implicated in decline (Varner *et al.*, 2007). The effects of fire on root-feeding insect populations and subsequent mortality have been investigated with inconsistent results (Hanula *et al.*, 2002; Campbell *et al.*, 2008), and with little connection to fungal symbionts. Otrosina (2005) attributes longleaf pine decline to 'exotic ecosystems', in particular long term soil erosion which has rendered areas where longleaf pine once flourished unsuitable.

1.2.3. *Ecological modeling of decline*

Ecological modeling of forest disease and insect outbreaks is a common practice (Pukkala *et al.*, 2005), which has been greatly enhanced by technological advances such as geographic information systems (GIS) databases (Meentemeyer *et al.*, 2008; Manter *et*

al., 2005; BenDor *et al.*, 2006). Among the benefits of these models are identification of sites at greatest risk of presenting disease symptoms (Manter *et al.*, 2005), and the factors that are correlated with symptom development (Meentemeyer *et al.*, 2008).

The cryptic nature of forest declines has led many researchers to use these tools to investigate ecological conditions that may predispose trees to decline. Understanding of sugar maple (*A. saccharum*) decline has also been enhanced by culling data on symptom presence or absence and superimposing site data including slope, aspect, soil chemistry and geology (Drohan *et al.*, 2002). Foliage and soil in plots with symptomatic trees had lower base cations than in plots where trees were asymptomatic, especially in soil <50 cm in depth, consistent with hypotheses suggesting base cation leaching. Also, declining plots had lower clay and higher sand and rock in their soil horizons, as well as tending to be at higher topographic positions in the landscape. These characteristics suggest that droughty sites are at increased risk of developing decline symptomatology. However, droughty sites only tended to display symptoms when base cations were lower, suggesting a synergistic effect (Drohan *et al.*, 2002).

Decline of silver fir (*Abies alba* Mill.) and Norway spruce (*Picea alba* Karst.) in central Europe has also been observed and hypotheses about causal factors tested. An investigation into the conditions correlated with the yellowing and defoliation of *A. alba* showed that altitude, stand age, and aspect of slope were all positively correlated with decline symptoms (Thomas *et al.*, 2002). As in sugar maple decline, plant available water holding capacity was inversely correlated with altitude, suggesting that droughty soil may be an important predisposing factor. More recent analyses focusing on actual

and modeled tree ring growth, weather and air pollution show a significant effect of sulfur dioxide emissions in silver fir decline (Elling *et al.*, 2009), consistent with symptom development at higher elevation sites. However, frost events and available soil water were found to be important decline predictors in this system.

Yellow-cedar (*Callitropsis nootkatensis*) decline has occurred across 200,000 hectares of its range in southeastern Alaska. While several biotic agents have been screened as potential contributing agents, none have been consistently associated with declining trees (Hennon and Shaw, 1994). Dendrochronological data indicate that changes in climatic conditions may be important in this decline (Beier *et al.*, 2008). Climatological data, including daily temperature maxima and minima, total precipitation and snowfall, were used to model thaw-freeze events which were thought to be important inciting stressors. These climate data were compared to tree growth ring data in areas where decline was present or absent. The increase in thaw-freeze events, coupled with decreased snowfall, was found to be strongly correlated with declining yellow-cedar (Beier *et al.*, 2008).

Baccala *et al.*, (1998) investigated decline of Cordilleran cypress (*Austrocedrus chilensis* (D.Don) Florin et Boutelje in Argentina. In their study, several site and climatic variables were measured from 32 sites in Nahuel Huapi National Park that had varying incidence of *A. chilensis* decline symptomatology. Using univariate, bivariate, and multivariate analyses, they found disease incidence positively correlated with high precipitation, and negatively correlated with high elevation and steep slopes. These data suggest that poor soil drainage was an important predisposing factor in *A. chilensis*

decline (in contrast with sugar maple decline and silver fir decline) and led to subsequent research into the potential role of *Phytophthora* spp. de Bary as contributing factors (Greslebin *et al.*, 2005).

In loblolly pine decline in the southeastern U.S., disease incidence was investigated in four physioregions in central Alabama and was shown to be positively correlated with predisposing site factors such as slope and aspect (Eckhardt and Menard, 2008). Steep slopes with south-southwestern facing aspects had increased disease incidence allowing for the development of a risk model using available spatial data (Eckhardt, 2003). The combination of steep, south-facing slopes suggests that water deficit may be an important inciting factor in loblolly pine decline. Subsequent application of the model to loblolly pine stands at Fort Benning Military Reservation, GA (FB) showed a high correlation of loblolly pine decline to predicted high risk sites, with an increased incidence of *Leptographium* root infection and increased captures of beetle vectors (Menard, 2007).

1.3. Ophiostomatoid fungi

Ophiostomatoid fungi (Ascomycota, Ophiostomataceae) include species of *Ophiostoma* Syd. & P. Syd., *Ceratocystiopsis* Upadhyay & Kendr., and *Grosmannia* Goid., with anamorphs of the form-genera *Leptographium*, *Hyalorhinocladiella* Upadhyay & Kendr., *Sporothrix* Hektoen & C.F. Perkins and *Pesotum* J.L. Crane & Schokn. (Wingfield *et al.*, 1993; Zipfel *et al.*, 2006). Previously thought to be closely related to the genus *Ceratocystis* Ellis &Halstead, fine morphological characteristics (de

Hoog, 1974), as well as biochemical (Smith *et al.*, 1967; Jewell, 1974; Harrington, 1981), and DNA sequence data (Hausner *et al.*, 1993; Spatafora and Blackwell, 1994) indicate that similarities between *Ceratocystis* and the Ophiostomataceae sensu stricto are the result of convergent evolution.

Until recently members of the Ophiostomataceae producing *Leptographium* anamorphs were considered species of *Ophiostoma* (Jacobs and Wingfield, 2001). The form-genus *Leptographium* has been shown to be a monophyletic lineage (Jacobs *et al.*, 2001; Zipfel *et al.*, 2006), and all *Ophiostoma* species with *Leptographium* anamorphs have been transferred to *Grosmannia*, with *Ophiostoma* sensu stricto and *Ceratocystiopsis* applied to species with *Pesotum*, *Sporothrix*, or *Hyalorhinocladiella* anamorphs (Zipfel *et al.*, 2006).

1.3.1. *Mycological description*

Ascomata in members of the Ophiostomataceae are typically darkly pigmented, with spherical bases and long necks (Upadhyay, 1981; Jacobs and Wingfield, 2001), but not produced in stromata (Kirk *et al.*, 2001). The asci are evanescent and ascospores are released through the ostiole at the perithecial apex in a gelatinous matrix facilitating vectoring by insects. All anamorphic states are hyphomycetous, varying from micronemata (*Sporothrix*), to synnemata (*Pesotum*) to mononemata (*Leptographium*).

Leptographium spp. typically have darkly pigmented mycelia and mononematous conidiophores that produce either hyaline or light-colored single-celled conidia from one to several ranks of metulae (branches) (Kendrick, 1962; Wingfield, 1985). The conidia in

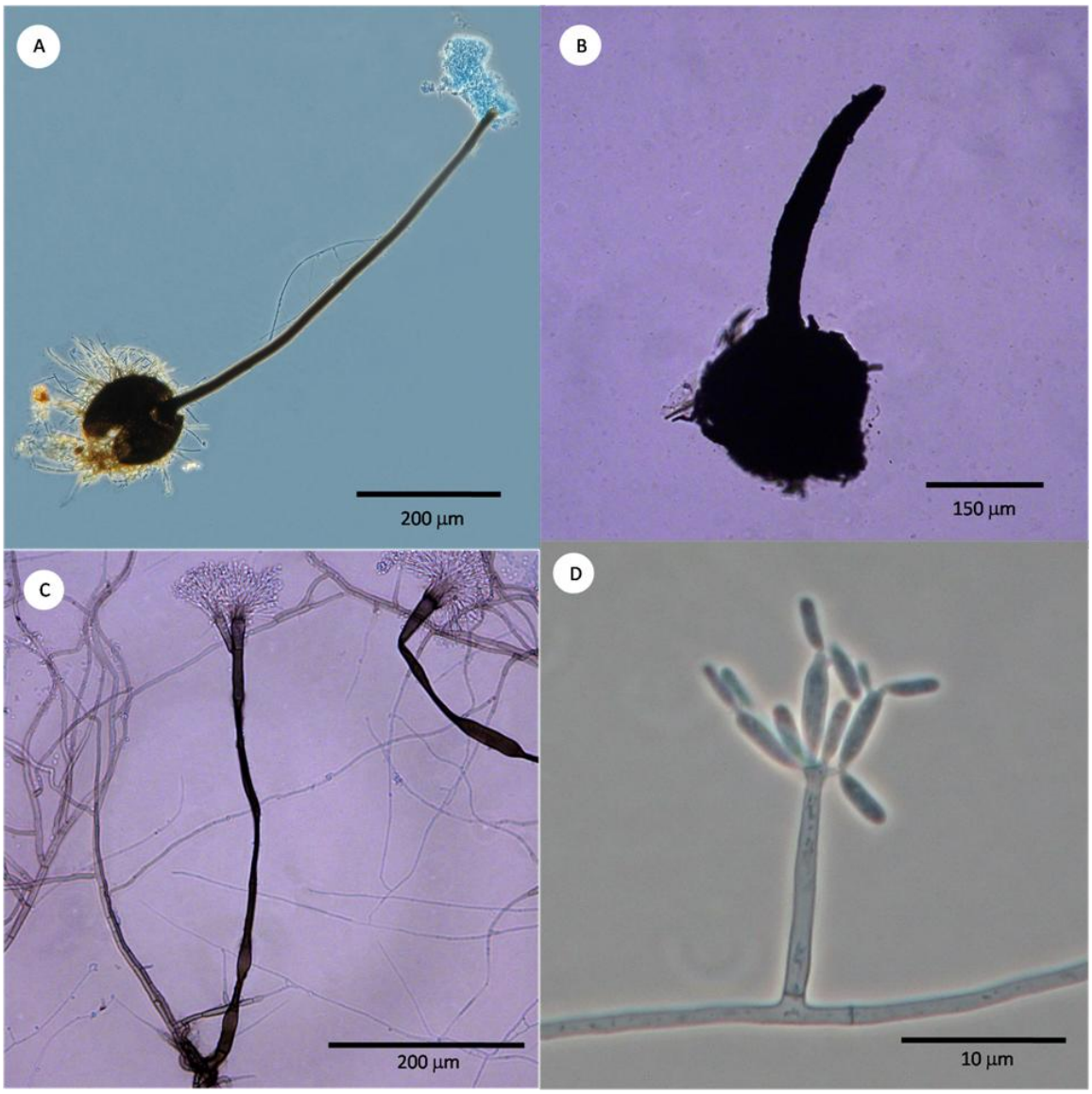

Figure 1.2. Ophiostomatoid fungi. A. Perithecium of *Ophiostoma* sp. with long neck divergent ostiolar hyphae. B. Perithecium of *Grosmannia huntii*, with shorter neck and convergent ostiolar hyphae. C. Mononematous conidiophore of *Leptographium serpens*. D. Micronematous conidiophore of *Sporothrix* sp.

Leptographium spp. (and *Pesotum* spp.) are borne in slimy masses which are also well suited to insect vectors, particularly bark beetles (Molnar, 1965; Malloch and Blackwell, 1993; Wingfield, 1993).

1.3.2. *Association with trees and wood products*

Called 'blue-stain' fungi for their dark green to navy blue to black pigmentation, ophiostomatoid fungi do not have any wood degrading ability (Seifert, 1993; Schirp *et al.*, 2003). While the stain can decrease the value of the commodity in some markets (Seifert, 1993), there are members of this family that cause disease in healthy trees. One of the most well-known plant diseases, Dutch elm disease (DED), is caused by the fungi *Ophiostoma ulmi* (Buism.) Nannf. and *O. novo-ulmi* Brasier. These two species are thought to have been introduced into Europe and North America from Asia, while attempts to find their native provenance did not succeed, it did yield a related species, *O. himal-ulmi* Brasier (Brasier and Mehrotra, 1995). Dutch elm disease has effectively removed the American elm (*Ulmus americana* L.) from the North American landscape.

Black stain root disease (BSRD), caused by the three varieties of *Grosmannia wageneri* (Goheen &F.W. Cobb) Zipfel, de Beer & Wingfield, presents a counterpoint to Dutch elm disease in its etiology. The disease was first reported from western pines (Wagener and Mielke, 1961) and the causal agent described by Kendrick (1962). Subsequently, the disease was found on Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco (Cobb and Platt, 1967).

In contrast with other species of *Leptographium*, which infect tracheids and ray parenchyma, *L. wageneri* is restricted to the tracheids (Wagener and Mielke, 1961), where it disrupts the flow of sap, leading to tree mortality (Hessburg and Hansen, 1987). Another contrasting feature of BSRD, relative to DED, is that *G. wageneri* is apparently native to the Pacific Northwest (Hansen *et al.*, 1988), while the DED pathogens are introduced to North America and Europe.

1.3.3. Leptographium *species associated with southern pines*

In the following sections, the biologies and pathologies of four ophiostomatoid species of interest in longleaf pine decline are reviewed. All of these species are associated with *Pinus* spp., and with the exception of *Grosmannia huntii*, have been previously isolated from the roots of declining pines in the southeastern U.S.

1.3.3.1. Leptographium procerum *(Kendrick) Wingfield*

Originally described from jack pine (*P.banksiana* Lamb.) in Quebec (Kendrick, 1962), *Verticicladiella procera* Kendrick was later transferred to *Leptographium* by M.J. Wingfield (Wingfield, 1985). *Leptographium procerum* has since been isolated from approximately 20 pinaceous host species and a similar number of curculionid vector species (Jacobs and Wingfield, 2001). Many studies have examined the interactions between *L. procerum*¸its vectors, and eastern white pine in white pine root decline (WPRD) (Dochinger, 1967; Houston, 1969; Towers, 1977; Weaver *et al.*, 1981; Lackner and Alexander, 1983; Meyer *et al.*, 1983; Lackner and Alexander, 1984; Horner *et al.*,

1987) and questions raised as to the efficacy of *L. procerum* as a pathogen (Wingfield, 1983b; Harrington, 1988). While trees infected with the fungus may express symptoms of WPRD, such as delay in bud break, needle wilt, reddening or loss of needles, and profuse exudation of resin, the conditions that predispose or incite these symptoms has been subject to debate.

Leptographium procerum has been isolated from roots of declining red pine (Klepzig *et al.*, 1991), loblolly pine (Eckhardt *et al.*, 2004b) and longleaf pine (Otrosina *et al.*, 1999) and is frequently isolated from root-feeding bark beetles and weevils associated with these hosts (Klepzig *et al.*, 1995; Eckhardt *et al.*, 2007) and other tree species (Table 1.1).

1.3.3.2. Leptographium terebrantis *Barras & Perry*

The black turpentine beetle (BTB, *Dendroctonus terebrans* [Olivier], Section 1.4.1,) though not as damaging as its congener, the southern pine beetle (SPB, *D. frontalis* Zimmermann), has also been of interest to forest entomologists in the southeastern U.S. Forest Service researchers S. Barras and T. Perry examined the mycota of the BTB (Barras and Perry, 1971) and SPB (Barras and Perry, 1972). They described *L. terebrantis*, which they recovered from BTB and infested *P. taeda* (Barras and Perry 1971). This fungus has been reported from several other *Pinus* spp. and beetle spp. including SPB, red turpentine beetle (RTB, *D. valens* LeConte), *Hylurgops* LeConte spp., *Ips pini* (Say), and *Hylobius* Germar spp. (Jacobs and Wingfield 2001) (Table 1.2).

Table 1.1. Previous reports of *Leptographium procerum*, their provenances, hosts, and vectors.

Provenance/Country	State/Province	Host (*Pinus* spp.)	Insect Vectors	References
North America				
Canada	Ontario	*P. strobus*		Kendrick, 1962
	Quebec	*P. banksiana*		Kendrick, 1962*
			Tomicus piniperda	Hausner *et al.*, 2005
	Alabama	*P. taeda*	*Colopterus unicolor, Corthylus punctatissimus, Hylastes salebrosus, H. tenuis, Hylobius pales, Pachylobius picivorus, Pityophagus cephalotes*	Eckhardt *et al.*, 2007
	Florida	*P. clausa*	*D. terebrans, Hylastes* spp *, Hb.pales,*	Barnard and Meeker, 1995
		P. elliottii	*P. picivorus*	Wingfield, 1986
		P. palustris		
	Georgia	*P. taeda*	*D. terebrans, H. salebrosus, H. tenuis, Hb. pales, P. picivorus*	Menard, 2007
	Illinois	*P. resinosa*		Wingfield, 1986
	Iowa	*P. strobus*		Wingfield, 1986
	Massachusetts	*P. sylvestris*	*D. terebrans*	Rane and Tattar, 1987
		P. thunbergiana		
	Michigan	*P. resinosa*	*Hb. radicis*	Wingfield, 1983a
	Minnesota	*P. banksiana, P. nigra, P. resinosa, P. strobus, P. sylvestris*	*D. valens, Hb. pales,P. picivorus, Pissodes approximatus*	Wingfield 1983a, 1986

*= Original species description and type locality

Table 1.1. Continued

Provenance/Country	State/Region	Host (Pinus spp.)	Insect Vectors	References
North America				
USA	New York	*P. resinosa, P. strobus, P. sylvestris*		Kendrick, 1962 Sinclair and Hudler, 1980
	Pennsylvania	*P. strobus*		Towers, 1977
	South Carolina	*P. palustris*		Otrosina *et al.*, 1999
	Virginia	*P. strobus,* Christmas trees	*Hb.pales, Ips* spp., *Orthotomicus caelatus, Pi. nemorensis, Pityogenes hopkinsi, Pityophthorus* spp., *Xyleborus* spp.	Wingfield, 1986 Nevill and Alexander, 1992a, b Lackner and Alexander, 1984
	Wisconsin	*P. banksiana, P. nigra, P. ponderosa, P. resinosa, P. sylvestris*	*D.valens, H.porculus, Hb. pales, Hb. radicis, Hb. rhizophagus, P.picivorus*	Wingfield, 1983a Klepzig *et al.*, 1991
Europe				
Croatia		*P. strobus*		Wingfield, 1986 Halambek, 1981
France	Orleans	*P. sylvestris*	*Hb.abietis, Pi. piceae*	Levieux *et al.*, 1994 Piou, 1993
Norway		"timber wood"		Hausner *et al.*, 2005
Sweden	Sodermanland	*P.* sp.	*Pi. strobi* gallery	Kendrick, 1962
United Kingdom	East Anglia	*P. sylvestris* (billets)	*H.opacus, Hylurgops palliatus*	Wingfield and Gibbs, 1991
Asia-Pacific				
Korea		*P. radiata* logs		Kim *et al.*, 2005
New Zealand		*P. strobus* , *P. nigra, P. taeda, P. radiata*	*H.ater*	Wingfield, 1986 Shaw and Dick, 1980 Mackenzie and Dick, 1984

Table 1.2. Previous reports of *Leptographium terebrantis*, their provenances, hosts and vectors.

Provenance	State/Region	Host (Pinus spp.)	Insect Vectors	References
North America				
USA	Alabama	*P. taeda*	*Colopterus unicolor, Dendroctonus frontalis, D. terebrans, Hylastes salebrosus, H. tenuis, Hylobius pales, Ips* spp, *Pachylobius picivorus, Pityophagus cephalotes*	Eckhardt *et al.*, 2007
	California	*P. ponderosa*	*D. brevicomis, D. ponderosae, D. valens, Hylurgops porosus*	Six *et al.*, 2003 Harrington and Cobb, 1983
	Georgia	*P. taeda*	*D. terebrans, H. salebrosus, H. tenuis ,Hb. Pales, P. picivorus*	Menard, 2007
	Louisiana*	*P. taeda*	*D.terebrans*	Barras and Perry, 1971*
	Massachusetts	*P. sylvestris P. thunbergiana*	*D. terebrans*	Rane and Tattar, 1987 Highley and Tattar, 1985
	Minnesota	*P. banksiana, P. nigra, P. resinosa, P. sylvestris*	*D. valens*	Wingfield, 1983a
	Mississippi	*P. taeda*	*D. terebrans*	Barras and Perry, 1971
	Oregon		*D. valens*	Six *et al.* 2003
	South Carolina	*P. palustris*		Otrosina *et al,*. 1999
	Vermont	*P. strobus*	*D. valens*	Jacobs *et al.*, 2004
	Wisconsin	*P. resinosa, P. strobus*	*D. valens, H. porculus, Hb. Radicis*	Wingfield, 1983a, 1986 Klepzig *et al.,* 1991
	Wyoming	*P. contorta*		Six *et al.*, 2003
Canada	British Columbia	*P. contorta*		Six *et al.*, 2003
Europe				
Germany		"Gymnosperm wood"		Hausner *et al.,* 2005

*= Original species description and type locality

Leptographium terebrantis was also shown to be an important component of the red pine decline pathosystem (Klepzig *et al.*, 1991). In the southeastern U.S., Eckhardt *et al.* (2007) reported on several other vectors for *L. terebrantis*, including *Hylastes* Erichson spp., *Pachylobius picivorus* (Germar), *Hylobius pales* Herbst, and *Pityophagus cephalotes* LeConte. Inoculation studies with *L. terebrantis* and *L. procerum* have shown the former to produce significantly larger lesions than the latter on *P. strobus* (Wingfield, 1983b, 1986), and *P. taeda* (Nevill *et al.*, 1995; Eckhardt *et al.*, 2004b)

1.3.3.3. Grosmannia huntii *(R.C. Rob.Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf.*

Originally described as *Ceratocystis huntii* (Robinson-Jeffrey and Grinchenko, 1964), this species has a *Leptographium* anamorph (Wingfield, 1985) that is less frequently observed. The holotype was collected from *Pinus contorta* Dougl. ex Loudon var. *latifolia* Engelm. in British Columbia, and has since been found in New Zealand, England, Australia, and other areas of the U.S. (Davidson and Robinson-Jeffrey, 1965; Gibbs and Inman, 1991; Jacobs and Wingfield, 2001; Reay *et al.*, 2005) (Table 1.3). Hosts include *P. ponderosa* C. Lawson, *P. monticola* Douglas ex D. Don, *P. banksiana* Lamb., *P. strobus*, and *Picea mariana* (Mill.) Britton, Stearns & Poggenburg. Associated insects include *D. ponderosae* Hopkins, *H. ater* (Paykull), *H. macer* LeConte, *Ips pini* (Say), and *Tomicus piniperda* (L.) (Jacobs and Wingfield, 2001) (Table 1.3).

Table 1.3. Reports of *Grosmannia huntii*, their provenances, hosts, and vector relationships.

Provenance/Country	State/Region	Hosts (Pinus spp.)	Insect vectors	References
Americas				
Canada	Alberta			Lee *et al.*, 2005
	British Columbia	*Pinus contorta*	Trees attacked by *Dendroctonus monticolae*	Robinson-Jeffrey and Grinchenko, 1964* Lee *et al.*, 2005
	Ontario	*P. resinosa*		Griffin, 1968
USA	Arizona	*P. ponderosa*	*Dendroctonus* sp?	Davidson and Robinson-Jeffrey, 1965
	Colorado	*P. ponderosa*	Trees attacked by *Ips pini*	
	New York	*P. strobus*	*Dendroctonus* sp.	
	Oregon	*P. ponderosa*		
	Washington			
Chile		*P. radiata*	*Hylastes ater*	Zhou *et al.*, 2004
Europe				
Italy				Jacobs *et al.*, 1998
Portugal				
United Kingdom		*P. sylvestris*	*Tomicus piniperda*	Gibbs and Inman, 1991
Asia-Pacific				
Australia		*P. radiata*	*Hylastes ater*	Jacobs *et al.*, 1998
New Zealand		*P. taeda*		Reay *et al.*, 2005 Thwaites *et al.*, 2005
Korea		*Pinus radiata* logs		Kim *et al.*, 2005

*= Original species description

Although other fungi share morphological characters with *G. huntii* (R.C. Rob.Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., including *G. penicillata* (Grosmann) Goid. and *G. piceaperda* (Rumbold) Goid., *G. huntii* has morphological differences that discriminate itself from these species, and molecular evidence to support its distinction as a valid species (Jacobs *et al.*, 2001; Jacobs *et al.*, 2004; Zipfel *et al.*, 2006). A noteworthy characteristic often overlooked is the presence of serpentine hyphae, which are typically present in isolates collected from the southeastern U.S. These similarities make differentiation from *L. serpens* difficult.

While reports of *G. huntii* have come from many regions, Jacobs and Wingfield (2001) note that nothing is known about the pathogenicity of this species. Given the frequency with which *G. huntii* has been found in the world's pine forests, an assessment of its pathogenicity is certainly due.

1.3.3.4. Leptographium serpens *Goidanich*

This species, originally described as *G. serpens* Goidanich and having a *Scopularia* anamorph (Goidanich, 1936), has wended its way through several other genera and synonyms (*Ceratocystis serpens*, *Ophiostoma s.*, *Verticicladiella s.*, *L. s.*, *V. alacris*, *L. alacre*, *L. gallaeciae*) (Jacobs and Wingfield, 2001) only to be restored, in the light of molecular evidence, to its original taxonomic designation (Zipfel *et al.*, 2006). The rarity of the teleomorph has led one author to retain the anamorph name (Upadhyay, 1981), a convention adopted in this dissertation.

The holotype of *L. serpens*, now in a degraded condition, was collected from *P. sylvestris* L. in Italy (Goidanich, 1936). Collections have been made in other western European countries (Table 1.4); France (Morelet, 1988), the United Kingdom (Wingfield and Gibbs, 1991), Spain, Portugal (de Ana Magan, 1982; Jacobs and Wingfield, 2001), and Poland (Siemaszko, 1939, cited in Harrington, 1988). More recent surveys of ophiostomatoid fungi from pines and scolytines in Poland do not report this species being present (Jankowiak, 2005, 2006; Jankowiak and Bilanski, 2007). Cultures from Siemaszko's original report are not known to be extant.

Reports of *L. serpens* have also come from South Africa (Wingfield and Knox-Davies, 1980), where the arboreal plant hosts and scolytine insect vectors are known to be introduced, and thus likely that the fungus is introduced. Early studies on the pathogenicity of *L. serpens* in South Africa suggested that this fungus may be an important agent in tree mortality (Wingfield and Knox-Davies, 1980). However, subsequent research has indicated limited virulence, when comparing lesion length with other ophiostomatoid species (Zhou *et al.*, 2002).

The authenticity of some reports of *L. serpens*, particularly from the western U.S. is questionable (Harrington, 1988). Reports of this fungus from the southeastern U.S. suggest that the fungus may be the most aggressive of the *Leptographium* species found in pine roots (Eckhardt *et al.*, 2004b). Increased aggressiveness coupled with other lines of evidence, suggests that *L. serpens* may be a recent introduction into the southeastern U.S.

Table 1.4. Previous reports of *Leptographium serpens*, provenances, hosts, and vector relationships.

Provenance/Country	State/Region	Hosts (Pinus spp.)	Insect vectors	References
Europe				
France	Orleans	*P. nigra* *P. sylvestris*		Morelet, 1988
Italy		*P. sylvestris*		Goidanich, 1936 *
Italy	Tombolo	*P. pinea*		Lorenzini and Gambogi, 1976
Poland			*Tomicus piniperda*	Siemaszko 1939 in Harrington, 1988
Spain, Portugal		*P. pinaster*		de Ana Magan, 1982
United Kingdom	East Anglia	*P. sylvestris* (billets)	*Hylastes ater*	Wingfield and Gibbs, 1991
North America				
USA	Alabama	*P. taeda*	*Colopterus unicolor*, *Corthylus punctatissimus*, *Gnathotrichus materiorius*, *Hylastes salebrosus*,*H. tenuis*, *Monarthrum mali Orthotomicus caelatus*, *Pityophagus cephalotes*, *Xyleborinus saxesenii*, *Xylosandrus compactus*, *X. crassiusculus*,	Eckhardt *et al.*, 2007
	Mississippi	*P. taeda*		Zambino and Harrington, 1992
	Virginia	*P. strobus*		Zambino and Harrington, 1992
Africa				
South Africa	Western Cape	*P. pinaste* *P. radiata*		Wingfield and Knox-Davies, 1980 Wingfield and Marasas, 1980
	Mpumalanga, KwaZulu Natal	*P. patula*, *P. elliottii*	*H. angustatus*, *Hylurgops porosus*	Zhou *et al.*, 2001; Zhou *et al.*, 2002

*= Original species description

As the species epithet suggests, *L. serpens* is characterized as having serpentine hyphae. This trait is not unique to *L. serpens* (see previous section on *G. huntii*), and it has also been suggested that the trait is not consistent in culture (Kendrick, 1962). This characteristic has led to confusion between these two species, which have previously been found in sympatry in Europe and the U.K. (Gibbs and Inman, 1991; Wingfield and Gibbs, 1991; Jacobs *et al.*, 1998).

1.4. Beetles, vectors of ophiostomatoid fungi

Ophiostomatoid fungi, including *Ophiostoma*, *Ceratocystis*, and *Leptographium* spp. are most commonly vectored by beetles (Coleoptera) of the family Curculionidae, especially members of the subfamilies Scolytinae (Figure 1.3) (Klepzig *et al.*, 1991; Paine *et al.*, 1997; Hanula *et al.*, 2002, Eckhardt *et al.*, 2004a) and Molytinae (Klepzig *et al.*, 1991; Nevill and Alexander, 1992b; Levieux *et al.*, 1994; Hanula *et al.*, 2002). Known as the bark and ambrosia beetles, scolytines occupy a wide variety of ecological niches (Wood, 1982), but are typically associated with forest tree hosts (Paine *et al.*, 1997). Molytines also vary in their niche, but include many taxa reliant upon woody plants as hosts (Arnett *et al.*, 2002).

While the most destructive members of the Scolytinae are noted for boring into the boles of live, healthy trees and killing them, most scolytines are not primary pests (Paine *et al.*, 1997). The major tree killers are the most researched, but they are not the only insects that may impact living trees of commercial importance.

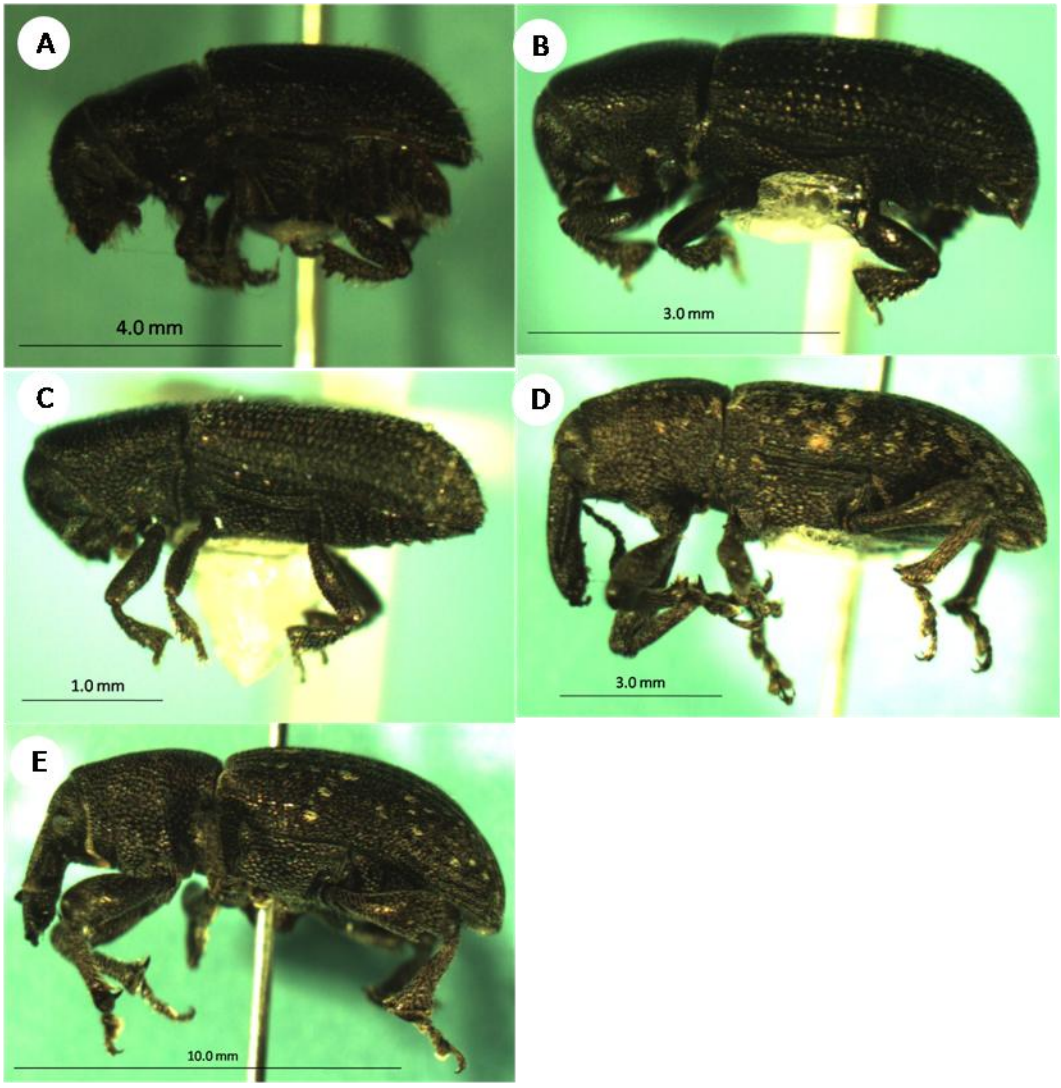

Figure 1.3. Root-feeding bark beetles and regeneration weevils. A. *Dendroctonus terebrans*. B. *Hylastes salebrosus*. C. *H. tenuis*. D. *Hylobius pales* (note straight metatibia) E. *Pachylobius picivorus* (note flanged metatibia).

Many members of the genus *Dendroctonus* have earned their generic epithet as killers of trees. In the southeastern U.S., SPB is the preeminent threat to pine forestry (Thatcher *et al.*, 1980). For this reason, SPB is the most researched forest insect pest in the region. As this dissertation considers the ophiostomatoid fungi and vectors associated with the roots and lower stems of longleaf pine, and since SPB and its associated mycota are not known to attack these tissues, they will not be considered further here.

1.4.1. *Black turpentine beetle,* Dendroctonus terebrans *(Olivier)*

Less aggressive toward healthy trees is BTB (Figure 1.3A). This species infrequently attacks healthy trees, and typically does not kill its host. One of the largest members of the genus, adult BTBs are 5.0-7.5 mm in length with a body about 2.2 times as long as wide (Wood, 1982). Similar in size and morphology is RTB, which has a reddish cast rather than the black cast of BTB. The range of BTB extends from eastern Massachusetts south to Florida and east to Texas (Wood, 1982; Rane and Tattar, 1987; Clarke *et al.*, 2000). In contrast with SPB, BTB attacks the lower stems of larger trees, and may burrow through the phloem downward into the roots (Wood, 1982).

1.4.2. Hylastes *spp. Erichson*

Members of the genus *Hylastes* are phloeophagous insects typically feeding on, and breeding in, conifer roots and lower stems, or in felled trees or stumps (Blackman, 1941). There are approximately 27 species globally; 15 of these occur in Northern and Central America (Wood, 1982). These insects are typically found on pine, but may also

infest spruce, Douglas-fir, and true firs (Blackman, 1941). They are not believed to kill healthy trees within their native ranges, but are often associated with trees that may be either declining or dying due to other causes.

In the southeastern U.S., the most frequently encountered members of this genus are *H. salebrosus* Eichhoff (Figure 1.3B), and *H. tenuis* Eichhoff (Figure 1.3C) (Wood, 1982; Atkinson and Peck, 1994; Bauman, 2003; Eckhardt *et al.*, 2004a; Eckhardt *et al.*, 2007). Another species collected in the southeast is *H. porculus* Erichson (Blackman, 1941; Wood, 1982, Miller and Rabaglia, 2009). The range of this species extends through much of the eastern U.S. The genus, in the subtribe Hylastina (fide Arnett *et al.*, 2002), is morphologically similar to *Hylurgops* LeConte, which also has root- and stump-feeding members.

Hylastes tenuis is the smaller of the two most commonly captured southern species, approximately 2.1-2.7 mm long in both sexes, and is about 3.0 times as long as wide (Wood, 1982). Its range extends from Hidalgo, Mexico, north and east to New York State, and with rare exceptions, is found exclusively on pines (Wood, 1982) in stumps and roots, as well as being attracted to freshly sawn pine timber (Blackman, 1941).

Hylastes salebrosus is approximately 3.3-5.0 mm long, and about 2.5 times as long as wide in both sexes (Wood, 1982). It has been captured throughout Texas east to Florida, and north to New Jersey (Wood, 1982), with similar affinity to loblolly, longleaf and shortleaf, and freshly sawn timber (Blackman, 1941).

The fungal relations of several *Hylastes* spp. have been investigated, as the fungi they vector may be of economic consequence. *Hylastes nigrinus* (Mannerheim) has been shown to be an important vector of *L.wageneri* in the BSRD pathosystem (Witcosky and Hansen, 1985; Witcosky *et al.*, 1986). In red pine decline, *H. porculus* has been shown to vector *Leptographium* spp. (Klepzig *et al.*, 1995). *Hylastes ater* and *H. angustatus* (Herbst), introduced into southern hemisphere pine plantations, have been shown to vector blue stain fungi (Tribe, 1992; Zhou *et al.*, 2001; Reay *et al.*, 2002; Reay *et al.*, 2005; Mausel *et al.*, 2007). *Hylastes* spp. in the United Kingdom have also been shown to vector *Leptographium* spp. (Wingfield and Gibbs, 1991).

1.4.3. *Regeneration weevils*

Pinophagous weevils (Coleoptera: Curculionidae, Molytinae) have been demonstrated to be vectors of ophiostomatoid fungi (Nevill and Alexander, 1992c; Levieux *et al.*, 1994). In the southeastern U.S., the most common representatives of the molytine weevils are the pales weevil, *Hylobius pales* (Herbst) (Figure 1.3D), pitch-eating weevil, *Pachylobius picivorus* (Germar) (Figure 1.3E), and *Pissodes nemorensis* Germar (Neidhardt, 1969). The two former species overlap greatly in niche and range in the eastern U.S. (with *H. pales* having a slightly greater range), have similar morphology, and are frequently treated together by forest entomologists (Dixon and Foltz, 1990). A key distinguishing characteristic is the presence of a distal flange in the metathoracic

tibiae in *P. picivorus*, giving the legs a "bell-bottomed" appearance, when compared with *H. pales*, whose metathoracic tibiae are parallel-sided for most of their length (Kissinger, 1964).

1.5. Longleaf pine

Longleaf pine (*Pinus palustris*, Figure 1.4) was historically the dominant tree species throughout the forests of the coastal plain in the southeastern U.S. Once occupying a range estimated to be between 28 and 37 million ha (Boyer, 1990; Frost, 2006), longleaf pine extended from Virginia south into peninsular Florida, and west to Texas. This range has been reduced to approximately 1.2 million ha (Frost 2006). The reduction is attributable to shifts in land use including; exploitation for forest products, fire suppression, rooting by feral hogs, lack of natural regeneration and harvest with subsequent transition to other species such as loblolly pine and slash pine (*P. elliottii* Engelm.) (Frost, 2006). In 1997, longleaf pine was designated the state tree of Alabama, selected from the generic "southern pines" which had been the state tree since 1948 (Alabama Department of Archives and History 2003).

The wood of longleaf pine is considered to be of high value, preferred by foresters in old growth and second growth stands (Croker and Boyer, 1975). Because the stems are self pruning, longleaf poles have fewer knots than other pine species. Longleaf pine has been exploited for many products in addition to traditional wood and fiber. Shed longleaf needles may be used for basketry or for mulch as pine "straw". One of the earliest uses of longleaf pine following European settlement was products derived from

Figure 1.4. Longleaf pine at Fort Benning, GA.

the resin of longleaf pine, including pine tar, rosin and turpentine. These products were indispensible during the era of sailing ships and were a valued commodity from the longleaf forest. The naval store industry employed over 27,000 workers during its peak in the early 20th century (Hodges, 2006). With the decline in area of longleaf pine, sources of pine-derived turpentine have shifted to overseas production, mainly from slash pine (Hodges, 2006).

1.5.1. *Longleaf pine and fire*

Longleaf pine is a pyrophytic species, requiring frequent fires and with several adaptations to protect it from fire. Longleaf pine cones are not serotinous, as in some pyrophytic pine species, but the seeds do require contact with a mineral soil to successfully establish. This can be accomplished by using fire or mechanical removal of duff (Boyer, 1990). The grass-stage and the dense needles act as insulation for the apical bud, and development of roots help longleaf withstand fires. Also longleaf pine is susceptible to brown spot needle blight, which fire helps to mitigate (Boyer, 1990). Seedlings that have begun bolting become susceptible to fire until they grow to approximately 1 m in height, at which point they become fire tolerant again (Boyer, 1990).

1.5.2. *Health issues and longleaf pine*

Longleaf pine is considered tolerant to the main disease and insect problems of southern yellow pines including: annosum root disease, fusiform rust (Walkinshaw, 1978), littleleaf disease (Campbell and Copeland, 1954), and attack by southern pine beetle (Moser *et al.*, 2003). One disease problem prevalent on longleaf pine is brown spot needle blight (Siggers, 1932; Wakeley, 1970), caused by *Mycosphaerella dearnessii* (teleomorph= *Scirrhia acicola* [Dearn.] Siggers). This disease frequently affects the needles of grass-stage longleaf. Brown spot on young longleaf can have negative effects on growth that last into maturity (Wakeley, 1970). Genetic resistance has been reported (Derr and Melder, 1970), and cultural practices to encourage early height growth in longleaf reduce the period of susceptibility. Feral hogs (*Sus scrofa* L.) may also cause substantial losses to longleaf regeneration by either uprooting young longleaf pine, or damaging lateral roots of mature longleaf pine (Lipscomb, 1989).

The main enemies of longleaf pine are abiotic, and include drought, lightning and high winds. Longleaf pine typically succumbs via a suite of these agents (Wahlenberg, 1946). As for diseases of the stem and roots, Hepting (1971) noted infection of longleaf pine by ophiostomatoid fungi such as *Ophiostoma ips* (Rumb.) Nannf. and *O. minus* (Hedgc.) H. & P. Sydow, which are vectored by bark beetles (*O. ips* on *Ips* spp. and *O. minus* on SPB) (Rumbold, 1931). Also attacking longleaf roots are some common wood decay fungi of the southeast, *Heterobasidion annosum* (Fr.) Bref, *Phaeolus schweinitzii* (Fr.) Pat., *Armillaria tabescens* (Scop.) Emel, and *Porodaedalea pini* (Brot.) Murrill. Several genera of potentially pathogenic nematodes may also be found on roots of

longleaf (Ruehle, 1964), although no wide scale damage has been reported. The "conventional wisdom" is that longleaf pine is a heartier tree than other southern pines such as loblolly, shortleaf, and slashes when it comes to diseases and insect pests (Hepting, 1971).

1.6. Central theme

The central theme of this dissertation is the biology and ecology of insect vectors and ophiostomatoid fungi that are potentially important in longleaf pine decline and loblolly pine decline. The LPDRM, developed to identify high-risk loblolly pine decline sites (Eckhardt, 2003; Eckhardt and Menard, 2008), is applied to longleaf pine in this dissertation. Tree growth and crown condition will be measured and compared between predicted high and low risk sites. The ophiostomatoid mycota of longleaf pine roots were investigated and the population dynamics of vector insects and their ability to transfer these fungi. Molecular markers, in the form of DNA sequence data, are also used to confirm the presence of recently isolated ophiostomatoid fungi, and to identify a novel species associated with pine roots and vector insects.

1.7. Literature Cited

Alabama Department of Archives and History. 2003. Alabama Tree: Southern Longleaf Pine. Online: http://www.archives.state.al.us/emblems/st_tree.html, accessed 7 May 2008

Arnett, R.H.J., Thomas, M.C., Skelley, P.E., Frank, J.H., 2002. American Beetles, Volume II: Polyphaga: Scarabaeoidea through Curculionoidea: Books:. CRC Press, Boca Raton, FL.

Atkinson, T.H., Peck, S.B., 1994. Annotated checklist of the bark and ambrosia beetles (Coleoptera, Platypodidae and Scolytidae) of tropical southern Florida. Florida Entomologist 77, 313-329.

Baccala, N.B., Rosso, P.H., Havrylenko, M., 1998. *Austrocedrus chilensis* mortality in the Nahuel Huapi National Park (Argentina). Forest Ecology and Management 109, 261-269.

Barnard, E.L., Meeker, J.R., 1995. *Leptographium* root infections of pines in Florida., Florida Dept. of Agriculture and Consumer Services, Division of Plant Industry. Plant Pathology Circular No. 369

Barras, S.J., Perry, T., 1971. *Leptographium terebrantis* sp nov associated with *Dendroctonus terebrans* in loblolly pine. Mycopathologia et Mycologia Applicata 43, 1-10.

Barras, S.J., Perry, T., 1972. Fungal symbionts in the prothoracic mycangium of *Dendroctonus frontalis* (Coleoptera: Scolytidae). Zeitschrift fur Angewandte Entomologie 71, 95-104.

Bauce, E., Allen, D.C., 1991. Etiology of a sugar maple decline. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 21, 686-693.

Bauman, T.A., 2003. Interaction of fire and insects in the restoration and management of longleaf pine. MS Thesis, Louisiana State University, Baton Rouge, LA.

Beier, C.M., Sink, S.E., Hennon, P.E., D'Amore, D.V., Juday, G.P., 2008. Twentieth-century warming and the dendroclimatology of declining yellow-cedar forests in southeastern Alaska. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 38, 1319-1334.

BenDor, T.K., Metcalf, S.S., Fontenot, L.E., Sangunett, B., Hannon, B., 2006. Modeling the spread of the Emerald Ash Borer. Ecological Modelling 197, 221-236.

Blackman, M.W., 1941. Bark beetles of the genus *Hylastes* Erichson in North America. US Department of Agriculture Miscellaneous Publication No. 417, Washington D.C.

Boyer, W.D., 1979. Regenerating the natural longleaf pine forest. Journal of Forestry 77, 572-575.

Boyer, W.D., 1990. *Pinus palustris* Mill.-longleaf pine. In: Burns, R.M., Honkala, B.H. (Eds.), Silvics of North America. Volume 1. Conifers. U.S. Department of Agriculture, Forest Service, Washington DC, pp. 405-412.

Brasier, C.M., Mehrotra, M.D., 1995. *Ophiostoma himal-ulmi* sp nov, a new species of Dutch elm disease fungus endemic to the Himalayas. Mycological Research 99, 205-215.

Buchanan, T.S., Harvey, G.M., Welch, D.S., 1951. Pole blight of western white pine,-a numerical evaluation of the symptoms. Phytopathology 41, 199-208.

Campbell, J.W., Hanula, J.L., Outcalt, K.W., 2008. Effects of prescribed fire and other plant community restoration treatments on tree mortality, bark beetles, and other saproxylic Coleoptera of longleaf pine, *Pinus palustris* Mill., on the Coastal Plain of Alabama. Forest Ecology and Management 254, 134-144.

Campbell, W.A., Copeland, O.L., 1954. Littleleaf disease of shortleaf and loblolly pines. U.S.D.A. Forest Service Circular No. 940. Washington, DC.

Carter, M.C., Foster, C.D., 2004. Prescribed burning and productivity in southern pine forests: a review. Forest Ecology and Management 191, 93-109.

Ciesla, W.M., 1970. Forest disease conditions at Anniston Army Depot, Alabama. U.S. D.A. Forest Service, Southeastern Area, State & Private Forestry, Division of Forest Pest Control, Pineville, LA, 3 pp.

Clarke, S.R., Evans, R.E., Billings, R.F., 2000. Influence of pine bark beetles on the West Gulf Coastal Plain. Texas Journal of Science 52, 105-126.

Cobb, F.W., Platt, W.D., 1967. Pathogenicity of *Verticicladiella wageneri* to Douglas-fir. Phytopathology 57, 998-999.

Croker, T.C.J., Boyer, W.D., 1975. Regenerating longleaf pine naturally. USDA Forest Service, Southern Forest Experiment Station, Research Paper SO-105. New Orleans, LA, 26 p.

Davidson, R.W., Robinson-Jeffrey, R.C., 1965. New records of *Ceratocystis europhioides* and *C. huntii* with *Verticicladiella* imperfect stages from conifers. Mycologia 57, 488-490.

de Ana Magan, F.J.F., 1982. Las hogueras en el monte y el ataque del hongo *Leptographium gallaeciae* sp. nv. sobre *P. pinaster* Ait. Bol. Serv. Plagas 8, 69-82.

de Hoog, G.S., 1974. The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. Studies in Mycology 7, 84p.

Derr, H.J., Melder, T.W., 1970. Brown-spot resistance in longleaf pine. Forest Science 16, 204-209.

Dixon, W.N., Foltz, J.L., 1990. Pine reproduction weevils, *Hylobius pales* (Herbst) & *Pachylobius picivorus* (Germar) (Coleoptera: Curculionidae). Florida Department of Agriculture and Consumer Services, Dept. of Plant.Industry.

Dochinger, L.S., 1967. *Leptographium* root decline of eastern white pine. Phytopathology 57, 809-809.

Drohan, P.J., Stout, S.L., Petersen, G.W., 2002. Sugar maple (*Acer saccharum* Marsh.) decline during 1979-1989 in northern Pennsylvania. Forest Ecology and Management 170, 1-17.

Eckhardt, L.G., 2003. Biology and ecology of *Leptographium* species and their vectors as components of loblolly pine decline. PhD Dissertation, Louisiana State University, Baton Rouge, LA.

Eckhardt, L.G., Goyer, M.A., Klepzig, K.D., Jones, J.P., 2004a. Interactions of *Hylastes* species (Coleoptera : Scolytidae) with *Leptographium* species associated with loblolly pine decline. Journal of Economic Entomology 97, 468-474.

Eckhardt, L.G., Jones, J.P., Klepzig, K.D., 2004b. Pathogenicity of *Leptographium* species associated with loblolly pine decline. Plant Disease 88, 1174-1178.

Eckhardt, L.G., Menard, R.D., 2008. Topographic features associated with loblolly pine decline in central Alabama. Forest Ecology and Management 255, 1735-1739.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

Elling, W., Dittmar, C., Pfaffelmoser, K., Rotzer, T., 2009. Dendroecological assessment of the complex causes of decline and recovery of the growth of silver fir (*Abies alba* Mill.) in Southern Germany. Forest Ecology and Management 257, 1175-1187.

Filip, G.M., Rosso, P.H., 1999. Cypress mortality (mal del cipres) in the Patagonian Andes: comparisons with similar forest diseases and declines in North America. European Journal of Forest Pathology 29, 89-96.

Frost, C., 2006. History and future of the longleaf pine ecosystem. In: Jose, S., Jokela, E., Miller, D. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 9-48.

Gibbs, J.N., Inman, A., 1991. The pine shoot beetle *Tomicus piniperda* as a vector of blue-stain fungi to windblown pine. Forestry 64, 239-249.

Goidanich, G., 1936. Il genere di Ascomiceti *Grosmannia* G. Goid. Bollettino della Stazione di Patologia Vegetale di Roma 16, 26-60.

Greslebin, A.G., Hansen, E.M., Winton, L.M., Rajchenberg, M., 2005. *Phytophthora* species from declining *Austrocedrus chilensis* forests in Patagonia, Argentina. Mycologia 97, 218-228.

Griffin, H.D., 1968. The genus *Ceratocystis* in Ontario. Canadian Journal of Botany 46, 689-718.

Halambek, M., 1981. *Verticicladiella procera* Kendrick, causal organism of Eastern white pine wilting in conifers culture (English summary). Plant Protection 32, 312-323.

Hansen, E.M., Goheen, D.J., Hessburg, P.F., Witcosky, J.J., Schowalter, T.D., 1988. Biology and management of black-stain root disease in Douglas-fir. In: Harrington, T.C., Cobb Jr, F.W. (Eds.), *Leptographium* root diseases on conifers. American Phytopathological Society Press, St. Paul, Minn., pp. 63-80.

Hanula, J.L., Meeker, J.R., Miller, D.R., Barnard, E.L., 2002. Association of wildfire with tree health and numbers of pine bark beetles, reproduction weevils and their associates in Florida. Forest Ecology and Management 170, 233-247.

Harrington, T.C., 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73, 1123-1129.

Harrington, T.C., 1988. *Leptographium* species, their distributions, hosts and insect vectors. In: Harrington, T.C., Cobb, F.W. (Eds.), *Leptographium* root diseases on conifers. American Phytopathological Society Press, St. Paul, MN, pp. 1-39.

Harrington, T.C., Cobb, F.W., 1983. Pathogenicity of *Leptographium* and *Verticicladiella* spp isolated from roots of western North American conifers. Phytopathology 73, 596-599.

Hausner, G., Iranpour, M., Kim, J.J., Breuil, C., Davis, C.N., Gibb, E.A., Reid, J., Loewen, P.C., Hopkin, A.A., 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis*, *Leptographium truncatum*, and *Leptographium wingfieldii*. Canadian Journal of Botany-Revue Canadienne De Botanique 83, 1222-1237.

Hausner, G., Reid, J., Klassen, G.R., 1993. On the phylogeny of *Ophiostoma*, *Ceratocystis* ss, and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. Canadian Journal of Botany-Revue Canadienne De Botanique 71, 1249-1265.

Haywood, J.D., 2005. Effects of herbaceous and woody plant control on *Pinus palustris* growth and foliar nutrients through six growing seasons. Forest Ecology and Management 214, 384-397.

Henderson, J.P., Grissino-Mayer, H.D., 2009. Climate-tree growth relationships of longleaf pine (*Pinus palustris* Mill.) in the Southeastern Coastal Plain, USA. Dendrochronologia 27, 31-43.

Hennon, P.E., Shaw, C.G., 1994. Did climatic warming trigger the onset and development of yellow-cedar decline in southeast Alaska. European Journal of Forest Pathology 24, 399-418.

Hennon, P.E., Shaw, C.G., Hansen, E.M., 1990. Symptoms and fungal associations of declining *Chamaecyparis nootkatensis* in southeast Alaska. Plant Disease 74, 267-273.

Hepting, G.H., 1971. Diseases of forest and shade trees of the United States. U.S.D.A. Forest Service. U.S. Govt. Print. Off., Washington, DC, p. 658 p.

Hessburg, P.F., Hansen, E.M., 1987. Pathological anatomy of black stain root disease of Douglas-fir. Canadian Journal of Botany-Revue Canadienne De Botanique 65, 962-971.

Highley, L., Tattar, T.A., 1985. *Leptographium terebrantis* and black turpentine beetles associated with blue stain and mortality of black and scots pines on Cape Cod, Massachusetts. Plant Disease 69, 528-530.

Hodges, A.W., 2006. The naval stores industry. In: Jose, S., Jokela, E.J., Miller, D.L. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 43-48.

Horner, W.E., Alexander, S.A., Lewis, K.J., 1987. Colonization patterns of *Verticicladiella procera* in scots and eastern white pine and associated resin soaking, reduced sapwood moisture content, and reduced needle water potential. Phytopathology 77, 557-560.

Houston, D.R., 1969. Basal canker of white pine. Forest Science 15, 66-83.

Jacobs, K., Bergdahl, D.R., Wingfield, M.J., Halik, S., Seifert, K.A., Bright, D.E., Wingfield, B.D., 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycological Research 108, 411-418.

Jacobs, K., Wingfield, M.J., 2001. *Leptographium* species: tree pathogens, insect associates, and agents of blue-stain. APS Press, St. Paul, MN.

Jacobs, K., Wingfield, M.J., Wingfield, B.D., 2001. Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. Canadian Journal of Botany-Revue Canadienne De Botanique 79, 719-732.

Jacobs, K., Wingfield, M.J., Wingfield, B.D., Yamaoka, Y., 1998. Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov. Mycological Research 102, 289-294.

Jankowiak, R., 2005. Fungi associated with *Ips typographus* on *Picea abies* in southern Poland and their succession into the phloem and sapwood of beetle-infested trees and logs. Forest Pathology 35, 37-55.

Jankowiak, R., 2006. Fungi associated with *Tomicus piniperda* in Poland and assessment of their virulence using Scots pine seedlings. Annals of Forest Science 63, 801-808.

Jankowiak, R., Bilanski, P., 2007. Fungal flora associated with *Tomicus piniperda* L. in an area close to a timber yard in southern Poland. Journal of Applied Entomology 131, 579-584.

Jewell, T.R., 1974. Qualitative study of cellulose distribution in *Ceratocystis* and *Europhium*. Mycologia 66, 139-146.

Jose, S., Merritt, S., Ramsey, C.L., 2003. Growth, nutrition, photosynthesis and transpiration responses of longleaf pine seedlings to light, water and nitrogen. Forest Ecology and Management 180, 335-344.

Jung, T., Blaschke, H., Osswald, W., 2000. Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. Plant Pathology 49, 706-718.

Kendrick, W.B., 1962. The *Leptographium* complex: *Verticicladiella*. Canadian Journal of Botany 40, 771-797.

Kim, G.H., Kim, J.J., Lim, Y.W., Breuil, C., 2005. Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. Canadian Journal of Botany-Revue Canadienne De Botanique 83, 272-278.

Kirk, P.M., Cannon, P.F., David, J.C., Stalpers, J.A., 2001. Ainsworth & Bisby's dictionary of the fungi, 9th edition. CABI Pub., Oxon, UK.

Kissinger, D.G., 1964. Curculionidae of America north of Mexico; a key to the genera. Taxonomic Publications, South Lancaster, MA.

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Science 37, 1119-1139.

Klepzig, K.D., Smalley, E.B., Raffa, K.F., 1995. *Dendroctonus valens* and *Hylastes porculus* (Coleoptera, Scolytidae) - vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. Great Lakes Entomologist 28, 81-87.

Knapp, B.O., Wang, G.G., Walker, J.L., 2008. Relating the survival and growth of planted longleaf pine seedlings to microsite conditions altered by site preparation treatments. Forest Ecology and Management 255, 3768-3777.

Lackner, A.L., Alexander, S.A., 1981. Association of root disease and insect infestations with eastern white pines expressing sensitivity to air pollution. Phytopathology 71, 769-769.

Lackner, A.L., Alexander, S.A., 1983. Root disease and insect infestations on air pollution sensitive *Pinus strobus* and studies of pathogenicity of *Verticicladiella procera*. Plant Disease 67, 679-681.

Lackner, A.L., Alexander, S.A., 1984. Incidence and development of *Verticicladiella procera* in Virginia Christmas tree plantations. Plant Disease 68, 210-212.

Leaphart, C.D., 1956. Physiological studies of some fungi associated with pole blight of western white pine. Mycologia 48, 25-40.

Leaphart, C.D., Gill, L.S., 1959. Effects of inoculations with *Leptographium* spp. on western white pine. Phytopathology 49, 350-353.

Leaphart, C.D., Johnson, H.E., 1973. Pole blight: also a disease of western white pine plantations. Plant Disease Reporter 57, 948-951.

Leaphart, C.D., Stage, A.R., 1971. Climate: factor in origin of pole blight of *Pinus monticola* Dougl. Ecology 52, 229-239.

Lee, S., Kim, J.J., Breuil, C., 2005. *Leptographium longiclavatum* sp nov., a new species associated with the mountain pine beetle, *Dendroctonus ponderosae*. Mycological Research 109, 1162-1170.

Levieux, J., Piou, D., Cassier, P., Andre, M., Guillaumin, D., 1994. Association of phytopathogenic fungi for the scots pine (*Pinus sylvestris* L.) with the European pine weevil *Hylobius abietis* (L) (Col- Curculionidae). Canadian Entomologist 126, 929-936.

Lipscomb, D.J., 1989. Impacts of feral hogs on longleaf pine regeneration. Southern Journal of Applied Forestry 13, 177-181.

Lorenzini, G., Gambogi, P., 1976. Un caso di "moria" di *Pinus pinea* associata alla presenza di *Verticicladiella* sp. (nota preliminare). Informatore Fitopatologico 5, 5-8.

Mackenzie, M., Dick, M., 1984. *Verticicladiella* root disease. Forest Pathology in New Zealand 8, 1-4.

Malloch, D., Blackwell, M., 1993. Dispersal biology of the ophiostomatoid fungi. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), Ceratocystis and Ophiostoma: taxonomy, ecology and pathogenicity. American Phytopathological Society Press, St. Paul, MN, pp. 195-206.

Manion, P.D., 1981. Tree disease concepts. Prentice-Hall, Englewood Cliffs, N.J.

Manter, D.K., Reeser, P.W., Stone, J.K., 2005. A climate-based model for predicting geographic variation in Swiss needle cast severity in the Oregon Coast Range. Phytopathology 95, 1256-1265.

Matusick, G., Eckhardt, L.G., Enebak, S.A., 2008. Virulence of *Leptographium serpens* on longleaf pine seedlings under varying soil moisture regimes. Plant Disease 92, 1574-1576.

Mausel, D.L., Gara, R.I., Lanfranco, D., Ruiz, C., Ide, S., Azat, R., 2007. The introduced bark beetles *Hylurgus ligniperda* and *Hylastes ater* (Coleoptera : Scolytidae) in Chile: seasonal flight and effect of *Pinus radiata* log placement on colonization. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 37, 156-169.

Meentemeyer, R., Rizzo, D., Mark, W., Lotz, E., 2004. Mapping the risk of establishment and spread of sudden oak death in California. Forest Ecology and Management 200, 195-214.

Menard, R.D., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. MS Thesis, Louisiana State University, Baton Rouge, LA.

Meyer, G.J., Hindal, D.F., Quinn, D.O., 1983. Occurrence of white pine root decline in West Virginia. Phytopathology 73, 967-967.

Miller, D.R., Rabaglia, R.J., 2009. Ethanol and (-)-alpha-pinene: attractant kairomones for bark and ambrosia beetles in the southeastern U.S. Journal of Chemical Ecology 34, 435-448.

Molnar, A.C., 1965. Pathogenic fungi associated with a bark beetle on alpine fir. Canadian Journal of Botany 43, 563-570.

Morelet, M., 1988. Observations sur trois Deuteromycetes infeodes aux pins [Observations of three deuteromycetes from pines]. Annales de la Societe des Sciences Naturelles et d'Archeologie de Toulon et du Var 40, 41-45.

Moser, W.K., Treiman, T., Johnson, R., 2003. Species choice and the risk of disease and insect attack: evaluating two methods of choosing between longleaf and other pines. Forestry 76, 137-147.

Neidhardt, K.F.E., 1969. Ecology and behavior of two pine reproduction weevils, *Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) (Coleoptera: Curculionidae), MS Thesis, Auburn University, Auburn, AL.

Nevill, R.J., Alexander, S.A., 1992a. Distribution of *Hylobius pales* and *Pissodes nemorensis* (Coleoptera, Curculionidae) within Christmas tree plantations with procerum root disease. Environmental Entomology 21, 1077-1085.

Nevill, R.J., Alexander, S.A., 1992b. Root- and stem colonizing insects recovered from eastern white pines with procerum root disease. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 22, 1712-1716.

Nevill, R.J., Alexander, S.A., 1992c. Transmission of *Leptographium procerum* to eastern white pine by *Hylobius pales* and *Pissodes nemorensis* (Coleoptera, Curculionidae). Plant Disease 76, 307-310.

Nevill, R.J., Kelley, W.D., Hess, N.J., Perry, T.J., 1995. Pathogenicity to loblolly pine of fungi recovered from trees attacked by southern pine beetles. Southern Journal of Applied Forestry 19, 78-83.

Oak, S., Tainter, F., Williams, J., Starkey, D., 1996. Oak decline risk rating for the southeastern United States. Annales Des Sciences Forestieres 53, 721-730.

Otrosina, W.J., 2005. Exotic ecosystems: where root disease is not a beneficial component of temperate conifer forests. In: Lundquist, J.E., Hamelin, R.C. (Eds.), Forest Pathology: From Genes to Landscapes. American Phytopathological Society Press, St. Paul, MN, pp. 121-126.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant and Soil 217, 145-150.

Paine, T.D., Baker, F.A., 1993. Abiotic and biotic predisposition. In: Schowalter, T.D., Filip, G.M. (Eds.), Beetle-Pathogen Interactions in Conifer Forests. Academic Press, London, UK, pp. 61-80.

Paine, T.D., Raffa, K.F., Harrington, T.C., 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annual Review of Entomology 42, 179-206.

Piou, D., 1993. Possible role of *Hylobius abietis* (L) in the transport of *Leptographium procerum* (Kendr) Wingf and in the infection of Scots pine. Annales Des Sciences Forestieres 50, 297-308.

Pukkala, T., Moykkynen, T., Thor, M., Ronnberg, J., Stenlid, J., 2005. Modeling infection and spread of *Heterobasidion annosum* in even-aged Fennoscandian conifer stands. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 35, 74-84.

Raffa, K.F., Hall, D.J., 1988. Seasonal occurence of pine root collar weevil, *Hylobius radicis* (Coleoptera, Curculionidae), in red pine stands undergoing decline. Great Lakes Entomologist 21, 69-74.

Rane, K.K., Tattar, T.A., 1987. Pathogenicity of blue-stain fungi associated with *Dendroctonus terebrans*. Plant Disease 71, 879-883.

Reay, S.D., Thwaites, J.M., Farrell, R.L., 2005. A survey of *Ophiostoma* species vectored by *Hylastes ater* to pine seedlings in New Zealand. Forest Pathology 35, 105-113.

Reay, S.D., Walsh, P.J., Ram, A., Farrell, R.L., 2002. The invasion of *Pinus radiata* seedlings by sapstain fungi, following attack by the black pine bark beetle, *Hylastes ater* (Coleoptera : Scolytidae). Forest Ecology and Management 165, 47-56.

Robinson-Jeffrey, R.C., Grinchenko, A.H.H., 1964. A new fungus in the genus *Ceratocystis* occurring on blue-stained lodgepole pine attacked by bark beetles. Canadian Journal of Botany 42, 527-532.

Roth, E.R., Peacher, P.H., 1971. Alabama loblolly pine die-off evaluation. U.S.D.A. Forest Service, Southeastern Area, State & Private Forestry, Forest Pest Management Group, Report 72-2-9, Pineville, LA.

Ruehle, J.L., 1964. Plant parasitic nematodes associated with pine species in southern forests. Plant Disease Reporter 48, 60-61.

Rumbold, C.T., 1931. Two blue-staining fungi associated with bark-beetle infestation of pines. Journal of Agricultural Research 43, 847-873.

Sayer, M.A.S., Brissette, J.C., Barnett, J.P., 2005. Root growth and hydraulic conductivity of southern pine seedlings in response to soil temperature and water availability after planting. New Forests 30, 253-272.

Sayer, M.A.S., Haywood, J.D., 2006. Fine root production and carbohydrate concentrations of mature longleaf pine (*Pinus palustris* P. Mill.) as affected by season of prescribed fire and drought. Trees-Structure and Function 20, 165-175.

Schirp, A., Farrell, R.L., Kreber, B., 2003. Effects of New Zealand sapstaining fungi on structural integrity of unseasoned radiata pine. Holz Als Roh-Und Werkstoff 61, 369-376.

Seifert, K., 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. American Phytopathological Society, St. Paul, MN, pp 141-152.

Shaw, C.G., Dick, M., 1980. *Verticicladiella* root disease of *Pinus strobus* in New Zealand. Plant Disease 64, 96-98.

Siemaszko, W., 1939. Zespoly grzybów towarzyszacych kornikom polskim [Fungi associated with bark beetles in Poland]. Planta Polonica 7, 1-54.

Siggers, P.V., 1932. The brown-spot needle blight of longleaf pine seedlings. Journal of Forestry 30, 579-593.

Sinclair, W.A., Hudler, G.W., 1980. Tree and shrub pathogens new or noteworthy in New York state. Plant Disease 64, 590-592.

Six, D.L., Harrington, T.C., Steimel, J., McNew, D., Paine, T.D., 2003. Genetic relationships among *Leptographium terebrantis* and the mycangial fungi of three western *Dendroctonus* bark beetles. Mycologia 95, 781-792.

Smith, M.J., Patik, C.M., Rosinski, M.A., 1967. A comparison of cellulose production in the genus *Ceratocystis*. Mycologia 59, 965-969.

Spatafora, J.W., Blackwell, M., 1994. The polyphyletic origins of Ophiostomatoid fungi. Mycological Research 98, 1-9.

Thatcher, R.C., Searcy, J.L., Coster, J.E., Hertel, G.D., 1980. The Southern Pine Beetle. U.S.D.A. Forest Service, Science and Education Administration, Expanded Southern Pine Beetle Research and Applications Program.

Thomas, A.L., Gegout, J.C., Landmann, G., Dambrine, E., King, D., 2002. Relation between ecological conditions and fir decline in a sandstone region of the Vosges mountains (northeastern France). Annals of Forest Science 59, 265-273.

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C., McNew, D., 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. New Zealand Journal of Botany 43, 653-663.

Towers, B., 1977. Occurrence of *Verticicladiella procera* in Pennsylvania -1976. Plant Disease Reporter 61, 477-477.

Tribe, G.D., 1992. Colonization sites on *Pinus radiata* logs of the bark beetles, *Orthotomicus erosus*, *Hylastes angustatus* and *Hylurgus ligniperda* (Coleoptera, Scolytidae). Journal of the Entomological Society of Southern Africa 55, 77-84.

Upadhyay, H.P., 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, GA.

Varner, J.M., Hiers, J.K., Ottmar, R.D., Gordon, D.R., Putz, F.E., Wade, D.D., 2007. Overstory tree mortality resulting from reintroducing fire to long-unburned longleaf pine forests: the importance of duff moisture. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 37, 1349-1358.

Wagener, W.W., Mielke, J.L., 1961. A staining fungus root disease of ponderosa, jeffrey and pinyon pines. Plant Disease Reporter 45, 831-835.

Wahlenberg, W.G., 1946. Longleaf pine: its use, ecology, regeneration, protection, growth, and management. Charles Lathrop Pack Forestry Foundation in cooperation with the U.S.D.A. Forest service, Washington, D. C.

Wakeley, P.C., 1970. 30-year effects of uncontrolled brown spot on planted longleaf pine. Forest Science 16, 197-202.

Walkinshaw, C.H., 1978. Cell necrosis and fungus content in fusiform rust-infected loblolly, longleaf, and slash pine seedlings. Phytopathology 68, 1705-1710.

Weaver, M.J., Stipes, R.J., Amos, D.F., Heikkenen, H.J., 1981. Factors associated with declining eastern white pine (*Pinus strobus*) on Virginia landscapes. Phytopathology 71, 772-772.

Williams, R.A., Tainter, F.H., 1971. Pine decline in southern Arkansas. Plant Disease Reporter 55, 1036-1036.

Wingfield, M.J., 1983a. Association of *Verticicladiella procera* and *Leptographium terebrantis* with insects in the Lake States. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 13, 1238-1245.

Wingfield, M.J., 1983b. Pathogenicity of *Verticicladiella procera* and *Leptographium terebrantis* in greenhouse and field inoculations. Phytopathology 73, 838-839.

Wingfield, M.J., 1985. Reclassification of *Verticicladiella* based on conidial development. Transactions of the British Mycological Society 85, 81-93.

Wingfield, M.J., 1986. Pathogenicity of *Leptographium procerum* and *Leptographium terebrantis* on *Pinus strobus* seedlings and established trees. European Journal of Forest Pathology 16, 299-308.

Wingfield, M.J., 1993. *Leptographium* species as anamorphs of *Ophiostoma*: progress in establishing acceptable generic and species concepts. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. American Phytopathological Society Press, St. Paul, MN, pp. 43-51.

Wingfield, M.J., Gibbs, J.N., 1991. *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. Mycological Research 95, 1257-1260.

Wingfield, M.J., Knox-Davies, P.S., 1980. Root disease, associated with *Verticicladiella alacris*, of pines in South Africa. Plant Disease 64, 569-571.

Wingfield, M.J., Marasas, W.F.O., 1980. *Verticicladiella alacris* sp. nov. associated with a root disease of pines in South Africa. Transactions of the British Mycological Society 75, 21-28.

Wingfield, M.J., Seifert, K.A., Webber, J.F., 1993. *Ceratocystis* and *Ophiostoma* : taxonomy, ecology, and pathogenicity. American Phytopathological Society, St.Paul, MN.

Witcosky, J.J., Hansen, E.M., 1985. Root-colonizing insects recovered from Douglas-fir in various stages of decline due to black-stain root disease. Phytopathology 75, 399-402.

Witcosky, J.J., Schowalter, T.D., Hansen, E.M., 1986. *Hylastes nigrinus* (Coleoptera, Scolytidae), *Pissodes fasciatus*, and *Steremnius carinatus* (Coleoptera, Curculionidae) as vectors of black-stain root disease of Douglas-fir. Environmental Entomology 15, 1090-1095.

Wood, S.L., 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. Great Basin Naturalist Memoirs 6, 1-1359.

Zak, B., Campbell, W.A., 1958. Susceptibility of southern pines and other species to the littleleaf pathogen in liquid culture. Forest Science 4, 156-161.

Zambino, P.J., Harrington, T.C., 1992. Correspondence of isozyme characterization with morphology in the asexual genus *Leptographium* and taxonomic implications. Mycologia 84, 12-25.

Zarnoch, S.J., Ruehle, J.L., Belanger, R.P., Marx, D.H., Bryan, W.C., 1994. Growth and crown vigor of 25 year old shortleaf pine progenies on a littleleaf disease site. USDA Forest Service Southeastern Forest Experiment Station Research Paper, 1-12.

Zhou, X.D., De Beer, W., Wingfield, B.D., Wingfield, M.J., 2002. Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. Fungal Diversity 10, 229-240.

Zhou, X.D., de Beer, Z.W., Ahumada, R., Wingfield, B.D., Wingfield, M.J., 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Diversity 15, 261-274.

Zhou, X.D., de Beer, Z.W., Wingfield, B.D., Wingfield, M.J., 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. Sydowia 53, 290-300.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Studies in Mycology 55, 75-97.

CHAPTER II APPLICATION OF THE LOBLOLLY PINE DECLINE RISK MODEL TO LONGLEAF PINE STANDS AT FORT BENNING, GA.

2.1. Abstract

Longleaf pine decline (LLPD), like other tree declines, has interfered with management goals of this species. Decline symptoms typically include a reduction in height and radial growth, an increase in foliar transparency, and decrease in foliar density which precede premature mortality. A loblolly pine decline risk model (LPDRM) was applied to longleaf pine (*Pinus palustris*) stands at Fort Benning, Georgia. Longleaf pine data collected included tree heights and diameters, age, crown condition, increment growth, standing resin reserves, and root infection. Stand factors measured included slope, aspect, elevation, land form, and stand basal area. In loblolly pine decline and other pine declines, fungi of the form-genus *Leptographium* and their beetle vectors have been identified as contributing agents, and prior studies suggest that these agents play an important role in LLPD, thus fungal isolations were made from longleaf pine roots and from captured bark beetles and regeneration weevils. *Leptographium procerum, L. terebrantis,* and other ophiostomatoid fungi were recovered from longleaf pine roots. Infected longleaf pine trees rarely displayed crown symptoms during the observation

period. *Hylastes tenuis* was the most abundant root-feeding *Leptographium* vector recovered overall, although the most frequently recovered fungus, *Grosmannia huntii* was only isolated from roots of a single tree during this study. In most age classes, predicted asymptomatic and predicted symptomatic plots did not significantly differ in crown density, foliar transparency, dieback, or standing resin reserves. Trees in the predicted symptomatic class did not differ significantly in height, radial or increment growth from trees in the predicted asymptomatic class, though some exceptions were observed in the 20-40 year age class.

These data suggest that the slope and aspect do not predict symptom development consistent with LLPD in most stands of longleaf pine. Trees of all age classes may harbor *Leptographium* spp. and other ophiostomatoid fungi. *Grosmannia huntii*, previously unreported from the area, was isolated from a single symptomatic individual, which subsequently died. This fungus may potentially be an important contributing factor in longleaf pine decline.

2.2. Introduction

Forest decline refers to a reduction in growth that may lead to premature death in trees (Manion, 1981). Typical decline symptoms include a reduction in annual height and radial growth, thinning or wilting of leaves or needles, and premature death when compared with either asymptomatic or historical stands. Declines have been recorded in several hardwood and conifer tree species, (Oak *et al.*, 1996; Filip and Rosso, 1999), and are attributed to factors that predispose (permanent factors), incite (shorter term

stressors) and contribute (opportunistic biotic agents) to tree decline (Manion, 1981). Ecological modeling has frequently been used to identify predisposing and inciting factors in forest declines (Baccala *et al.*, 1998; Thomas *et al.*, 2002).

Pine decline syndromes have been reported in several species including eastern white pine (*Pinus strobus* L.) (Dochinger, 1967), red pine (*P. resinosa* Aiton) (Klepzig *et al.*, 1991), and western white pine (*P. monticola* Douglas ex D. Don) (Leaphart and Johnson, 1973), and the role of *Leptographium* spp. Lagerb.& Melin (teleomorph= *Grosmannia* Goid.) (Weaver *et al.*, 1981; Wingfield, 1986; Klepzig *et al.*, 1991; Eckhardt *et al.*, 2004b), and their beetle vectors explored (Nevill and Alexander, 1992; Klepzig *et al.*, 1995; Eckhardt *et al.*, 2004a).

In the southeastern U.S., decline has been observed in several pine species, including shortleaf, loblolly, and longleaf pines (*P. echinata* Mill., *P. taeda* L., and *P. palustris* Mill., respectively). Shortleaf pine is affected by littleleaf disease, which results in decline-like symptoms (Campbell and Copeland, 1954). Littleleaf disease also affects loblolly pine, although typically only in stands where the two species occur together. In littleleaf disease, clayey soils and areas adjacent to roads were demonstrated to be important predisposing and inciting factors. *Phytophthora cinnamomi* Rand was investigated as a contributing factor (Campbell and Copeland 1954).

Although the symptoms are similar (Eckhardt *et al.*, 2007), loblolly pine decline occurs in areas that have no shortleaf pine and under different stand and soil conditions than littleleaf disease (Eckhardt and Menard, 2008). In a study of loblolly pine decline, declining stands were found more frequently on sites with slopes greater than 5% and

with south-southwestern aspects, suggesting that drought-prone sites are at increased risk for decline (Eckhardt and Menard, 2008). A risk model was developed using these topographic factors from data collected in four physiographic regions in central Alabama (Eckhardt and Menard, 2008) and has been successfully applied to loblolly pine at Fort Benning Military Reservation (FB) in western Georgia (Menard, 2007), as well as loblolly pine stands in Louisiana, Mississippi and Texas (Eckhardt and Menard, unpublished data). In these studies, *L. procerum* (Kendrick) Wingfield, *L. terebrantis* Barras & Perry, and *L. serpens* (Goid.) Siemaszko were recovered from roots in symptomatic loblolly pine stands more frequently than asymptomatic loblolly pine stands. Root-feeding beetles including *Dendroctonus terebrans* (Olivier), *Hylastes salebrosus* Eichhoff, *H. tenuis* Eichhoff, *Hylobius pales* (Herbst), and *Pachylobius picivorus* (Germar) were more abundant on symptomatic sites, and were found to be vectoring these fungi (Eckhardt *et al.*, 2007). Unlike the southern pine beetle (*D. frontalis* Zimmermann), which is capable of mass-attacking and killing healthy trees, these other curculionids are considered secondary (Paine *et al.*, 1997) and are typically found in roots of either stressed trees or stumps (Blackman, 1941). In addition to predisposing factors (e.g. host genetics, or planting "off-site"), many potential inciting factors may stress trees and allow attack by secondary species, including overmaturity, drought, fire, or increased silvicultural activities (Manion, 1981).

Longleaf pine, once the dominant pine species in the southeastern U.S., is considered to be more tolerant of insect and disease threats than its sympatric congeners (Wahlenberg, 1946; Hepting, 1971). Longleaf pine's robustness and high quality wood

make it an important tree species for landowners (Boyer, 1990) and, as area where mature longleaf pine is the dominant species diminish, its value as habitat for endangered species such as the red cockaded woodpecker (*Picoides borealis* [Viellot]) increases (Conner and O'Halloran, 1987).

Decline has also been observed in longleaf pine. In an investigation at the Savannah River Site in South Carolina, foliar symptoms were the first indication of decline (Otrosina *et al.*, 1999). As the disease symptoms progressed in the 35 year old stand, trees exhibited thin crowns, needle discoloration, needle loss, stress cone crops, then tree death (Otrosina *et al.*, 1999). In declining trees, *L. procerum*, *L. terebrantis* and *Heterobasidion annosum* (Fr.) Bref. were recovered. At that time the authors suggest the role of these fungi in LLPD requires further study (Otrosina *et al.*, 1999).

The objective of this study is to test the loblolly pine decline risk model (LPDRM) and its ability to predict LLPD sites at Fort Benning (FB), Georgia. It is hypothesized that trees in predicted symptomatic plots, when compared with predicted asymptomatic plots, will have decreased growth, foliar density, standing resin reserves, and increased foliar transparency, dieback, captures of vector insects, and isolation of ophiostomatoid fungi from roots and insects. Additional objectives are to examine the abundance and distribution of ophiostomatoid fungi in the roots of longleaf pine across different age classes and to compare the abundance and distribution of the root-feeding beetles that vector these fungi by their risk classes and age classes.

2.3. Methods and Materials

2.3.1 *Plot establishment*

Plot locations were chosen using the LPDRM as described by Eckhardt (2003) and Eckhardt and Menard (2008). In the LPDRM, slope and aspect are correlated with decline risk. For slope, 0-5% indicates minimal risk, 5-10% indicates low risk, 11-15% indicates moderate risk, >15% indicates high risk. These classes were coded as 1-4, for minimal to high risk for subsequent analyses. Aspect of slope was also correlated with decline risk, with risk categories designated as minimal (337.5° to 67.5°), low (67.6°-112.5° and 292.6°-337.4°), moderate (247.6°-292.5°) and high (112.6°-247.5°) (Eckhardt 2003; Eckhardt and Menard 2008). Slope and aspect were previously used to develop a geographic information system (GIS) with four risk classes at FB (Menard, 2007).

For the current study, the four decline classes of the LPDRM (high risk, moderate risk, low risk, and minimal risk) were reclassified into two decline classes using ArcMap 9.2 (ESRI 1996), with high and moderate risk sites combined into predicted symptomatic (Decline, or "D" sites), and low and minimal risk sites combined into predicted asymptomatic (Healthy or "H" sites) (Figure 2.1). To explore the relationship between stand age and decline symptomatology, four age classes were also delineated at the start of data collection; <10 years, 10-19 years, 20-40 years, and >40 years. Four replicates of each combination of stand class (D and H, n=2) and age class (n=4) were installed, for a total of 32 plots.

Potential plot locations were identified by querying the Fort Benning Forest Inventory GIS layer for longleaf pine stands within the appropriate age classes.

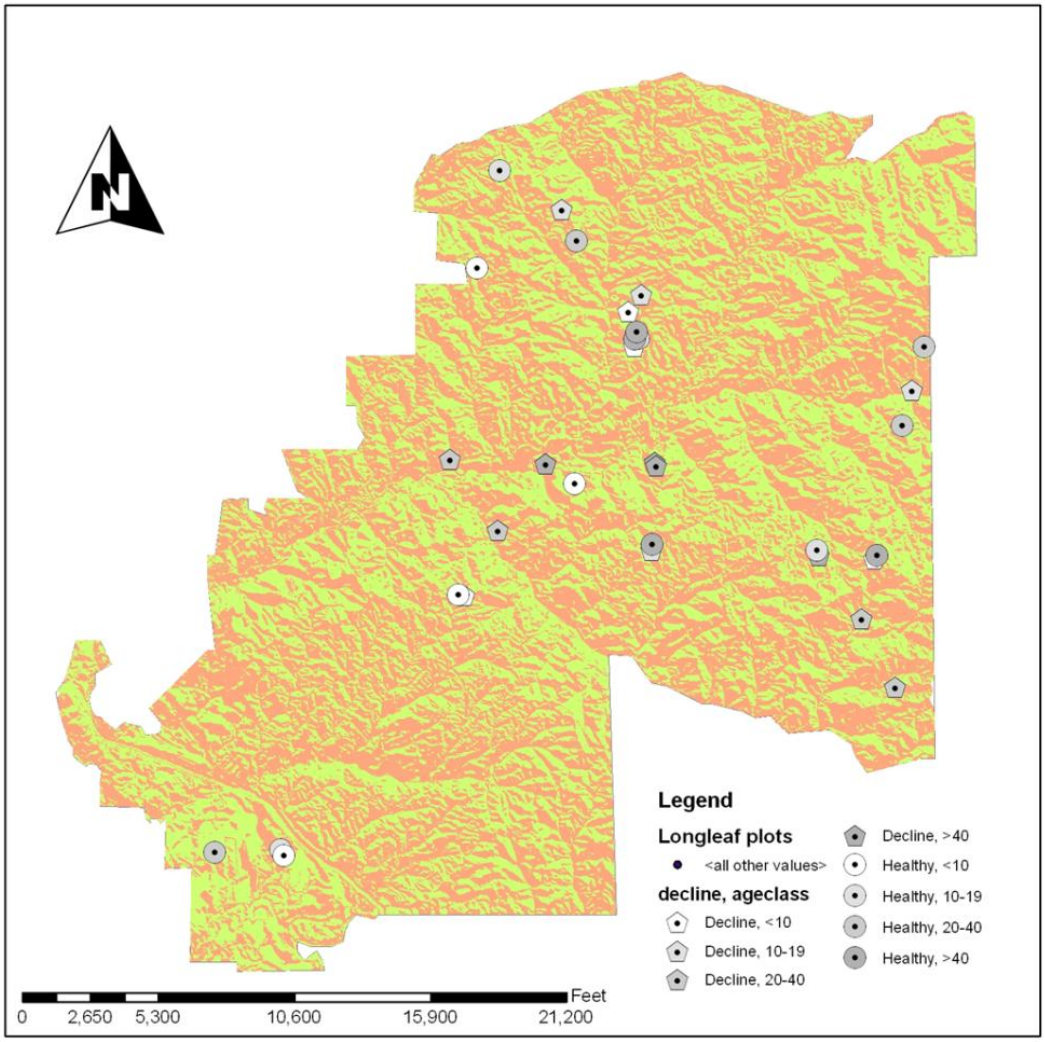

Figure 2.1. Loblolly pine decline risk model as applied at Fort Benning, GA with longleaf pine decline plots.

Approximately 60 potential plot locations were identified and these sites were located using a Garmin GPS V receiver (Garmin, Olathe, KS) connected to a laptop computer running ArcGIS 3.2a (ESRI, 1996), with the DNR Garmin extension version 5.1.1 (Minnesota Department of Natural Resources, 2005), for real time location. Potential plots were then locally assessed for slope and aspect, and plot centers installed. Selected plots were marked with a stake and the central point mapped with a Garmin GPS V receiver, with no fewer than 100 waypoint observations collected for averaging.

The plot layout was similar to the Forest Service's Forest Inventory and Analysis plots (USDA Forest Service 1998), with a central 7.62 m (25-foot) radius circle, and three similar satellite subplots at 120°, 240°, and 360° (0°) azimuth from the central focus, and with foci 36.6 m (120 feet) distant from the central focus (Figure 2.2). Thus each plot described four circular subplots with a total area of 0.073 ha (0.18 acres). Destructive sampling (root excavation and resin sampling, see below) was restricted to the central subplot.

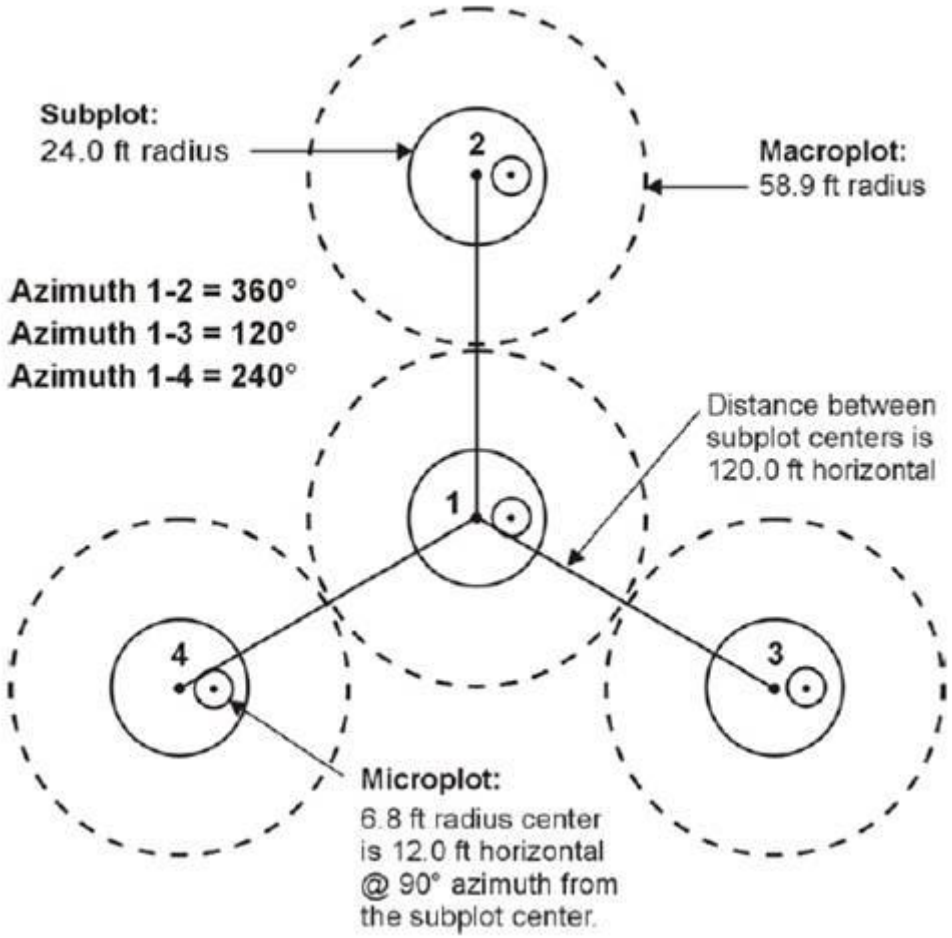

Figure 2.2. Plot layout (after USDA Forest Service, 1998).

2.3.2. *Assessment of host symptomatology and site factors*

All trees > 12.7 cm (5.0 in) diameter at breast height (DBH ,137 cm, 4.5 ft) in the central and subplots were tallied and DBH measured. Tree heights were measured using an electronic clinometer (Häglof Inc, Madison, MS). Increment cores were collected from the three excavated trees in the central plots at breast height to estimate tree age. Annual growth rings were counted, and seven added to estimate age growth from germination to 1.37 m. Age in years (a continuous variable) was used in addition to age class (a categorical variable) in several subsequent analyses to test for trends that may be associated with age and to account for the variability in plots over 40 years old.
Five and ten year growth increment was determined by measuring the outermost annual growth rings with a digital caliper. Increment growth parameters (5/10 year growth increment ratio, 5 year growth/annum, 10 year growth/annum) were then compared to decline indicators (*Leptographium* infection, *Leptographium* Incidence Rating, see p. 77), other growth parameters (height and DBH) and site parameters using PROC CORR and PROC STEPWISE in SAS 9.1 (SAS Institute, 2003).

All pine trees in the four subplots of each plot were crown-rated according to the protocol of the USDA Forest Service Forest Inventory and Analysis (USDA Forest Service, 1998) by observers certified to meet Forest Service minimum quality objectives. Six crown parameters were assessed; live crown ratio, crown density, crown light, crown position, crown dieback and foliar transparency. Live crown ratio is the proportion of the main stem with live foliage. Crown density is the proportion of live crown silhouette with foliage. Foliar transparency is an estimate of the proportion of light blocked by the

foliated limbs; higher foliar transparency indicates more light transmitted through the foliage. Crown light is the number of vertices (out of five) receiving light from above. Crown position is a coded categorical variable which serves as a surrogate for dominant, co-dominant, and understory. A crown position value of 1 is assigned to superstory trees, 2 to overstory, and 3 to understory. Crown dieback is the proportion of the foliated branches with recent (current year's) loss of foliage. For each tree, each parameter is assessed by two observers, on level grade or uphill, examining the tree from orthogonal vantage points from a distance between 0.5 to 1.0 tree length. Tree damage and severity was also determined for disease and injury, including but not limited to; cankers, galls, parasitic plants, large wounds (>30% of diameter), broken tops and loss of apical dominance.

Crown rating was completed in all plots in summer 2006 and repeated in summer 2007. Crown rating data were compared with decline class and other variables using PROC CORR. Changes in crown parameters for indexed trees between years were compared using PROC GLM in SAS 9.1 (SAS Institute, 2003). Mean separation for ANOVA was achieved using the Tukey-Kramer method.

Stand basal area (total BA and pine BA) was estimated with a 10 basal area factor prism, and also calculated by pooling diameters of similar species (longleaf, loblolly, shortleaf, and pooled hardwood spp.) for each plot. Other site factors for each plot were also noted, including; landform (concave, convex, or flat) and order of the nearest road (paved road, major unpaved road, tank trails).

2.3.3. *Resin sampling to assess host defense response*

To compare standing resin reserves, a subset of trees (30 trees/age class/stand category) from the central subplots in each plot (excluding trees <10 years) were tapped for 24 h (± 1.5 h). Resin was sampled on the south face of each tree. The bark was shaved before tapping through the cambium approximately 1.37 m above ground with a 1.9 cm (¾") dia arch punch (No. 149, C.S. Osborne & Co., Harrison, NJ). A tared 15 ml centrifuge tube with a spout was then attached to the tree to collect resin (Figure 2.3). After resin collection, the tubes were capped and stored at 4 °C until weighed. Resin weights were compared using PROC GLM to compare the main effects of age class and decline class, plot, and interactions between these effects in SAS 9.1 (SAS Institute, 2003). Resin weight was also compared with age using PROC GLM in SAS (SAS Institute, 2003). Means were separated using the Tukey-Kramer method.

2.3.4. *Infection of roots and soil by* Leptographium *spp.*

Lateral roots of three dominant or codominant longleaf pine trees in the central subplot were sampled by manual excavation. For each tree, two roots were excavated outward from the root collar to the drip line using a protocol modified from Otrosina *et al.*, (1999) and Eckhardt *et al.* (2007). Excavated roots were sampled approximately every 30 cm (1 ft) from the base of the tree to drip line, and the sampled segments kept at 4 °C until processing at Auburn University School of Forestry and Wildlife Sciences (AUSFWS). The root segments were washed in tap water and then cut into pieces

Figure 2.3. Resin collection apparatus, in situ.

approximately 6 mm × 6 mm (¼ ” × ¼ ”) and surface sterilized in a solution of 80% ethanol, 10% bleach (active sodium hypochlorite 0.3%), and 10% distilled water (v:v:v). The surface sterilized pieces were then plated on 1% malt-extract agar (MEA) and 1% MEA amended with cycloheximide (800 mg/L) and streptomycin (200 mg/L) (CSMA) to foster the growth of ophiostomatoid fungi. *Leptographium* spp. and other ophiostomatoid fungi are cycloheximide-tolerant (Harrington, 1981), though unamended MEA was included to allow for rare intolerant species of interest to be observed and isolated.

Four root segments were plated on 20 plates of CSMA and MEA, for a total of 160 segments per tree. Plates were incubated under fluorescent lights at 22 °C. After one week and periodically thereafter, plates were examined under a dissecting microscope for the presence of fungal taxa of interest for six weeks. Ophiostomatoid fungi growing on the media or root segments were transferred to CSMA or MEA amended with streptomycin only (SMEA) and serially transferred until axenic cultures were obtained. Isolates were then transferred to MEA for identification and archiving on agar slants. The number of trees per plot yielding *Leptographium* spp. (out of the three) provided the *Leptographium* Incidence Rating (LIR), corresponding to no infection (0), low (1), moderate (2), or high infection (3) at the plot level (Eckhardt, 2003). One half (16) of the plots were excavated summer 2006, and the remainder during summer 2007.

At the time of root excavation, soil samples were taken every 30 cm (1 ft) from the area adjacent to the root with a soil auger, with 6 additional samples collected at the distal end of the excavated root (Lewis *et al.*, 1987). Soil samples were stored at 4 °C

until processing at AUSFWS. The soil samples were thoroughly mixed before a representative 10 g subsample was suspended in 40 mL of sterile 0.5% water agar, and 1 mL aliquoted onto 10 plates of CSMA and MEA (adapted from Johnson and Curl, 1972) to test for the presence of ophiostomatoid fungi.

Relationships among age class, age in years, decline class, slope class, aspect class, elevation, and root infection were analyzed using the Pearson Correlation Coefficients (PROC CORR) and logistic regression (PROC LOGIST) in SAS (SAS Institute, 2003). Distribution of fungi by decline and age classes was tested using Fisher's exact test (PROC FREQ) in SAS (SAS Institute, 2003).

In addition to LPDRM testing, the effects of *Leptographium* root infections on growth and crown parameters were tested. For each fungal taxon, infection (presence=1) was tested for effects on the change in growth parameters between 2006 and 2007 (height and DBH), and for effects on crown parameters (live crown ratio, crown density, crown dieback, and foliar transparency) using the 2007 crown rating data. Comparisons were made by ANOVA using PROC GLM in SAS (SAS Institute, 2003).

2.3.5. *Incidence of pinophagous beetle activity and infestation with ophiostomatoid fungi.*

Insect pitfall traps were placed in each subplot (3/plot) to collect beetle vectors. The traps (adapted from Klepzig *et al.*, 1991) were constructed of sections of PVC tubing 4" (10 cm) dia and 8" (20 cm) long and capped at one end (Figure 2.4). Eight radial holes were drilled to allow insects to enter and fall into a cup which sits inside the

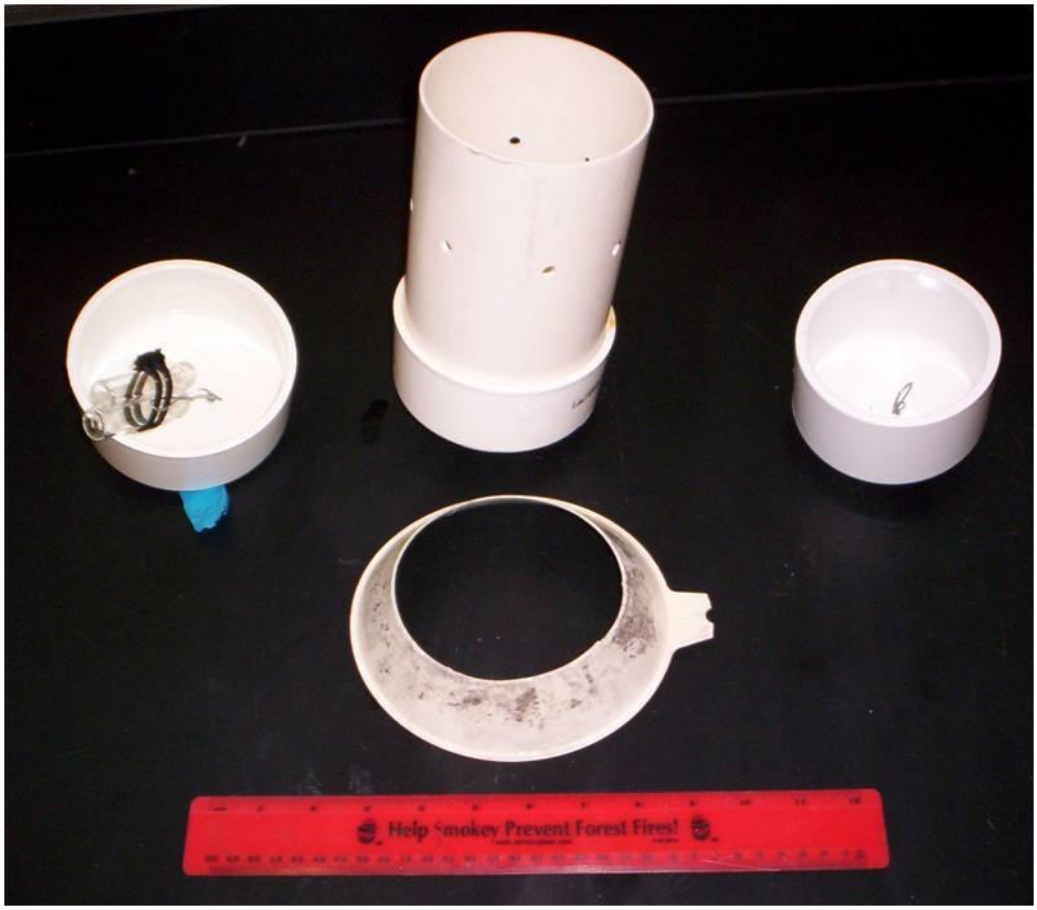

Figure 2.4. Pitfall trap used to collect root-feeding beetles. Modified from Klepzig *et al.*, (1991) by Menard (2007). Clockwise from left: lid containing 2 dram vials for baits, trap body, removable cup, plastic skirt to limit flooding of trap.

trap and a plastic skirt was fitted on the trap to reduce the risk of flooding (Menard, 2007). Steam-distilled southern pine turpentine (Hercules, Wilmington, DE) and 95% ethanol (in two 8 mL vials) were used as an insect attractant. Three sections of longleaf pine twig, approximately 5 cm long × 2 cm dia (2"×5/8") were also placed in the cup as substrate for the captured insects and as additional bait.

Insect trap collection was performed weekly from 4 March to 5 May 2006 (10 collections) and from 24 August 2006 to 26 August 2007 (52 collections). Thus, data were collected for two springs (2006 and 2007), and one continuous year (2006-2007). During each trap collection, the contents of the pitfall trap were transferred to a clean specimen cup and stored at 4 °C for analysis at AUSFWS. The trap cup was cleaned with 70% ethanol and swabbed with liquid Teflon™ (Northern Products, Woonsocket RI) to deter insects from crawling out of the trap. Fresh twig sections were placed in the trap cup, and the ethanol and turpentine vials evacuated and replaced. At AUSFWS, insects were sorted from each specimen cup, identified to species and tallied. Each curculionid was then rolled on one plate of CSMA and one plate of MEA in a laminar flow hood to collect fungal propagules (Figure 2.5). Ophiostomatoid fungi were then serially transferred to CSMA until axeny, at which point the fungi were transferred to MEA for archiving and species determination. Insect numbers were pooled at the plot and species levels over the course of the sampling period and effects of tree age class and stand decline class on insect populations tested using PROC GLM in SAS 9.1 (SAS Institute, 2003). For ANOVA analyses, means were separated using the Tukey-Kramer procedure.

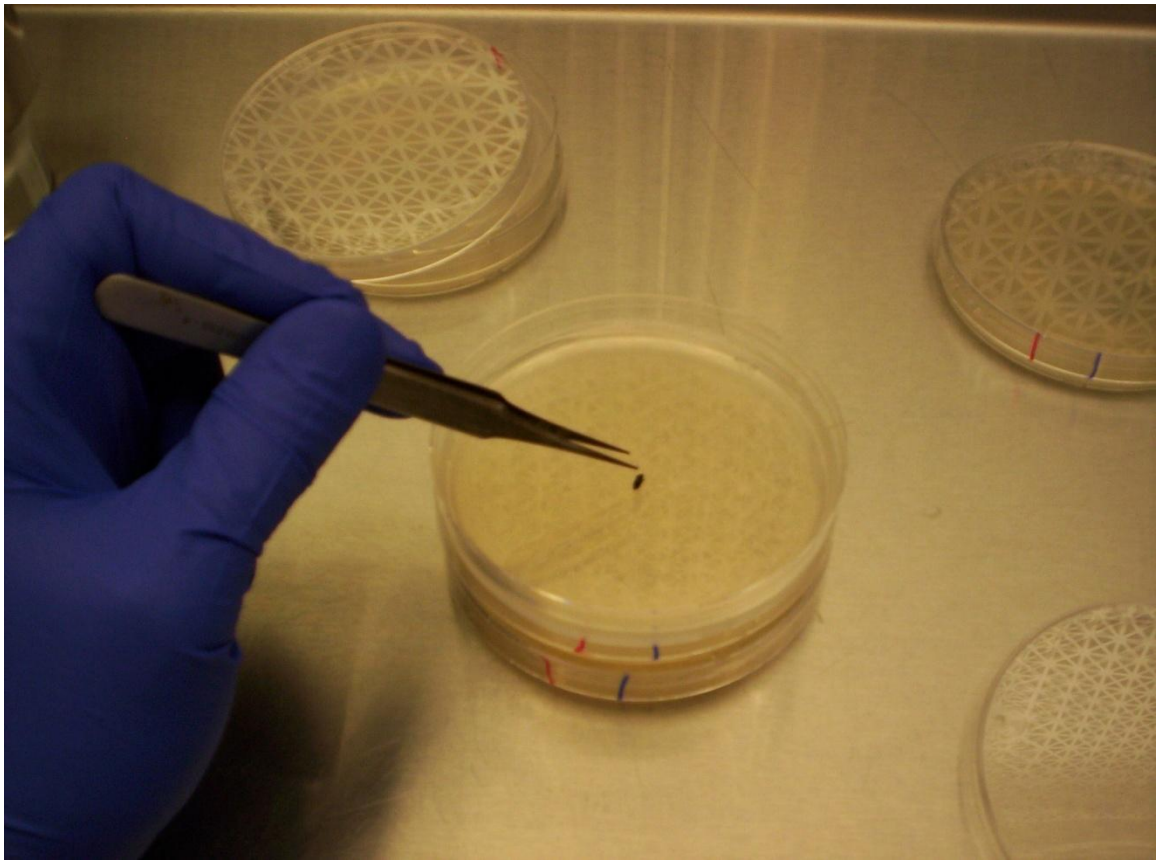

Figure 2.5. Insect rolling to collect fungal propagules.

2.3.6. *Application of the LPDRM to longleaf pine decline observations*

In the LPDRM, predicted symptomatic plots are expected to have (relative to predicted asymptomatic plots); increased foliar transparency, decreased crown density, decreased resin mass, lower increment, height and radial growth, an increased incidence in *Leptographium* spp. in the roots, and an increased number of insect vector captures. Comparisons were made using PROC CORR in SAS (SAS Institute, 2003) using both the assigned decline classes (H and D), as well as the slope and aspect classes from the LPDRM (see Section 2.3.1). Stepwise logistic regression (PROC LOGISTIC) was used to identify significant predictors of *Leptographium* infection, including decline class (H or D), slope class (1-4), aspect class (1-4), age class, and nearest road order.

2.4. Results

2.4.1. *Plot establishment*

Thirty-two plots were installed at FB, 29 in Georgia, and 3 in adjacent Alabama (Table 1.1). The youngest plot was planted to longleaf pine in 2000, and the oldest plot dates to 1898. Percent slope ranged from 0% to 22%. Elevation ranged from 72.5 to 194.5 m (239 to 638 feet) above sea level. Total basal area ranged from 0 to 22.96 m^2ha^{-1}.

Table 2.1. Longleaf pine decline study plot locations at Fort Benning, GA.

Plot *	Latitude	Longitude	Total Basal Area (m^2/ha)	Pine Basal Area (m^2/ha)	Age Class^	Land form#	Aspect (Degrees)	Slope (%)	Elev (m)
D1	32.3707	-84.8519	6.89	6.89	1	Cc	79	4.3	139
D2	32.4185	-84.8564	22.96	20.66	2	Cc	130	5.3	99
D3	32.4164	-84.817	4.59	0‡	1	Cc	341	1.4	81
D4	32.7520	-84.7759	16.07	16.07	4	Cc	354	12.0	128
D5	32.4692	-84.7814	13.78	13.78	2	Cc	156	21.4	128
D6	32.4570	-84.7795	9.18	9.18	2	Cc	163	10.3	131
D7	32.3934	-84.8373	16.07	16.07	4	Cv	339	6.2	123
D8	32.3595	-84.6875	11.48	11.48	4	Cc	116	19.4	149
D9	32.3353	-84.6743	22.96	22.96	3	Cv	207	21.5	177
D10	32.4396	-84.6648	18.37	18.37	3	Cv	264	17.9	116
D11	32.3805	-84.6818	0	0	2	Cc	233	11.3	191
D12	32.3821	-84.7047	2.30	2.30	1	Cc	132	15.8	159
D13	32.5055	-84.8081	9.18	9.18	1	Fl	75	6.0	146
D14	32.3852	-84.7739	18.37	16.07	3	Cv	18	22.0	162
D15	32.4159	-84.7719	4.59	4.59	3	Cc	239	8.3	137
D16	32.4149	-84.7712	16.07	16.07	3	Cc	265	9.9	135
H1	32.3709	-84.8542	16.07	16.07	1	Cv	316	1.1	143
H2	32.4090	-84.8052	0	0	1	Cc	116	0.6	129
H3	32.4595	-84.779	6.89	6.89	2	Cc	345	5.8	142
H4	32.4619	-84.7782	16.07	16.07	2	Cc	20	9.1	133
H5	32.4944	-84.8023	4.59	4.59	1	Cc	123	2.6	132
H6	32.4545	-84.6594	16.07	16.07	3	Cc	150	3.9	156
H7	32.4272	-84.6692	11.48	11.48	4	Cc	142	1.6	152
H8	32.8178	-84.6806	2.30	0	2	Cc	313	4.3	194
H9	32.3839	-84.7054	11.48	2.30	1	Cc	18	8.4	169
H10	32.2914	-84.5062	18.37	18.37	4	Cc	26	4.0	149
H11	32.5196	-84.3345	11.48	11.48	4	Cc	47	6.8	156
H12	32.2826	-84.9298	18.37	18.37	3	Cc	259	1.1	80
H13	32.2805	-84.9285	13.78	13.78	3	Cc	83	1.6	73
H14	32.2821	-84.957	18.37	18.37	3	Cc	133	2.3	86
H15	32.3872	-84.7737	13.78	13.78	4	Fl	26	2.0	150
H16	32.4598	-85.7774	9.18	9.18	2	Cv	356	7.1	149

*Plots beginning with D are predicted symptomatic, plots beginning with H are predicted asymptomatic. ‡ Plots with 0 pine basal area contained only juvenile longleaf pine. ^- Ages classes: 1= <10 Years, 2= 10-19 Years, 3=20-40 Years, 4= >40 Years. #-Landform: Cc = Concave, Cv = Convex, Fl = Flat.

2.4.2. *Assessment of host symptomatology and site factors*

Mean values for growth measurements (DBH, height, changes in these values between years, and five- and ten-year increment growth) for each decline class and age class are presented in Table 2.2. Assessed trees ranged from 4.57-56.64 cm (1.8-22.3 in) DBH, and from 3.08-32.92 m (10.1 -108.0 ft) in height. Trees smaller than 12.7 cm (5.0 inches) diameter were selected for excavation on plots <10 years old, but were crown rated as saplings, such that crown density, foliar transparency, and dieback were not assessed for these trees. From all 32 plots, 294 longleaf pine trees (DBH ≥12.7 cm/ 5.0 in) and 46 longleaf saplings (DBH < 12.7 cm /5.0 in) were assessed. Seventeen shortleaf pine and 66 loblolly pine were also assessed. Among hardwood species (n=53 trees), the majority were oaks (*Quercus* spp. L.), including blackjack oak (*Q. marilandica* Münchh.), bluejack oak (*Q. incana* Bartram), turkey oak (*Q. laevis* Walter), water oak (*Q. nigra* L.), and willow oak (*Q. phellos* L.). Other hardwood species included sweetgum (*Liquidambar styraciflua* L.), hickory (*Carya* spp. Nuttall), flowering dogwood (*Cornus florida* L.), and cherry (*Prunus* sp. L.).

Height growth (difference in height between 2006 and 2007) was negatively correlated with age ($F_{1,244}$=9.38, p=0.0024) and age class ($F_{1,244}$ =10.60, p=0.0013), but was not correlated with stand decline class. Diameter growth was negatively correlated with decline class ($F_{3,250}$= 5.28, p=0.0015) indicating that trees in predicted decline stands experienced a greater increase in diameter than trees in the predicted healthy stands.

Table 2.2. Growth parameters means (SE) for comparisons between decline classes age classes, and change between years (2006 and 2007). Predicted symptomatic plots are designated "D", and predicted asymptomatic "H". Mean were separated by the Tukey-Kramer method. Cells bearing the same letter within a row are not significantly different at $p<0.05$.

	<10 Years		10-19 Years		20-40 Years		>40 Years		F(df)	p
	H	D	H	D	H	D	H	D		
DBH (cm), 2006	8.19 (1.30) ^{A}	8.64 (1.42) ^{A}	12.04 (0.81) ^{A}	12.29 (0.84) ^{A}	29.74 (0.99) ^{B}	22.23 (0.89) ^{C}	39.52 (0.91) ^{D}	37.34 (1.02) ^{D}	164.42 (7,246)	<0.0001
DBH (cm), 2007	8.71 (1.30) ^{A}	9.73 (1.42) ^{A}	12.80 (0.81) ^{A}	12.88 (0.84) ^{A}	30.35 (0.99) ^{B}	22.89 (0.89) ^{C}	39.98 (0.91) ^{D}	38.48 (0.91) ^{D}	172.10 (7,258)	<0.0001
Change in DBH	0.53 (0.25) ^{A}	1.09 (0.25) ^{A}	0.71 (0.15) ^{A}	0.58 (0.15) ^{AB}	0.64 (0.18) ^{A}	0.61 (0.18) ^{A}	0.48 (0.18) ^{A}	-0.18 (0.20) ^{B}	2.92 (7,246)	0.0059
Height (m), 2006	6.19 (1.89) ^{A}	6.19 (1.89) ^{A}	9.17 (2.80) ^{B}	10.30 (3.14) ^{B}	20.51 (6.25) ^{C}	16.49 (5.03) ^{D}	22.37 (6.83) ^{CE}	23.44 (7.13) ^{E}	167.35 (7,242)	<0.0001
Height (m), 2007	6.92 (0.67) ^{A}	6.86 (0.73) ^{A}	9.30 (0.43) ^{B}	10.49 (0.43) ^{B}	20.45 (0.52) ^{C}	16.12 (0.46) ^{D}	21.73 (0.49) ^{CE}	23.59 (0.49) ^{E}	173.16 (7,254)	<0.0001
Change in Height	0.71 (0.31) ^{A}	0.11 (0.34) ^{A}	0.08 (0.2) ^{A}	-0.33 (0.23) ^{A}	0.67 (0.34) ^{A}	0.18 (0.20) ^{A}	-0.35 (0.23) ^{B}	-0.04 (0.26) ^{AB}	2.1 (7,238)	0.044
Five Year IG*(mm)	16.7 (1.4) ^{A}	16.4 (1.5) ^{A}	15.5 (1.2) ^{AB}	13.8 (1.5) ^{AB}	11.2 (1.2) ^{BC}	8.8 (1.4)^{BC}	6.3 (1.4) ^{C}	7.3 (1.4) ^{C}	9.41 (7,68)	<0.0001
Ten Year IG(mm)	NA	NA	34.4 (2.6) ^{A}	33.9 (3.0) ^{A}	26.2 (2.1) ^{A}	16.7 (2.4) ^{B}	11.3 (2.4) ^{B}	15.9 (2.4) ^{B}	14.12 (6,48)	<0.0001

* Increment Growth

Mean values for crown rating parameters are presented in Table 2.3. Live crown ratio decreased with age, and was lower in predicted decline plots in the 10-19 and >40 age classes, and was negatively correlated with several age-related parameters: age in years (ρ= -0.0958, p=0.01), age class (ρ= -0.118, p=0.002), and height (ρ= -0.147, p< 0.0001). Live crown ratio was also negatively correlated with LIR (ρ= -0.1024, p= 0.006). Live crown ratio decreased in the younger age classes, and increased in older plots. Crown density overall was similar across age and decline classes. Crown density was positively correlated with DBH (ρ= 0.093, p=0.001) and crown light (ρ= 0.166, p<0.0001), and negatively correlated with crown position (ρ= -0.076, p=0.04), and dieback (ρ= -0.107, p=0.0043). Across all plots in the three oldest age classes (where density was measured), crown density increased from 2006 to 2007 (t=8.50, p<0.0001). Change in crown density was higher in predicted symptomatic plots than predicted asymptomatic plots ($F_{1,167}$=9.70, p=0.0022), although change in crown density was similar in most age/decline categories.

Dieback was similar across age and decline classes. Dieback was negatively correlated with DBH (ρ= -0.129, p= 0.0005), crown density (ρ= -0.106, p= 0.0043), crown light (ρ= -0.142, p= 0.0001), height (ρ= -0.151, p= 0.0001), age class (ρ= -0.106, p= 0.0044), age in years (ρ= -0.109, p= 0.0034), and positively correlated with crown position (ρ= 0.181, p< 0.0001), all of which suggest that younger trees and understory trees experienced more dieback than older trees. There were no correlations among

Table 2.3. Means for crown parameters (SE) compared between decline classes age classes, and change (between years 2006 and 2007) of measurement. Predicted symptomatic plots are designated "D", and predicted asymptomatic "H". Means were separated by the Tukey-Kramer method. Cells bearing the same letter within a row are not significantly different at $p<0.05$.

	<10 Years		10-19 Years		20-40 Years		>40 Years	
	H	D	H	D	H	D	H	D
Live Crown Ratio, 2006	74.3 (3.0)A	71.3 (3.0)A	65.1 (1.7)B	54.8 (1.7) C	48.5 (2.1) CD	43.4 (1.9) D	50.8 (1.9) C	47.2 (2.1) D
Live Crown Ratio, 2007	68.8 (2.7) A	72.3 (3.0) A	61.5 (1.7) B	52.2 (1.8) C	49.5 (2.1) D	46.7 (1.9) D	50.8 (1.9) D	52.8 (2.0) D
Change in LCR	-8.0 (2.1) A	1.0 (2.1) AB	-4.1 (1.2) A	-2.6 (1.2) A	1.0 (1.5) AB	3.8 (1.3) B	-0.6 (1.4) AB	3.14 (1.6) B
Crown Position, 2006	2.13 (0.05) A	2.4 (0.05) B	2.07 (0.03) A	2.07 (0.03) A	2.00 (0.04) A	2.00 (0.03) A	2.00 (2.04) A	2.00 (2.04) A
Crown Position, 2007	2.40 (0.11) AB	2.8 (0.11) A	2.13 (0.06) AC	2.14 (0.07) AC	2.00 (0.08) C	2.00 (0.07) C	2.00 (0.07) C	2.03 (0.07) C
Change in Crown Position	0.27 (0.08) AB	0.40 (0.08) A	0.07 (0.05) BC	0.07 (0.05) BC	0.00 (0.06) BC	0.00 (0.05) C	0.00 (0.05) BC	0.04 (0.06) BC
Crown Light, 2006	2.27 (0.26) AB	3.60 (0.26) C	2.24 (0.15) A	1.86 (0.16) A	2.32 (0.18) A	2.22 (0.17) A	3.11 (0.17) BC	2.28 (0.19) A
Crown Light, 2007	3.06 (0.28) AB	4.07 (0.30) A	2.78 (0.17) B	2.70 (0.18) B	3.35 (0.21) AB	3.18 (0.19) AB	3.81 (0.19) A	2.97 (0.20) B
Change in Crown Light	0.80 (0.24) A	0.47 (0.24) A	0.55 (0.14) A	0.84 (0.14) A	1.03 (0.17) A	1.00 (0.15) A	0.69 (0.16) A	0.41 (0.18) A

Table 2.3. Continued

	<10 Years		10-19 Years		20-40 Years		>40 Years	
	H	D	H	D	H	D	H	D
Crown Density, 2006	NA	NA	38.5 (1.5) A	35.2 (1.2) A	36.6 (1.1) A	36.9 (1.0) A	39.3 (1.0) A	36.7 (1.1) A
Crown Density, 2007	NA	NA	38.2 (1.6) A	39.6 (1.7) A	40.5 (1.5) A	50.7 (1.4) B	44.1 (1.4) A	42.4 (1.4) A
Change in Density	NA	NA	1.3 (2.1) A	4.6 (1.7) A	3.9 (1.5) A	13.7 (1.4) B	4.9 (1.4) A	4.1 (1.6) A
Crown Transparency, 2006	NA	NA	25.0 (0.7) A	25.2 (0.6) A	24.8 (0.5) A	25.7 (0.5) A	26.1 (0.5) A	25.3 (0.5) A
Crown Transparency, 2007	NA	NA	25.0 (0.7) AB	24.6 (0.7) AB	24.4 (0.6) AB	23.1 (0.6) A	24.7 (0.6) AB	26.6 (0.6) B
Change in Transparency	NA	NA	-0.7 (1.0) AB	-0.6 (0.8) AB	-0.5 (0.7) AB	-2.6 (0.7) A	-1.25 (-0.7) AB	1.1 (0.8) B
Crown Dieback, 2006	NA	NA	3.8 (1.1) A	4.4 (0.9) A	0.8 (0.8) B	1.9 (0.7) A	2.4 (0.7) A	1.7 (0.8) A
Crown Dieback, 2007	NA	NA	1.8 (0.7) A	0.1 (0.7) A	0.2 (0.7) A	0.8 (0.6) A	0.4 (0.6) A	1.0 (0.6) A
Change in Crown Dieback	NA	NA	-3.4 (1.1) AB	-4.5 (0.9) A	-0.6 (0.8) B	-1.6 (0.8) AB	-1.5 (0.8) AB	-0.6 (0.9) B
Vigor 2006 (Saplings only)	1.00 (0.05) A	1.08 (0.05) A	NA	NA	NA	NA	NA	NA
Vigor 2007 (Saplings only)	1.00 (0.00) A	1.00 (0.00) A	NA	NA	NA	NA	NA	NA

NA=Not applicable.

dieback and LIR, decline class, or root infection by any ophiostomatoid fungi. Foliar transparency was uniform across age and decline classes. Dieback and foliar transparency also did not change significantly between 2006 and 2007 in either decline class or age class.

2.4.3. *Resin sampling*

Resin mass was highly variable within and between plots, with a mean of 4.07 g tree^{-1}, and a standard deviation of 3.07 g tree^{-1}. Resin weight was positively correlated with age class ($F_{3,163}$= 3.92, p= 0.0097) and with age in years ($F_{1,168}$=14.35, p= 0.0002); older trees produced more resin than younger trees. Resin weight was not different between decline classes (Table 2.4). Resin weight was not correlated with LIR.

2.4.4. *Infection of roots and soil by* Leptographium *spp.*

Of the 96 trees excavated, 24 (25%) had *Leptographium* spp. recovered from their roots. *Leptographium procerum* was the most frequently recovered species (22 trees), with pooled *Pesotum* spp. (19 trees) and *L. terebrantis* (16 trees) also abundant (Table 2.5). *Grosmannia huntii* was isolated from a single tree which also yielded *L. procerum*, *L. terebrantis*, *Ophiostoma* spp. and *Pesotum* spp. This individual died shortly after excavation. *Leptographium serpens*, present on insects (see below) was not recovered from any excavated roots. Also recovered was an *Ophiostoma* sp. similar to *O. ips* (Rumb.) Nannf., but likely a novel species (M.J. Wingfield, personal communication).

Table 2.4. Mean mass (g) (SE) of standing resin reserves by age class and predicted decline class from longleaf pine at Fort Benning, GA. Predicted symptomatic plots are designated "D", and predicted asymptomatic "H". Rows with the same letter are not significantly different at p= 0.05. Mean separated by Tukey Kramer method.

Age class (years)	H	D
10 to 19	3.243 (2.992) [A]	2.756 (2.344) [A]
20 to 40	3.644 (2.643) [A]	4.320 (2.884) [A]
>40	5.090 (4.174) [A]	4.790 (3.001) [A]

Table 2.5. Incidence of ophiostomatoid fungi recovered from longleaf pine roots at Fort Benning, Georgia. For each Decline class/Age class combination, 12 trees were excavated.

Age Class	*L. procerum*	*L. terebrantis*	*G.aureum*-like	*G. huntii*	*O. ips*-like	*Ophiostoma* spp.	*Pesotum* spp.
			Predicted Asymptomatic 'H'				
<10 Years	3	2	1	0	0	1	2
10-19 Years	2	2	0	0	0	0	1
20-40 Years	3	3	0	0	0	0	3
>40 Years	5	2	0	0	0	0	3
Subtotal	13	9	1	0	0	1	9
			Predicted Symptomatic 'D'				
<10 Years	2	2	0	0	1	1	1
10-19 Years	3	2	0	0	0	0	1
20-40 Years	1	2	0	0	0	0	2
>40 Years	3	1	1	1	0	1	6
Subtotal	9	7	1	1	1	2	10
Total	22	16	2	1	1	3	19

Leptographium procerum and *L. terebrantis* were highly correlated (ρ=0.6872, p<0.0001), co-occurring in 13 trees. Other fungal species were also correlated with *L. procerum,* including *G. aureum-like* (ρ=0.2675, p<0.001) and *Pesotum* spp. (ρ=0.4134, p<0.001). None of the fungal species were correlated with stand decline class, and *Pesotum* spp. were positively correlated with age class (ρ =0.2455, p=0.016) and age in years (ρ= 0.2355, p=0.021). Slope class was negatively correlated with infection with *L. procerum* (ρ= -0.2082, p= 0.042) and *L. terebrantis* (ρ -0.2235, p= 0.0273). Aspect class was not correlated with either age class or decline class. Fisher's exact test was not significant when applied to applied to *L. procerum* (χ^2_3= 1.21, p= 0.75), *L. terebrantis* (χ^2_3= 0.28, p= 0.96), and *Pesotum* spp. (χ^2_3= 1.48, p= 0.69).

At the plot level, 16 plots yielded *Leptographium* spp. from excavated roots, and 16 had no *Leptographium* isolates recovered from roots (Figure 2.6). *Leptographium* Incidence Rating was not different in any decline or age class. Soil assays yielded one isolate of *L. procerum*. This isolate was recovered from a predicted symptomatic plot in the 10-20 age class with an LIR of 2.

Stepwise regression included *G. huntii* ($F_{1,93}$= 7.58, p=0.004) and *Pesotum* spp. ($F_{1,93}$= 4.20, p=0.04) as negative effectors of DBH. Stepwise regression did not include root infection by any species as effectors of height growth, five year growth increment, ten year growth increment, ratio of five to ten year growth increment, or crown density (Table 2.6). Infection with *Pesotum* spp. also predicted an increase in foliar transparency ($F_{1,53}$ =5.13, p=0.03). No other taxa were predictors of increase in foliar transparency.

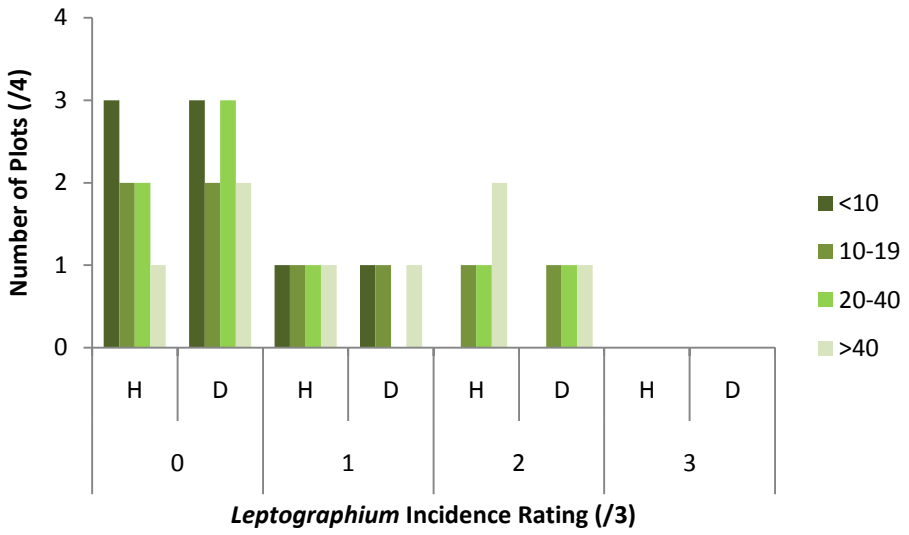

Figure 2.6. *Leptographium* incidence rating by decline class and age class.

Table 2.6. Test of effects of fungal infection on growth and crown parameters (2007 data). Significant effects ($p<0.05$) are in bold text. Marginal effects ($p<0.1$) are italicized.

Parameter *	*L. procerum*		*L. terebrantis*		*G. aureum-like*		*O. ips*-like		*Ophiostoma* spp.		*Pesotum* spp.	
	F (df)	p	F (df)	p	F (df)	p	F (df)	p	F (df)	p	F (df)	p
ΔDBH	**3.95 (1,93)**	**0.05**	1.20 (1.93)	0.28	**4.55 (1,93)**	**0.04**	0.08 (1,93)	0.78	*3.65 (1,93)*	*0.06*	**6.37 (1,93)**	**0.01**
ΔHt	0.30 (1,91)	0.58	1.73 (1,91)	0.19	0.01 (1,91)	0.92	0.34 (1,91)	0.56	0.03 (1,91)	0.87	1.82 (1,91)	0.18
LCR	0.56 (1,91)	0.46	0.25 (1,91)	0.62	1.40 (1,91)	0.24	0.74 (1,91)	0.39	0.29 (1,91)	0.59	2.46 (1,91)	0.12
CDn	0.37 (1,58)	0.54	0.05 (1,58)	0.82	NA	NA	NA	NA	NA	NA	**4.39 (1,58)**	**0.04**
CDb	0.65 (1,64)	0.42	0.28 (1,64)	0.60	NA	NA	NA	NA	NA	NA	0.19 (1,64)	0.67
CT	1.78 (1,58)	0.19	0.16 (1,58)	0.69	NA	NA	NA	NA	NA	NA	**8.45 (1,58)**	**0.01**

* ΔDBH-Change in DBH, ΔHt-Change in Height, LCR-Live Crown Ratio, CDn- Crown Density, CDb-Crown Dieback, CT-Crown Transparency

Infection with *L. procerum* and *L. terebrantis* did not affect any crown parameters. Infection with *L. procerum* did affect diameter growth, but neither species was associated with reduced height growth (Table 2.6). The effects of *G. huntii* on tree growth and crown health could not be tested due to the single occurrence.

2.4.5. *Incidence of pinophagous beetle activity and infestation with ophiostomatoid fungi.*

Five root-feeding beetles were captured most frequently; *H. tenuis*, *H. salebrosus*, *P. picivorus, Hb. pales,* and *D. terebrans* (in order of frequency) (Figure 2.7A-E). *Hylastes porculus* Erichson were also captured but were scored as *H. salebrosus* due to morphological similarity. The approximate proportion of *H. salebrosus*: *H. porculus* in post hoc diagnoses was 3:1.

None of these were greater in abundance in predicted symptomatic plots in comparison with predicted asymptomatic plots (Table 2.7). *Hylastes salebrosus* was captured more frequently in predicted asymptomatic plots (Table 2.7). The interaction term between decline and age class for *D. terebrans* was caused by the disparity in capture number in the >40 year age class. Removal of this age class from the model for *D. terebrans* captures rendered all effects (decline class, age class, and interaction term) not significant ($F_{5,18}$= 0.77, p=0.58).

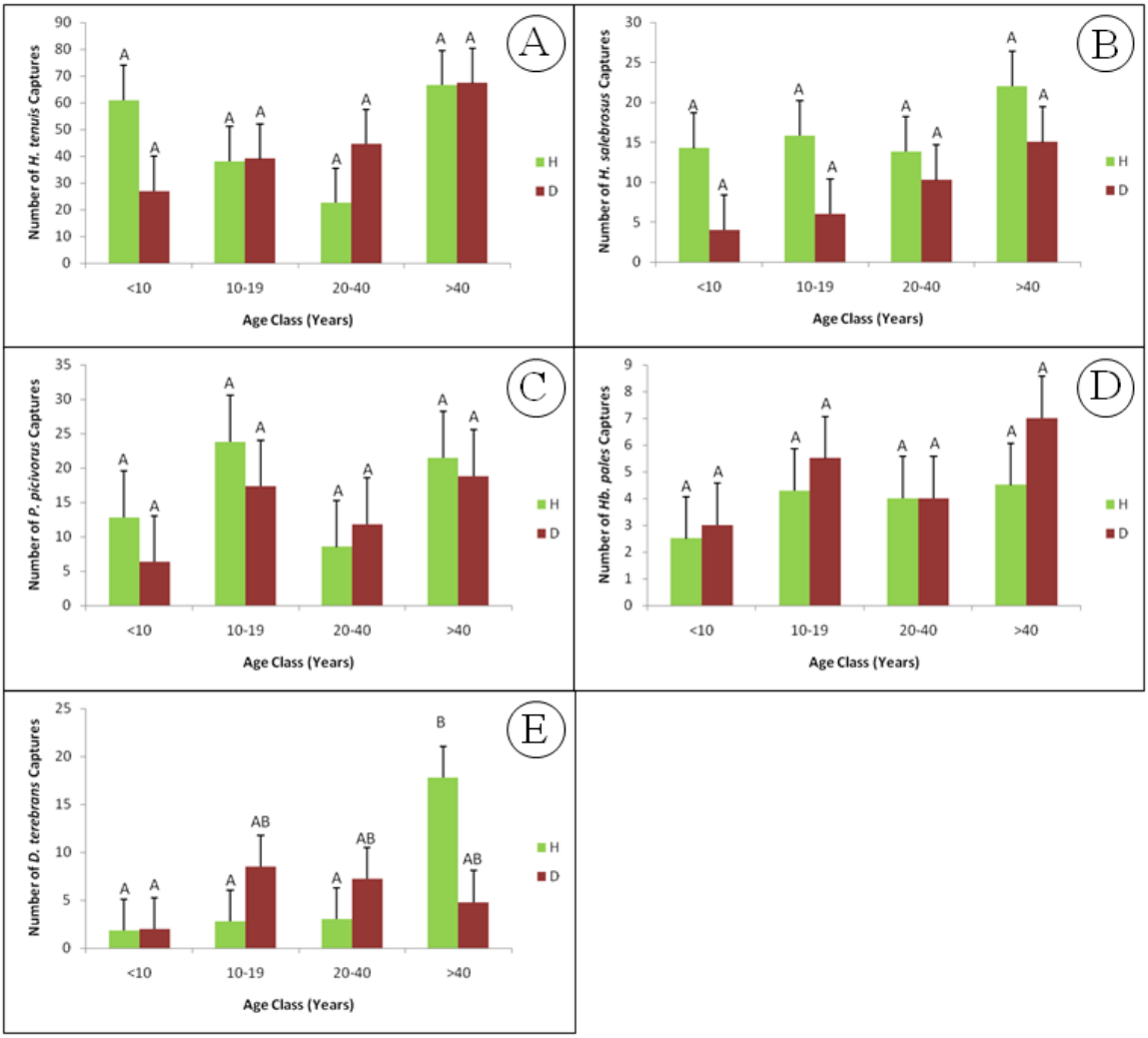

Figure 2.7. Pooled mean insect captures by decline class and age class. Columns within charts bearing the same letter are not significantly different ($p < 0.05$). Predicted symptomatic plots are designated "D", and predicted asymptomatic "H".Means separated by Tukey-Kramer Method. (A) *H. tenuis*, (B) *H. salebrosus*, (C) *P. picivorus*, (D) *Hb. pales*, (E) *D. terebrans*.

Table 2.7. ANOVA Comparison of root-feeding insect captures by decline class, age class, and mixed effects at Fort Benning, GA. Significant effects ($p < 0.05$) are in bold text, and marginal effects ($p<0.1$) are italicized.

Insect species	df	F, Model	p	Decline Class	p	Age Class	p	Decline × Age	p
D. terebrans	7,24	**2.69**	**0.03**	0.09	0.77	*2.84*	*0.06*	**3.41**	**0.03**
H. salebrosus	7,24	1.68	0.16	**5.92**	**0.02**	1.7	0.19	0.24	0.86
H. tenuis	7,24	1.77	0.14	0.08	0.78	*2.54*	*0.08*	1.57	0.22
Hb.pales	7,24	0.81	0.59	0.92	0.35	1.34	0.29	0.24	0.87
P. picivorus	7,24	0.85	0.55	0.43	0.52	1.62	0.21	0.23	0.87

In total, 979 isolates of ophiostomatoid fungi were recovered from 3352 individuals of the five principle insect vectors. The most frequently recovered fungal species from insects were, *L. procerum*, *L. terebrantis*, *L. serpens*, *G. huntii*, the *G. aureum*-like fungus, the *O. ips*-like fungus, other *Ophiostoma* spp. and *Pesotum* spp (Tables 2.8-2.12). Neither decline class nor age class were correlated with numbers of isolates recovered from insects.

2.5. Discussion

The LPDRM did not accurately predict the relationship of LLPD associated factors for most of the tested parameters in most of the age classes. Distribution of infected trees, numbers of insect vectors, mass of standing resin reserves, growth increment, crown density and transparency, did not differ between predicted symptomatic and predicted asymptomatic plots.

The 20-40 year age class did present several differences from the other three age classes, and the LPDRM was most effective in this age class. Height, DBH and ten-year increment growth were significantly higher in predicted asymptomatic trees in this age class. Crown dieback observed in 2006 was greater in predicted symptomatic plots, but contrary to the predictions of the LPDRM, crown density observed in 2007 was greater in predicted symptomatic plots of this age class. As these trends were not observed in stands > 40 years, age alone cannot explain these results. The time period in which trees in the 20-40 year age class were either planted or were regenerated naturally (1966-1986) coincided with a range wide growth reduction of old-growth trees (West *et al.*, 1993), as

Table 2.8. *Dendroctonus terebrans* captures and ophiostomatoid fungi infestation percentage by age class and decline class.

Age Class	Total Captures	*L. procerum*	*L. serpens*	*L. terebrantis*	*G. aureum*-like	*G. huntii*	*Ophiostoma* spp.	*O. ips*-like	*Pesotum* spp.
Predicted Asymptomatic ('H')									
<10 Years	8	0.0%	0.0%	0.0%	12.5%	0.0%	0.0%	12.5%	12.5%
10-19 Years	16	0.0%	0.0%	12.5%	6.3%	6.3%	0.0%	12.5%	6.3%
20-40 Years	15	0.0%	0.0%	0.0%	6.7%	0.0%	0.0%	20.0%	6.7%
>40 Years	78	1.3%	0.0%	2.6%	19.2%	0.0%	1.3%	9.0%	2.6%
Predicted Symptomatic ('D')									
<10 Years	8	0.0%	0.0%	0.0%	25.0%	0.0%	0.0%	0.0%	0.0%
10-19 Years	39	0.0%	0.0%	0.0%	20.5%	0.0%	2.6%	15.4%	2.6%
20-40 Years	32	3.1%	0.0%	0.0%	6.3%	0.0%	6.3%	12.5%	3.1%
>40 Years	19	10.5%	0.0%	5.3%	15.8%	5.3%	10.5%	15.8%	5.3%

Table 2.9. *Hylastes salebrosus* captures and ophiostomatoid fungi infestation percentage by age class and decline class.

Age Class	Total Captures	*L. procerum*	*L. serpens*	*L. terebrantis*	*G. aureum*-like	*G. huntii*	*Ophiostoma* spp.	*O. ips*-like	*Pesotum* spp.
Predicted Asymptomatic ('H')									
<10 Years	65	4.6%	0.0%	3.1%	0.0%	7.7%	0.0%	3.1%	1.5%
10-19 Years	79	2.5%	0.0%	3.8%	0.0%	3.8%	0.0%	1.3%	2.5%
20-40 Years	107	2.8%	0.0%	5.6%	0.0%	2.8%	0.0%	2.8%	2.8%
>40 Years	140	0.7%	0.0%	0.7%	0.0%	2.9%	1.4%	3.6%	0.7%
Predicted Symptomatic ('D')									
<10 Years	28	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.6%	3.6%
10-19 Years	34	5.9%	0.0%	0.0%	0.0%	0.0%	0.0%	5.9%	2.9%
20-40 Years	61	0.0%	0.0%	1.6%	0.0%	0.0%	1.6%	3.3%	3.3%
>40 Years	82	1.2%	1.2%	2.4%	0.0%	5.3%	0.0%	1.2%	2.4%

Table 2.10. *Hylastes tenuis* captures and ophiostomatoid fungi infestation percentage by age class and decline class.

Age Class	Total Captures	*L. procerum*	*L. serpens*	*L. terebrantis*	*G. aureum*-like	*G. huntii*	*Ophiostoma* spp.	*O. ips*-like	*Pesotum* spp.
Predicted Asymptomatic ('H')									
<10 Years	279	10.0%	1.1%	6.8%	0.0%	10.4%	1.4%	1.8%	8.6%
10-19 Years	155	4.5%	0.6%	3.2%	0.6%	9.0%	0.6%	0.0%	4.5%
20-40 Years	156	4.5%	3.2%	5.8%	0.0%	14.1%	1.3%	0.6%	8.3%
>40 Years	279	3.6%	1.4%	6.1%	0.0%	13.6%	1.1%	1.4%	9.3%
Predicted Symptomatic ('D')									
<10 Years	130	10.8%	0.8%	5.4%	0.0%	6.2%	7.7%	0.0%	7.7%
10-19 Years	177	3.4%	1.1%	4.5%	0.0%	8.5%	0.0%	0.6%	2.3%
20-40 Years	213	8.0%	3.3%	5.2%	0.5%	13.6%	0.5%	1.9%	7.0%
>40 Years	337	8.9%	2.4%	5.6%	0.0%	11.0%	0.9%	1.2%	8.9%

Table 2.11. *Hylobius pales* captures and ophiostomatoid fungi infestation percentage by age class and decline class.

Age Class	Total Captures	*L. procerum*	*L. serpens*	*L. terebrantis*	*G. aureum*-like	*G. huntii*	*Ophiostoma* spp.	*O. ips*-like	*Pesotum* spp.
Predicted Asymptomatic ('H')									
<10 Years	19	5.3%	0.0%	31.6%	0.0%	0.0%	0.0%	0.0%	0.0%
10-19 Years	29	10.3%	0.0%	13.8%	0.0%	0.0%	0.0%	0.0%	0.0%
20-40 Years	32	6.3%	0.0%	6.3%	0.0%	0.0%	3.1%	0.0%	0.0%
>40 Years	31	22.6%	0.0%	12.9%	0.0%	3.2%	0.0%	0.0%	3.2%
Predicted Symptomatic ('D')									
<10 Years	39	12.8%	0.0%	17.9%	0.0%	0.0%	0.0%	2.6%	0.0%
10-19 Years	38	21.1%	0.0%	10.5%	0.0%	0.0%	0.0%	0.0%	5.3%
20-40 Years	22	0.0%	0.0%	4.5%	0.0%	4.5%	0.0%	0.0%	0.0%
>40 Years	37	16.2%	5.4%	8.1%	0.0%	5.4%	2.7%	5.4%	0.0%

Table 2.12. *Pachylobius picivorus* captures and ophiostomatoid fungi infestation percentage by age class and decline class.

Age Class	Total Captures	*L. procerum*	*L. serpens*	*L. terebrantis*	*G. aureum*-like	*G. huntii*	*Ophiostoma* spp.	*O. ips*-like	*Pesotum* spp.
Predicted Asymptomatic ('H')									
<10 Years	55	12.7%	0.0%	10.9%	0.0%	0.0%	0.0%	1.8%	0.0%
10-19 Years	104	17.3%	0.0%	9.6%	0.0%	1.0%	0.0%	1.0%	0.0%
20-40 Years	42	16.7%	0.0%	2.4%	0.0%	0.0%	2.4%	0.0%	0.0%
>40 Years	99	9.1%	0.0%	4.0%	0.0%	0.0%	0.0%	0.0%	1.0%
Predicted Symptomatic ('D')									
<10 Years	27	7.4%	0.0%	11.1%	0.0%	0.0%	0.0%	0.0%	0.0%
10-19 Years	69	10.1%	0.0%	8.7%	0.0%	2.9%	0.0%	1.4%	0.0%
20-40 Years	47	23.4%	0.0%	14.9%	0.0%	2.1%	2.1%	0.0%	0.0%
>40 Years	75	9.3%	0.0%	13.3%	0.0%	0.0%	2.7%	0.0%	0.0%

well as a reduction in cone production (Brockway *et al.*, 2006). Also during this time, loblolly pine and slash pine (*P. elliottii* Mill.) were favored species to plant at FB, as longleaf pine regeneration was still considered a difficult species to regenerate. Any of these factors, or a combination of them, may have presented significant predisposing factors that would contribute to the differences observed in the 20-40 year age class trees.

Rates of infection with *Leptographium* spp. were not greater in this 20-40 year age class, nor were infected trees different from uninfected trees in growth or crown measurements (or in any age class). However, *L. procerum* and *L. terebrantis* are considered to be less aggressive species and thus physiological effects of infection may be subdued relative to species more recently reported from the southeastern U.S., such as *L. serpens* and *G. huntii*. The single tree which displayed typical decline symptomatology was infected with the latter species, and subsequently died.

Leptographium spp. and related fungi were found in tree roots in all four age classes and rarely from soil. This indicates that longleaf pine of all age classes may be successfully inoculated by bark beetles and weevils and suggests that vectors are the primary source of root infection, rather than by transfer through the soil.

If slope and aspect do not affect growth of longleaf pine at FB, then longleaf pine may be a suitable alternative species to plant on sites that are predisposed to loblolly pine decline. Slope and aspect are both correlated with droughty sites, especially within the sandhills region where FB is located. Longleaf pine is regarded as being more drought

tolerant that other *Pinus* spp. in the southeastern U.S., which is confirmed by the results observed in this study. However, while longleaf pine is more drought tolerant than other southern pines, drought effects are still measurable (Henderson and Grissino-Mayer, 2009)

As LLPD has been observed in other locations (Otrosina *et al.*, 1999), a more directly empirical approach to the site factors which coincide with declining longleaf pine is warranted. This study was not intended to test the survival of longleaf pine on high risk loblolly decline sites, thus a number of important hypotheses should be tested before adopting longleaf pine planting as a best management practice on such sites. Fort Benning is actively managing its forests to enhance longleaf pine, to fulfill its primary goal of providing a venue for the training of soldiers, as well as the secondary task of restoring habitat for species of conservation concern, such as the Federally-listed Endangered Species, red-cockaded woodpecker.

The problem of identifying ultimate rather than proximate contributors to decline is still difficult when examining only sites that are actively and visibly declining. The LPDRM examines a subset of predisposing and contributing factors, but the role of inciting factors is more difficult to incorporate into a short term study of a long term forest health issue. The effects of long term (multiple-year) drought, ongoing during the study period, are more difficult to measure. Catastrophic fire is another inciting factor, and fire is an important component of the longleaf pine ecosystem. While frequent fire is necessary to longleaf pine management, intense fire may kill trees directly or may weaken trees, allowing biotic agents to invade. The return of fire to long unburned sites

is considered to be an important inciting factor (Otrosina *et al.*, 1999; Varner *et al.*, 2005; Varner *et al.*, 2007), and the frequent and regular application of prescription burning at FB may have contributed to the observed results in this study. While several plots were burned during the course of study at FB, the effects of fire on trees were not evident from insect numbers or from root infestation data. This may be due in part to the haphazard burning of plots, but even in studies where fire intensity was directly investigated the effects of fire on insect populations have presented contrasting results (Hanula *et al.*, 2002; Sullivan *et al.*, 2003).

Ecological modeling of forest declines has proven of great benefit to land managers, and an empirical evaluation of longleaf pine decline is needed to inform restoration efforts. While the LPDRM has limited ability to predict LLPD symptomatology, some clues may be garnered from this study. The prevalence of *G.huntii* on *Hylastes* spp. and the recovery from a symptomatic individual demonstrates that the role of this fungus is not well characterized. Pathogenicity trials suggest that this fungus may be more aggressive towards longleaf and other southern pines (Matusick *et al*., unpublished data) than the fungi recovered from roots in situ in this study. The patchy distribution of *Leptographium* spp. revealed in this study suggests that larger plots may be necessary to accurately assess the mycota of longleaf pine roots in the landscape. The finding of the *O. ips*-like fungus also demonstrates the need to characterize what fungi are present, whether these fungi may be coevolved with native trees and insects, and which may be more likely to have effects on tree health.

The patchiness of *Leptographium* infection and variability in the resin reserves suggest that stand level genetic heterogeneity may be high in longleaf pine. Previous study of brown spot resistance in longleaf pine supports this hypothesis as phenotypically resistant tree were found to be distributed throughout natural stands (Wakeley, 1970). With little naturally occurring longleaf pine remaining, an understanding of the level of this heterogeneity may escape if timely research is not pursued.

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2.7. Literature Cited

Baccala, N.B., Rosso, P.H., Havrylenko, M., 1998. *Austrocedrus chilensis* mortality in the Nahuel Huapi National Park (Argentina). Forest Ecology and Management 109, 261-269.

Blackman, M.W., 1941. Bark beetles of the genus *Hylastes* Erichson in North America.. US Department of Agriculture Miscellaneous Publication No. 417, Washington D.C.

Boyer, W.D., 1990. *Pinus palustris* Mill.-longleaf pine. In: Burns, R.M., Honkala, B.H. (Eds.), Silvics of North America. Volume 1. Conifers. U.S. Department of Agriculture, Forest Service, Washington DC, pp. 405-412.

Brockway, D., Outcalt, K., Boyer, W., 2006. Longleaf pine regeneration ecology and methods. In: Jose, S., Jokela, E., Miller, D. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 95-133.

Campbell, W.A., Copeland, O.L., 1954. Littleleaf disease of shortleaf and loblolly pines. U.S. Department of Agriculture Forest Service, Washington, DC. Circular No. 540.

Conner, R.N., O'Halloran, K.A., 1987. Cavity-tree selection by red-cockaded woodpeckers as related to growth dynamics of southern pines. Wilson Bulletin 99, 398-412.

Dochinger, L.S., 1967. *Leptographium* root decline of eastern white pine. Phytopathology 57, 809-809.

Eckhardt, L.G., 2003. Biology and ecology of *Leptographium* species and their vectors as components of loblolly pine decline. In, Louisiana State University, Baton Rouge, LA, 158 p.

Eckhardt, L.G., Goyer, M.A., Klepzig, K.D., Jones, J.P., 2004a. Interactions of *Hylastes* species (Coleoptera : Scolytidae) with *Leptographium* species associated with loblolly pine decline. Journal of Economic Entomology 97, 468-474.

Eckhardt, L.G., Jones, J.P., Klepzig, K.D., 2004b. Pathogenicity of *Leptographium* species associated with loblolly pine decline. Plant Disease 88, 1174-1178.

Eckhardt, L.G., Menard, R.D., 2008. Topographic features associated with loblolly pine decline in central Alabama. Forest Ecology and Management 255, 1735-1739.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

ESRI, 1996. ArcView 3.2a. Redlands, CA.

Filip, G.M., Rosso, P.H., 1999. Cypress mortality (mal del cipres) in the Patagonian Andes: comparisons with similar forest diseases and declines in North America. European Journal of Forest Pathology 29, 89-96.

Hanula, J.L., Meeker, J.R., Miller, D.R., Barnard, E.L., 2002. Association of wildfire with tree health and numbers of pine bark beetles, reproduction weevils and their associates in Florida. Forest Ecology and Management 170, 233-247.

Harrington, T.C., 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. Mycologia 73, 1123-1129.

Henderson, J.P., Grissino-Mayer, H.D., 2009. Climate-tree growth relationships of longleaf pine (*Pinus palustris* Mill.) in the Southeastern Coastal Plain, USA. Dendrochronologia 27, 31-43.

Hepting, G.H., 1971. Diseases of forest and shade trees of the United States. U.S. Dept. of Agriculture Forest Service. U.S. Govt. Print. Off., Washington, DC, p. 658.

Johnson, L.F., Curl, E.A., 1972. Methods for research on the ecology of soil-borne plant pathogens. Burgess Pub. Co., Minneapolis, MN.

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Science 37, 1119-1139.

Klepzig, K.D., Smalley, E.B., Raffa, K.F., 1995. *Dendroctonus valens* and *Hylastes porculus* (Coleoptera, Scolytidae) - vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. Great Lakes Entomologist 28, 81-87.

Leaphart, C.D., Johnson, H.E., 1973. Pole blight: also a disease of western white pine plantations. Plant Disease Reporter 57, 948-951.

Lewis, K.J., Alexander, S.A., Horner, W.E., 1987. Distribution and efficacy of propagules of *Verticicladiella procera* in soil. Phytopathology 77, 552-556.

Manion, P.D., 1981. Tree disease concepts. Prentice-Hall, Englewood Cliffs, N.J.

Minnesota Department of Natural Resources, 2005. DNR Garmin version 5.1.1, St. Paul, MN.

Menard, R.D., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. Louisiana State University, Baton Rouge, LA, 54 p.

Nevill, R.J., Alexander, S.A., 1992. Root- and stem colonizing insects recovered from eastern white pines with procerum root disease. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 22, 1712-1716.

Oak, S., Tainter, F., Williams, J., Starkey, D., 1996. Oak decline risk rating for the southeastern United States. Annales Des Sciences Forestieres 53, 721-730.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant and Soil 217, 145-150.

Paine, T.D., Raffa, K.F., Harrington, T.C., 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annual Review of Entomology 42, 179-206.

SAS Institute, 2003. SAS Version 9.1. Cary, NC.

Sullivan, B.T., Fettig, C.J., Otrosina, W.J., Dalusky, M.J., Berisford, C.W., 2003. Association between severity of prescribed burns and subsequent activity of conifer-infesting beetles in stands of longleaf pine. Forest Ecology and Management 185, 327-340.

Thomas, A.L., Gegout, J.C., Landmann, G., Dambrine, E., King, D., 2002. Relation between ecological conditions and fir decline in a sandstone region of the Vosges mountains (northeastern France). Annals of Forest Science 59, 265-273.

USDA Forest Service, 1998. Forest Health Monitoring Field Methods Guide. In. National Forest Health Monitoring Program, Research Triangle Park, NC.

Varner, J.M., Gordon, D.R., Putz, E., Hiers, J.K., 2005. Restoring fire to long-unburned *Pinus palustris* ecosystems: Novel fire effects and consequences for long-unburned ecosystems. Restoration Ecology 13, 536-544.

Varner, J.M., Hiers, J.K., Ottmar, R.D., Gordon, D.R., Putz, F.E., Wade, D.D., 2007. Overstory tree mortality resulting from reintroducing fire to long-unburned longleaf pine forests: the importance of duff moisture. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 37, 1349-1358.

Wahlenberg, W.G., 1946. Longleaf pine: its use, ecology, regeneration, protection, growth, and management. Charles Lathrop Pack Forestry Foundation in cooperation with the U.S.D.A. Forest service, Washington, D. C..

Weaver, M.J., Stipes, R.J., Amos, D.F., Heikkenen, H.J., 1981. Factors associated with declining eastern white pine (*Pinus strobus*) on Virginia landscapes. Phytopathology 71, 772-772.

West, D.C., Doyle, T.W., Tharp, M.L., Beauchamp, J.J., Platt, W.J., Downing, D.J., 1993. Recent growth increases in old growth longleaf pine. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 23, 846-853.

Wingfield, M.J., 1986. Pathogenicity of *Leptographium procerum* and *Leptographium terebrantis* on *Pinus strobus* seedlings and established trees. European Journal of Forest Pathology 16, 299-308.

CHAPTER III. ECOLOGY OF ROOT-FEEDING CURCULIONIDS AND ASSOCIATED FUNGI IN LONGLEAF PINE FORESTS AT FORT BENNING, GA

3.1. Abstract

Root-feeding beetles, particularly of the curculionid subfamilies Scolytinae and Molytinae, are known to be effective vectors of ophiostomatoid fungi to their hosts. Attack by these insects and infection with these fungi may play an important role in accelerating tree death in pine declines. To examine the relationship between beetles and fungi in longleaf pine stands, root-feeding curculionids were collected in pitfall traps baited with ethanol and turpentine for 62 weeks, and rolled on agar media to isolate phoretic ophiostomatoid fungi. The most abundant root-feeding beetles captured were *Hylastes tenuis, H. salebrosus*, *Pachylobius picivorus*, *Hylobius pales,* and *Dendroctonus terebrans*. Vector captures peaked in spring and fall, although peaks for different insect taxa did not coincide. The most frequently isolated fungi were *Grosmannia huntii*, *Leptographium procerum*, *L. terebrantis,* and *L. serpens*. Other ophiostomatoid fungi recovered included *Ophiostoma* spp. and *Pesotum* spp. Insect infestation data

suggest that *Hylastes* spp. share an ecological niche, as do *Hb. pales* and *P. picivorus*, as the ratios of the fungal symbionts were similar. The mycota of *Dendroctonus terebrans* suggests that it does not share habitat with the other principle vectors.

3.2. Introduction

Bark beetles and regeneration weevils (Coleoptera: Curculionidae, subfamilies Scolytinae and Molytinae, respectively) are intimate associates of ophiostomatoid fungi (Ascomycota: Ophiostomatales) (Six, 2003), commonly called blue-stain fungi for the cosmetic damage some species cause in logs and lumber (Seifert, 1993). Most members bear their meiotically or mitotically derived spores in gelatinous matrices suitable for transfer by insects (Malloch and Blackwell, 1993), and include the sexual genera *Ophiostoma* Syd. & P. Syd., *Ceratocystiopsis* Upadhyay & Kendrick, and *Grosmannia* Goid., as well as the asexual form-genera *Pesotum* Crane & Schokn. emend Okada & Seifert, *Sporothrix* Hektoen & Perkins, *Hyalorhinocladiella* Upadhyay & Kendrick, and *Leptographium* Lagerb. & Melin (Zipfel *et al.*, 2006).

Some members of the genus *Grosmannia* (anamorph: *Leptographium*, sensu Zipfel *et al.*, 2006) are known for their role in forest diseases and declines. Black stain root disease affects several conifer species in the Pacific Northwest, and is caused by *G. wageneri* (Kendrick) Zipfel, de Beer & Wingfield (Wagener and Mielke, 1961) which is vectored by bark beetles including *Hylastes nigrinus* (Mannerheim) (Witcosky *et al.*, 1986) and *H. macer* LeConte (Goheen and Cobb, 1978). In South Africa, *L. serpens*

(Goid.) Siemaszko, *O. ips* (Rumbold) Nannf., *L. lundbergii* Lagerberg & Melin and other ophiostomatoid fungi have been isolated from pine roots and linked to the European bark beetles *H. angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius) and *Orthotomicus erosus* (Wollaston) (Zhou *et al.*, 2001; Zhou *et al.*, 2002). Pine plantations have been introduced in the southern hemisphere, followed by exotic pinophagous bark beetles and their associated fungi. Notably, *H. ater* (Paykull) has been found in New Zealand accompanied by *G. huntii* (Robinson-Jeffrey & Grinchenko) Zipfel, de Beer and Wingfield and *O. ips* (Reay *et al.*, 2002). In Chile *H. ater* and *Hg. ligniperda* have been found carrying *G. huntii*, *O. ips*, and other ophiostomatoid fungi (Zhou *et al.*, 2004).

In the northeastern U.S., white pine (*Pinus strobus* L.) root decline has been linked to the presence of *L. procerum* (Kendrick) Wingfield, *L. terebrantis* Barras & Perry (Wingfield, 1986), and their associated curculionid vectors including the weevils *Hylobius pales* (Herbst) and *Pissodes nemorensis* Germar, and a suite of scolytine beetles (Nevill and Alexander, 1992). The same fungi have been implicated in red pine (*P. resinosa* Aiton) decline (Klepzig *et al.*, 1991). The associated vectors were more typically bark beetles, *D. valens* LeConte and *H. porculus* Erichson (Klepzig *et al.*, 1995), although the weevils *Pachylobius picivorus* (Germar), *Hb. pales* and *Hb. radicis* Buchanan were also found to be infested with *L. procerum, L. terebrantis*, and other ophiostomatoid fungi including *O. ips* and *G. huntii* (Klepzig *et al.*, 1991).

In the southeastern U.S., ophiostomatoid fungi and their vectors have been found to be important contributing factors in loblolly pine (*P. taeda* L.) decline (Eckhardt *et al.*, 2007). In loblolly pine stands experiencing decline, *Hb.* pales and *P. picivorus*, and *Ps.*

nemorensis were captured as well as southern scolytines of the same guild; *H. salebrosus* Eichhoff, *H. tenuis* Eichhoff, and *D. terebrans* (Olivier). These insects were found to be infested with *L. procerum* and *L. terebrantis*, as well as *L. serpens* (Eckhardt *et al.*, 2007). In pathogenicity tests, *L. serpens* and *L. terebrantis* had greater effects on seedling growth than *L. procerum*, with *L. serpens* producing greater lesions than *L. procerum* or *L. terebrantis* in seedlings and mature trees (Eckhardt *et al.*, 2004b). Infestation with *Leptographium* spp. was shown to increase brood size in *Hylastes* spp., which in turn were effective in inoculating the fungi into uninfected root sections (Eckhardt *et al.*, 2004a).

Longleaf pine (*P. palustris* Mill.) has also been reported to experience decline (Otrosina *et al.*, 1999). Though a pyrophytic species, mature longleaf pine were dying after prescription burning. A survey of the roots revealed *L. procerum* and *L. terebrantis*, but no connection was made to insect vectors at that time (Otrosina *et al.*, 1999). The effects of fire on insect populations in longleaf pine stands have been examined after wildfire (Hanula *et al.*, 2002) and prescription fire (Sullivan *et al.*, 2003). *Hylastes* spp. and other phloem feeders were more abundant in unburned areas than in burned sites after a wildfire in Florida, while the trend with xylem feeding scolytines was an increase in populations with burn severity (Hanula *et al.*, 2002). In a similar study, applying prescription burns of differing intensity in southern Alabama, the opposite trend was observed in phloeophagous scolytines (Sullivan *et al.*, 2003). Some of the regeneration weevils were examined for the presence of ophiostomatoid fungi in the Florida study, and revealed the presence of *L. procerum* and *L. terebrantis* (Hanula et al., 2002).

In this investigation the distribution and abundance of root-feeding bark beetles and regeneration weevils, and their phoretic ophiostomatoid fungi, were studied at Fort Benning Military Reservation, located near the Fall Line separating the coastal plain from the piedmont region in west central Georgia. Fort Benning (FB) maintains an active prescription burning program to enhance longleaf pine habitat. In addition to investigating seasonal differences in beetle populations, the mycota of these insects was also evaluated to determine which fungi they vector.

3.3. Materials and Methods

3.3.1. *Plot setup and collection of pinophagous beetles.*

Thirty-two 0.07 ha plots were installed at FB, in Muskogee and Chattahoochee Counties in Georgia, and Russell County, Alabama. Plot design followed the protocol of the USDA Forest Service's Forest Inventory and Analysis program as described by the USDA Forest Service (1998) and modified in Chapter II. Plots were blocked into four age classes (<10years, 10-19 years, 20-40 years, and >40 years at the beginning of the study, 2006), for eight replicates of each age class.

Pitfall traps were placed in each subplot (3/plot) to collect vector beetles. The traps (adapted from Klepzig *et al.*, 1991) were constructed of sections of PVC tubing 4" (10 cm) dia and 8" (20 cm) long with a fixed capped at the bottom end and a loose cap at the top. Eight radial holes were drilled to allow insects to enter and fall into a cup which sits inside the trap. A plastic skirt was fitted on the trap to reduce the risk of flooding

(Menard, 2007). Steam-distilled southern pine turpentine (Hercules, Wilmington, DE) and 95% ethanol (in two 8 mL vials) were used as baits. Three sections of longleaf pine twig, approximately 5 cm long × 2 cm dia (2"×5/8") were also placed in the cup as substrate for the captured insects and as additional bait.

Pitfall trap collection was performed weekly from 4 March to 5 May 2006 (10 collections) and from 24 August 2006 to 26 August 2007 (52 collections). Thus, data were collected for two springs (2006 and 2007), and one continuous year (2006-2007). During each trap collection, the contents of the pitfall trap were transferred to a clean specimen cup and stored at 4 °C for analysis at Auburn University School of Forestry and Wildlife Sciences (AUSFWS). The trap cup was sprayed with 70% ethanol to disinfect it and swabbed with liquid Teflon™ (Northern Products, Woonsocket RI) to deter insects from crawling out of the trap. Fresh twig sections were placed in the trap cup, and the ethanol and turpentine evacuated and replaced. At AUSFWS, insects were sorted from each specimen cup and identified to species and tallied.

Insect numbers were pooled at the plot and species levels over the course of the sampling period and effects of tree age class and sampling week tested using repeated measures analysis (PROC MIXED) with the first order autoregressive model (AR1) for the covariance structure in SAS 9.1 (SAS Institute, 2003).

3.3.2. *Effects of weather variables*

Effects of temperature and precipitation were assessed using weather data from the National Climatic Data Center (http://cdo.ncdc.noaa.gov/dly/DLY). Data from the Columbus Airport weather station were used, as data from the FB weather station during the study period are incomplete. Average weekly maxima and minima were calculated from daily maxima and minima. Weekly precipitation was summed from the daily precipitation data during the period between collection dates. Weekly totals of *H. tenuis*, *H. salebrosus*, *Hb. pales*, *P. picivorus*, and *D. terebrans* were pooled by plot for correlation analysis with temperature and precipitation using PROC CORR in SAS 9.1 (SAS Institute, 2003). To assess seasonal effects, weekly capture numbers for each plot and week were compared by coding 13 week periods with dummy variables, and analysis with PROC CORR and PROC GLM. For ANOVA, means were separated using the Tukey-Kramer procedure.

3.3.3. *Infestation with ophiostomatoid fungi*

Each captured beetle of interest was rolled on one petri plate of cycloheximide and streptomycin-amended malt extract agar (CSMA) (Hicks *et al.*, 1980) and one petri plate of 2% w:v malt extract agar (MEA) to collect fungal propagules. Plates were checked after five days and periodically thereafter for one month for the presence of ophiostomatoid fungi, which were serially transferred via spore droplet or hyphal tipping to CSMA until pure cultures were obtained, at which point the fungi were transferred to

MEA for archiving prior to species determination. Identifications were made based on microscopic examination of morphological and cultural characters as described in (Jacobs and Wingfield, 2001) for *Leptographium* spp. Other ophiostomatoid fungi (*Pesotum* spp., *Ophiostoma* spp.) were identified to genus and pooled for analyses.

Distributions of fungi on insects were compared using Chi-squared tests (PROC FREQ) in SAS 9.1 (SAS Institute, 2003). Also tested were correlations between fungal isolation from beetles and weather variables in a similar manner to the above described procedures, using PROC CORR in SAS 9.1.

3.4. Results

3.4.1. *Incidence of pinophagous beetle activity and seasonal effects.*

A total of 4800 beetles were captured from four families (Table 3.1- 3.2). Four species of phloeophagous scolytine bark beetles (*H. tenuis*, *H. salebrosus*, *H. porculus* and *D. terebrans*) and two species of molytine weevils (*Hb. pales* and *P. picivorus*) were captured most frequently.

Hylastes tenuis was the most frequently captured insect with 1726 captures. A post-hoc examination of 250 randomly selected individuals did not recover any individuals of *H. opacus* Erichson, a morphologically similar but exotic insect. For most data analyses, *H. salebrosus* and *H. porculus* were pooled, as both were tallied as

H. salebrosus during the collection period. A post-hoc examination of intact captured specimens revealed 25% (97/385) of beetles previously identified as *H. salebrosus* were *H. porculus*. The ratio of these species varied by season (see p. 129).

Week of capture was a significant factor in all species tested by repeated measures analysis (Table 3.3). Stands in the >40 Year age class yielded more insect captures than the <10 Year age class in all species tested, and were higher than all other ages for *H. tenuis*, *H. salebrosus*, and *D. terebrans* (Figure 3.1).

Weekly temperature maxima ranged from 9 to 37 °C (48 to 99 °F) with an average of 25°C (77 °F), and the weekly temperature minima ranged from -4.4 to 25 °C (24 to 77 °F), with an average of 13 °C (55 °F) during the sampling period. Weekly precipitation peaked at 13.2 cm (5.2 inches), and averaged 2.3 cm (0.9 inches).

Comparison between captures in spring 2006 and 2007 (Figure 3.3) were similar for *D. terebrans* ($F_{1,8}$=7.37, p=0.03), and *H. salebrosus* ($F_{1,8}$=5.53, p=0.04). Comparisons between springs were dissimilar for *H. tenuis* ($F_{1,8}$ = 2.17, p=0.14), *Hb. pales* ($F_{1,8}$ =0.03, p=0.85), and *P. picivorus* ($F_{1,8}$=0.01, p=0.92).

Table 3.1. Most frequently captured root-feeding beetles, summed by age class.

	Age Class				
Insect species	<10 Years	10-19 Years	20-40 Years	>40 Years	Total
H. tenuis	409	332	369	616	1726
*H. salebrosus**	93	113	168	222	596
P. picivorus	82	184	122	179	567
Hb. pales	58	67	54	68	247
D. terebrans	16	55	47	97	215

*Includes *H. porculus*

Table 3.2. Other beetle species collected at Fort Benning, GA.

Taxon	Number of Captures
Curculionidae: Scolytinae	
Ipina	
Ips avulsus (Eichhoff)	67
Ips grandicollis (Eichhoff)	34
Ips calligraphus (Germar)	1
Orthotomicus caelatus (Eichhoff)	10
Xyleborina	
Xyleborinus saxesenii (Ratzeburg)	162
Xyleborus pubescens (Zimm.)	8
Xylosandrus compactus (Eichhoff)	7
Xylosandrus crassiusculus (Motschulsky)	84
Corthylina	
Gnathotrichus materiarius (Fitch)	22
Monarthrum mali (Fitch)	8
Curculionidae: Platypodinae	
Myoplatypus flavicornis (Fabricius)	4
Curculionidae: Molytinae	
Pissodes nemorensis Germar	4
Nitidulidae	
Colopterus unicolor (Say)	120
Cucujidae	35
Colydiidae	
Lasconotus spp. Erichson	883

Table 3.3. Summary statistics for repeated measures analysis for effects of age class and week of capture, August 2006-August 2007.

	Overall model			Age Class		Week	
Species	NMLRT*	p	AIC**	F(df)	p	F(df)	p
H. tenuis	130.84	<0.0001	6148	7.39 (3,28)	0.001	8.60 (50,1550)`	<0.0001
H. salebrosus	53.2	<0.0001	3598	3.67 (3,28)	0.02	4.87 (50,1550)	<0.0001
P. picivorus	81.47	<0.0001	4056	5.11 (3,28)	0.006	4.19 (50,1550)	<0.0001
Hb. pales	1.85	0.17	1349	1.97 (3,28)	0.14	1.74 (50,1550)	0.001
D. terebrans	77.52	<0.0001	2635	5.10 (3,28)	0.006	2.43 (50,1550)	<0.0001

*–Null Model Likelihood Ratio Test for overall model, χ^2_1 distributed. ** -Akaike Information Criterion

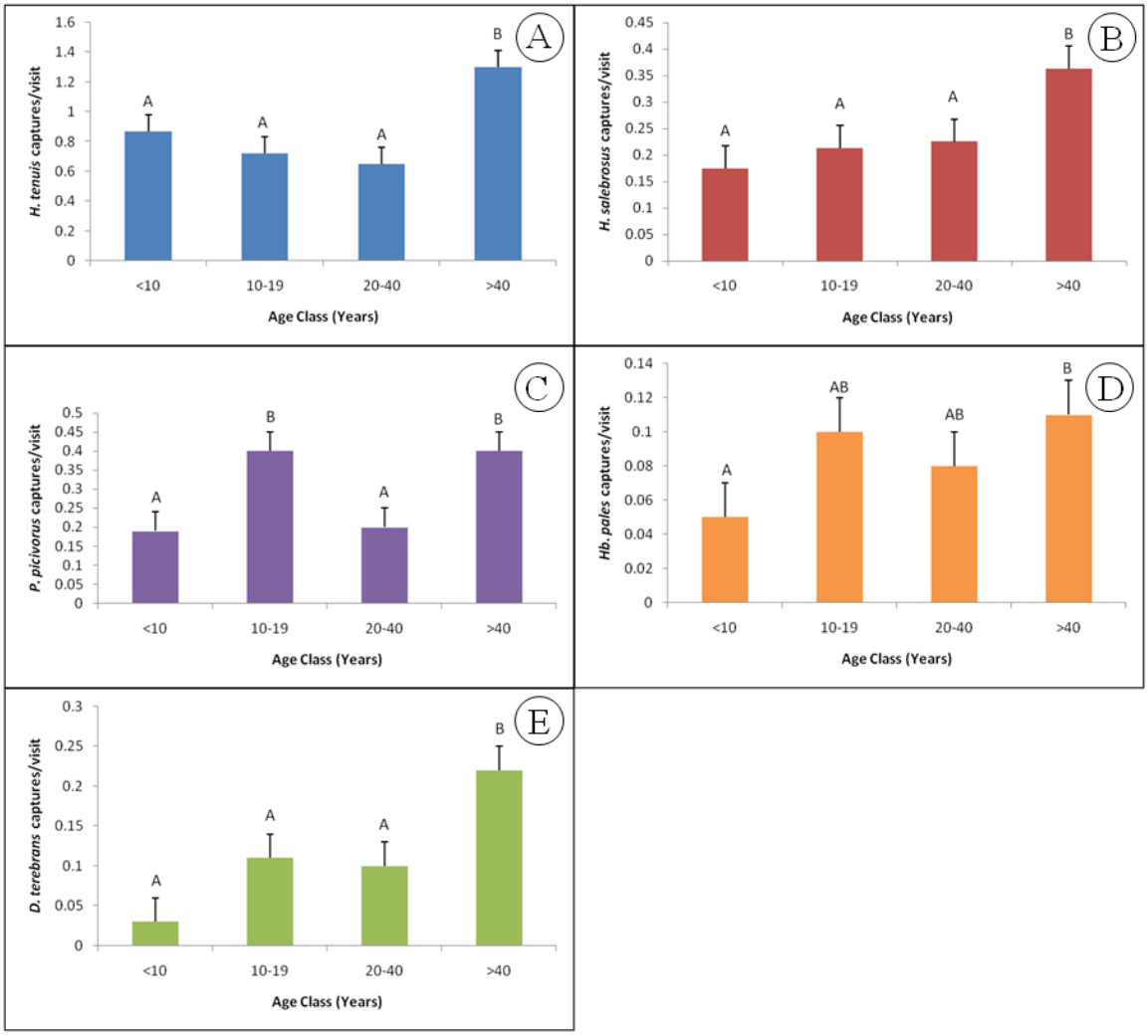

Figure 3.1. Mean number of captures per plot visit by age class, August 2006-August 2007. Columns within panes bearing the same letter are not significantly different at p=0.05. Mean separation by Tukey Kramer method. (A) *H. tenuis*, (B) *H. salebrosus*, (C) *P. picivorus*, (D) *Hb. pales*, (E) *D. terebrans*.

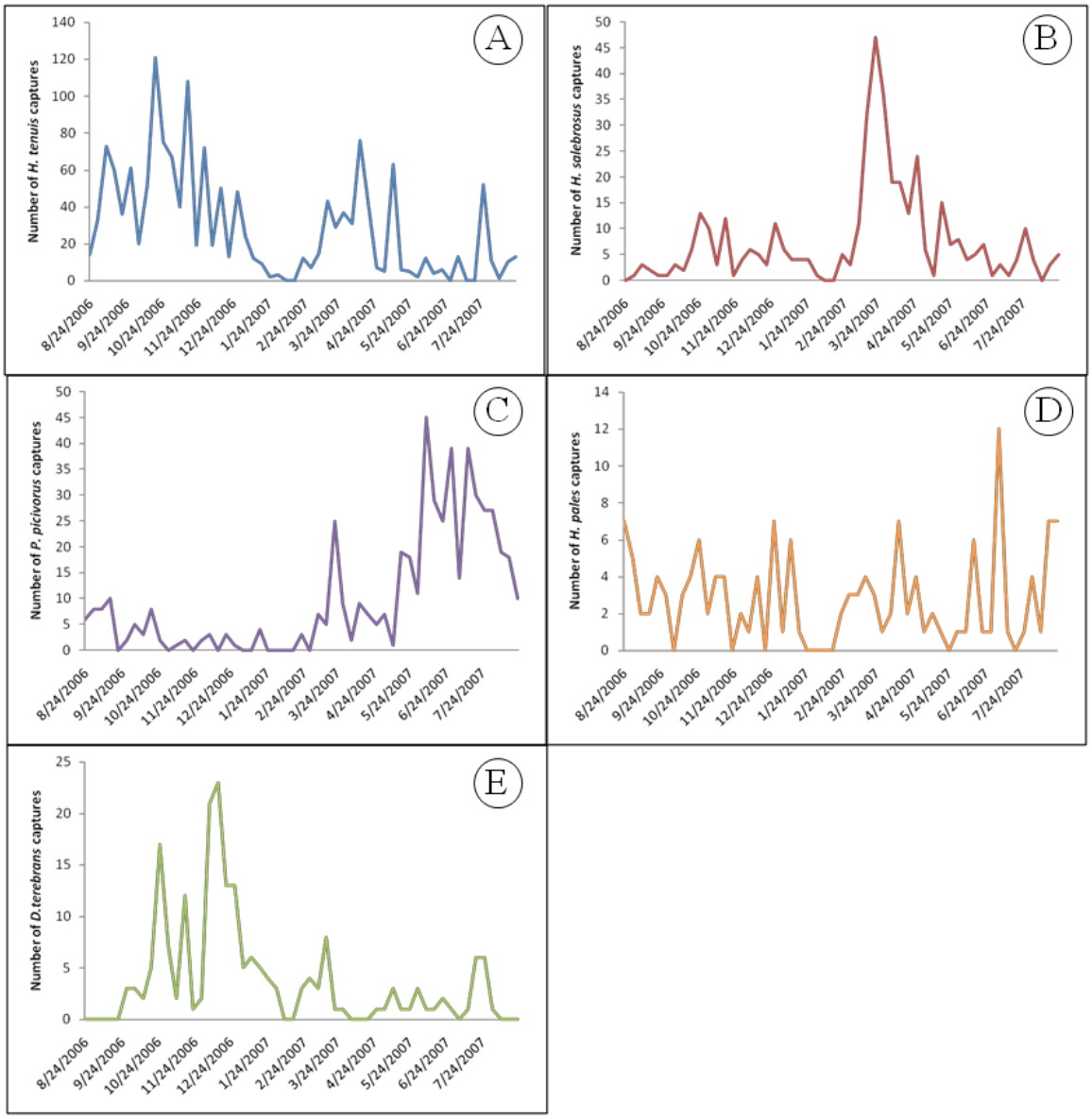

Figure 3.2. Weekly Captures of root-feeding beetles in longleaf pine stands at Fort Benning, GA from 24 August 2006-26 August 2007. (A) *H. tenuis*, (B) *H. salebrosus*, (C) *P. picivorus*, (D) *Hb. pales*, (E) *D. terebrans*

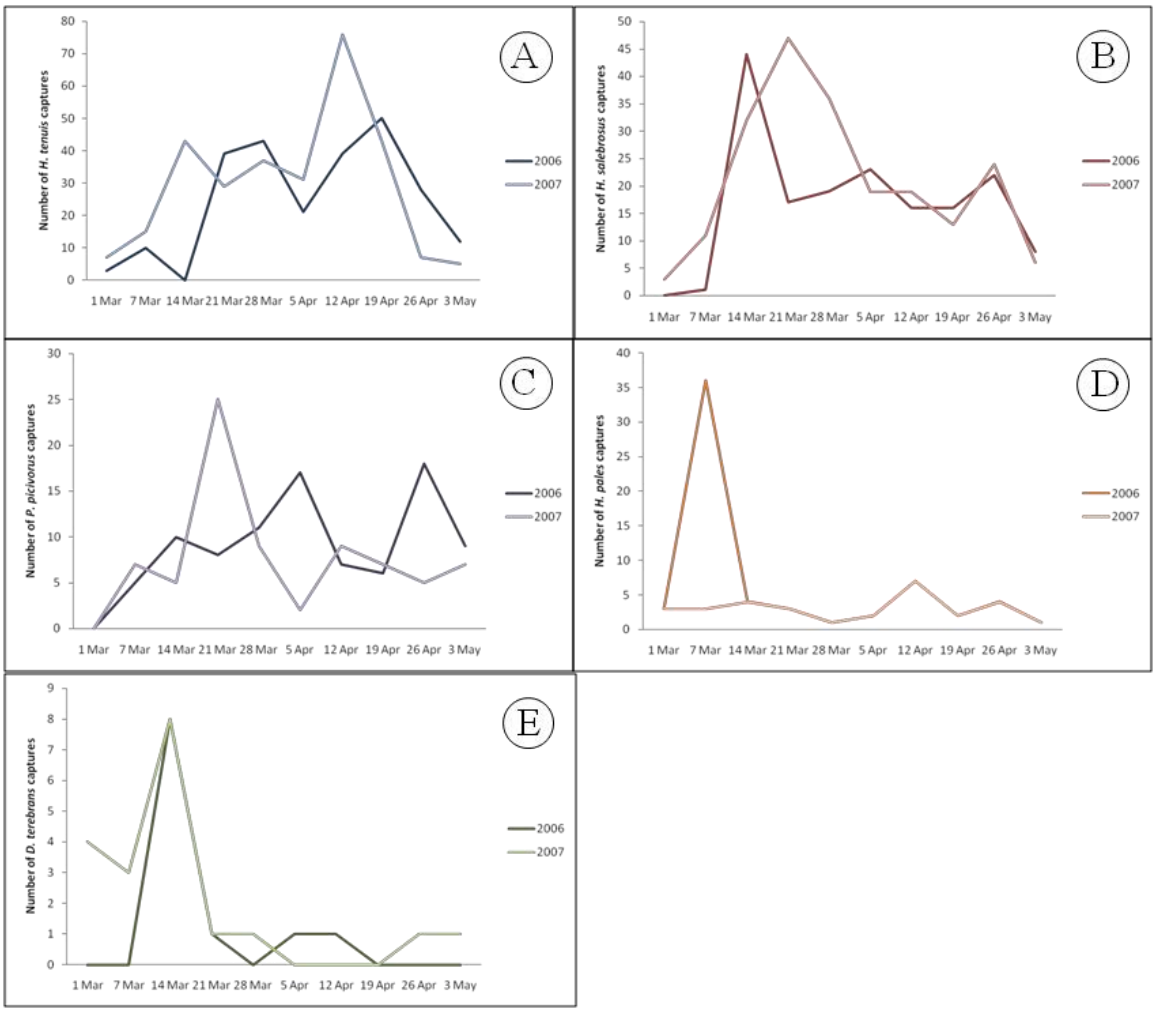

Figure 3.3. Weekly Captures of root-feeding beetles in longleaf pine stands at Fort Benning, GA, Spring (early March-early May) 2006 and 2007. (A) *H. tenuis*, (B) *H. salebrosus*, (C) *P. picivorus*, (D) *Hb. pales*, (E) *D. terebrans*

Vector captures peaked in spring and fall, with maxima of 100 vector captures in spring 2006, and 149 in fall 2006. All species were present during the spring trapping season in both years (Figure 3.3). Capture number dropped to zero for 3 weeks in February 2007, corresponding to temperature lows reported above. *Dendroctonus terebrans* was negatively correlated with weekly temperature maxima and minima (ρ= -0.368, p= 0.003 and ρ= -0.373, p= 0.003, respectively) which resulted in a strong positive correlation with winter trapping (ρ= 0.438, p=0.0004). While *Hylastes* spp. peaked in spring and fall, *H. tenuis* captures were higher in the fall (ρ= 0.552, p< 0.0001) and *H. salebrosus* captures peaked in the spring (ρ= 0.633, p< 0.0001, Figure 3.2 A,B). *Pachylobius picivorus* captures were positively correlated with temperature maxima and minima (ρ= 0.667, p< 0.0001 and ρ= 0.643, p< 0.0001, respectively), and were higher during the summer (ρ= 0.745, p< 0.0001, Figure 3.2 C), and lower in fall and winter (ρ= -0.285, p= 0.0249, ρ= -0.420, p= 0.0007, respectively). *Hylobius pales* captures were numerically higher, but not significantly so, in spring (ρ=0.214, p= 0.099).

The subset of *H. salebrosus* that were examined in the post-hoc diagnoses revealed the ratio of *H.salebrosus*:*H. porculus* to vary throughout the year. Spring and summer ratios ranged from 90% *H. salebrosus* (166:20 Hs:Hp) to 73% (60:13 Hs: Hp), but in winter the ratio shifted to 65% *H. porculus* (16:31 Hs:Hp). Differences between seasonal ratios were significant (χ^2_4 =.80.1, p <0.0001).

3.4.2. *Infestation with ophiostomatoid fungi*

The predominant species recovered from the principle beetle species were *L. procerum*, *L. terebrantis*, *G. huntii*, and *Pesotum* spp. (Table 3.4). Distribution of isolate recovery was similar between *Hylastes* spp. (χ^2_5= 5.58, p=0.35), and between weevils (χ^2_6= 8.14, p= 0.23). However, the distribution of isolate recovery from *D. terebrans* was different from both of these insect groups (χ^2_{14}= 623.33, p< 0.0001).

Overall, 27% of the major scolytine and molytine vectors yielded isolates of ophiostomatoid fungi, suggesting that these fungi are facultatively associated with their insect vectors. *Leptographium procerum* (24%), *G. huntii* (23%), *L. terebrantis* (20%), and pooled *Pesotum* spp. (16%) were the most frequently observed fungi infesting insects (Table 3.). The distribution of fungi was similar between the pine regeneration weevils, *H. pales* and *P. picivorus* (χ^2_6=8.14, p=0.23), and between *Hylastes* spp. (χ^2_5=5.58, p=0.35). The mycota of *D. terebrans* differed from the other most commonly captured beetles (χ^2_{14} =623.33, p<0.0001). Surprisingly, *D. terebrans* rarely yielded isolates of *L. terebrantis*, and had a higher frequency of recovering the *G. aureum*-like fungus and the *Ophiostoma ips*-like fungus (these last two likely new species, MJ Wingfield, personal communication). Other insects captured also yielded fungal isolates, although with decreased frequency relative to the predominant species (Table 3.5)

Table 3.4. Incidence of fungi isolated from exoskeletons of root-feeding curculionids at Fort Benning, GA.

Insect spp.(Spring 2006,Spring 2007,Total*)	*Lp***	*Lt*	*Ls*	*Ga*	*Gh*	*Oi*	*O.* spp.	*P.* spp.	Total by insect spp.	Percent infestation
H. tenuis (242,286,1726)	119	95	31	2	192	19	16	129	603	35
H. salebrosus (166,205,596)	11	15	1	0	26	17	3	13	86	14
P. picivorus (91,76, 567)	68	47	0	0	4	3	4	1	127	22
Hb. pales (98,26,247)	32	31	2	0	4	3	2	3	77	31
D. terebrans (11,15,215)	4	5	0	26	2	33	6	10	86	40
Total by fungal spp.	234	193	34	28	228	75	31	156	979	

*Total includes both springs (early Mar-early May), and Fall 2006-Fall2007 (late Aug 2006-late Aug 2007) data, ***Lp=L. procerum*, *Lt= L. terebrantis*, *Ga= G. aureum*-like, *Ls= L. serpens*, *Gh= G. huntii*, *Oi=O. ips*-like, *O.* spp. = other pooled *Ophiostoma* spp., *P.* spp.= pooled *Pesotum* spp.

Table 3.5. Isolates of ophiostomatoid fungi recovered from other beetle species at Fort Benning, GA.

Insect Species	*Lp*	*Lt*	*Ga*	*Oi*	*O.* spp.	*P.* spp.	Total
Ips spp.	1	2	4	7	3	14	102
O. caelatus	0	0	0	1	0	1	10
X. saxesenii	1	0	1	0	0	0	162
X. crassiusculus	1	0	0	0	1	1	84
G. materiorius	0	0	0	0	0	1	22
C. unicolor	0	1	0	0	1	0	127
Lasconotus spp.	0	0	0	19	1	2	883

Lp=L. procerum, *Lt= L. terebrantis*, Ga= *G. aureum*-like, *Ls= L. serpens*, *Oi=O. ips*-like, *O.* spp. = other pooled *Ophiostoma* spp., *P.* spp.= pooled *Pesotum* spp.

Table 3.6. Correlation table between weather variables and insect-derived fungal isolates. For each cell, the upper value is the Pearson Correlation Coefficient (ρ), and the lower value is the probability, p, of the absolute value of ρ=zero. Significant values ($p<0.05$) of ρ are indicated in bold and marginal values ($p<0.1$) are italicized.

	Lp	*Lt*	*Ls*	*Gh*	*Ga*	*Oi*	*O.* spp.	*P.* spp.	Insects
Weekly Max	0.19736	-0.00071	-0.04624	0.00605	-0.06092	*-0.23445*	0.11978	-0.09182	0.10169
Temp	0.1242	0.9956	0.7212	.9628	0.6381	*0.0666*	0.3538	0.4779	0.4316
Weekly Min	**0.27638**	-0.02521	-0.01137	0.03184	-0.03488	**-0.32334**	0.06045	-0.06965	0.07036
Temp	**0.0297**	0.8458	0.9301	0.8059	0.7878	**0.0104**	0.6407	0.5906	0.5869
Weekly	0.12075	-0.12445	-0.00524	-0.12050	0.02175	-0.13169	-0.13810	-0.15595	0.16807
Precipitation	0.3498	0.3352	0.9678	0.3509	0.8668	0.3076	0.2844	0.2261	0.1916

******Lp=L. procerum*, *Lt= L. terebrantis*, *Ga= G. aureum*-like, *Ls= L. serpens*, *Gh= G. huntii*, *Oi=O. ips*-like, *O.* spp. = other pooled *Ophiostoma* spp., *P.* spp.= pooled *Pesotum* spp.

Leptographium procerum isolates from insects were positively correlated with high weekly temperature minima (ρ= 0.197, p= 0.0297) and were most frequently collected during fall 2006 (ρ= 0.307, p= 0.0151). *Leptographium procerum* isolations were negatively correlated with the winter collection period (ρ= -0.279, p= 0.0278). *Leptographium terebrantis* isolates from insects were positively correlated with spring (ρ= 0.375, p= 0.0027), and negatively with summer and winter (ρ= -0.403, p= 0.0012, and ρ= -0.258, p= 0.043, respectively). *Ophiostoma* spp. isolates (excluding *O. ips*-like) were positively correlated with spring (ρ= 0.337, p= 0.0075) and negatively correlated with winter (ρ= -0.301, p= 0.013). *Ophiostoma ips*-like isolates were negatively correlated with weekly temperature minima (ρ= -0.323, p= 0.0104), though there were no correlations with season. Isolations of the *G.aureum*-like fungus were positively correlated with the fall (ρ= 0.259, p= 0.0417).

3.5. Discussion

Root-feeding insects are present throughout most of the year at FB, and carry a suite of fungi that include species previously unreported in the region. Several studies have examined the bark beetle and regeneration weevil fauna of southeastern forests (excluding southern pine beetle) (Fatzinger, 1985; Sullivan *et al.*, 2003; Campbell *et al.*, 2008a; Campbell *et al.*, 2008b). A smaller number have examined the ophiostomatoid mycota of these forests (Barnard *et al.*, 1991; Otrosina *et al.*, 1999).

Few studies have directly considered the relationship between phloeophagous root-feeding curculionids and their mycota in the southeastern U.S. (Hanula *et al.*, 2002; Eckhardt *et al.*, 2007; Menard, 2007).

Beetles were present during 59 of 62 weeks of sampling, indicating that root-feeding beetles and their fungi are active at FB almost year-round. In previous studies of the mycota of root-feeding beetles, collections were restricted to the spring, from early March to May (Eckhardt *et al.*, 2007; Menard, 2007). The results presented here indicate that year round sampling is necessary to assess population peaks in the curculionid fauna.

Numbers of insect captures in this study were lower than in studies using other types of traps, such as stovepipe traps (Fatzinger, 1985), or multiple funnel traps (Hanula *et al.*, 2002; Sullivan *et al.*, 2003). However, the need for live insects in order to maximize fungal isolation success precluded the use of lethal traps. Therefore direct comparisons can be made to studies in loblolly pine (Eckhardt *et al.*, 2007; Menard, 2007), with similar plot layouts, identical traps, and the latter study also performed at FB.

In general, the numbers of vector captures were similar to predicted asymptomatic plots in studies of loblolly pine decline (Eckhardt *et al.*, 2007; Menard, 2007). The number of *H. tenuis* captures was greater than the larger *Hylastes* spp. in contrast with other studies where the larger insects were predominant (Bauman, 2003; Eckhardt *et al.*, 2007) or similar in number (Menard, 2007). *Pachylobius picivorus* outnumbered *Hb. pales* by approximately 5:2. The range of these weevils overlaps to a great extent, with *Hb. pales* being predominant throughout much of that area, but the ratios are known to be

shifted in favor of *P. picivorus* in the southeast (Nord *et al.*, 1984). Fluctuations in the ratio of these weevils have been found in other studies in the southeastern U.S., with *P. picivorus* more abundant in some cases (Sullivan *et al.*, 2003; Menard, 2007), *Hb. pales* in others (Hanula *et al.*, 2002), or fluctuating between the two species (Eckhardt *et al.*, 2007). While correlations with weather observed in *D. terebrans* and *P. picivorus* were strong, the single year's data is not definitive, as other year-round studies have shown (Sullivan *et al.*, 2003). However, the occurrence of a winter peak in *D. terebrans* (and *H. porculus*) in the southeast reemphasizes the need for year-round survey for these species.

The overall isolation rate of ophiostomatoid fungi in the present study was lower than in previous studies (Eckhardt *et al.*, 2007). The proportion of fungal isolations from insects suggests niche overlap between the regeneration weevil species and between *Hylastes* spp. The rarity of *L. terebrantis* on its eponymous host was unexpected, as a previous study has shown 90% infestation (Eckhardt *et al.*, 2007), compared with approximately 2% in the present study. *Grosmannia huntii* and *L. serpens* were isolated more frequently from *Hylastes* spp. *Leptographium serpens*, previously reported from loblolly pine and associated insects in Alabama (Eckhardt *et al.*, 2007), was isolated infrequently from insects (<1% of all major vectors and 3.5% of all insect isolates). *Lasconotus* sp. was a frequently captured insect in the present study, and yielded the *O. ips*-like fungus. While little is known of the biology of these beetles in southeastern forests, its association with this fungus suggests these insects rarely share habitat with *Hylastes* spp. and regeneration weevils.

Pesotum spp. are cycloheximide tolerant fungi formerly assigned to the form-genus *Graphium*, and are insect vectored via similar structures to *Ophiostoma* and *Leptographium* (viz. sticky spores and elongate spore-bearing structures). *Pesotum* spp. are not known to be pathogenic, although members of the *Ophiostoma ulmi* complex do produce *Pesotum* anamorphs. As these fungi are not considered pathogenic, their role has been downplayed in previous studies with this pathosystems, and a rigorous attempt to identify isolates to species level was not pursued in this study. Future study may reinforce the observed niche differences between insect taxa in these fungi as well, and they might play an important role in longleaf pine decline (see Chapter II). *Ophiostoma* isolates were rarely recovered from beetles in this study. However, many of these isolates were associated with a *Sporothrix* anamorph, suggesting they may be members of the *Ophiostoma pluriannulatum* complex. Member of this complex have previously been found in the southeastern U.S., including *O. pluriannulatum* (Hedgcock) Syd & P. Syd. and *O. multiannulatum* (Hedgcock & Davidson) Hendr., and are associated with blue-stain of logs and lumber (Hedgcock, 1906; Davidson, 1935).

Grosmannia huntii, most frequently found on *Hylastes* spp., is here first reported from insects in the region. This fungus produces serpentine hyphae which may have led to confusion with *L. serpens*. Both fungi have only recently been found in the southeastern U.S., and the dominance of these fungal species on insects suggests that they may be recent introductions. Also, given their distinctive cultural character, their absence

from previous studies of ophiostomatoid fungi in the southeastern U.S. reinforces the hypothesis of recent introduction. The role of these fungi in pine decline in the southeast also merits future study.

Fungal recovery from insects varied by season. These trends followed host affinities in some cases; *O. ips*-like isolates were correlated with winter, as were *D. terebrans* captures which was the predominant vector for the *O. ips*-like fungus. In other cases, fungal isolations appeared to be less host-dependent; *L. terebrantis* isolations were positively correlated with spring, though vector captures overall were greater in fall. Whether temperature effects on seasonal changes are caused by temperature directly, or may result from seasonal changes in host phenology or vector behavior is not testable from the data presented. Further examination of seasonal effects on the transit of ophiostomatoid fungi would be of benefit to land managers in planning forestry activities such as thinning or harvesting.

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3.7. Literature Cited

Barnard, E.L., Gilly, S.P., Dixon, W.N., 1991. Incidence of *Heterobasidion annosum* and other root-infecting fungi in residual stumps and roots in thinned slash pine plantations in Florida. Plant Disease 75, 823-828.

Bauman, T.A., 2003. Interaction of fire and insects in the restoration and management of longleaf pine. MS Thesis, Louisiana State University, Baton Rouge, LA. 72 p.

Campbell, J.W., Hanula, J.L., Outcalt, K.W., 2008a. Effects of prescribed fire and other plant community restoration treatments on tree mortality, bark beetles, and other saproxylic Coleoptera of longleaf pine, *Pinus palustris* Mill., on the Coastal Plain of Alabama. Forest Ecology and Management 254, 134-144.

Campbell, J.W., Hanula, J.L., Waldrop, T.A., 2008b. Effects of prescribed fire and fire surrogates on saproxylic Coleoptera in the Southern Appalachians of North Carolina. Journal of Entomological Science 43, 57-75.

Davidson, R.W., 1935. Fungi causing stain in logs and lumber in the southern states, including five new species. Journal of Agricultural Research 50, 789-807.

Eckhardt, L.G., Goyer, M.A., Klepzig, K.D., Jones, J.P., 2004a. Interactions of *Hylastes* species (Coleoptera : Scolytidae) with *Leptographium* species associated with loblolly pine decline. Journal of Economic Entomology 97, 468-474.

Eckhardt, L.G., Jones, J.P., Klepzig, K.D., 2004b. Pathogenicity of *Leptographium* species associated with loblolly pine decline. Plant Disease 88, 1174-1178.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

Fatzinger, C.W., 1985. Attraction of the black turpentine beetle (Coleoptera, Scolytidae) and other forest Coleoptera to turpentine-baited traps. Environmental Entomology 14, 768-775.

Goheen, D.J., Cobb, F.W., 1978. Occurrence of *Verticicladiella wageneri* and its perfect state, *Ceratocystis wageneri* sp nov in insect galleries. Phytopathology 68, 1192-1195.

Hanula, J.L., Meeker, J.R., Miller, D.R., Barnard, E.L., 2002. Association of wildfire with tree health and numbers of pine bark beetles, reproduction weevils and their associates in Florida. Forest Ecology and Management 170, 233-247.

Hedgcock, G.G., 1906. Studies upon some chromogenic fungi which discolor wood. Mo. Bot. Gard. Ann. Rept. 17, 59-114.

Hicks, B.R., Cobb, F.W., Gersper, P.L., 1980. Isolation of *Ceratocystis wageneri* from forest soil with a selective medium. Phytopathology 70, 880-883.

Jacobs, K., Wingfield, M.J., 2001. *Leptographium* species : tree pathogens, insect associates, and agents of blue-stain. APS Press, St. Paul, Minn.

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Science 37, 1119-1139.

Klepzig, K.D., Smalley, E.B., Raffa, K.F., 1995. *Dendroctonus valens* and *Hylastes porculus* (Coleoptera, Scolytidae) - vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. Great Lakes Entomologist 28, 81-87.

Malloch, D., Blackwell, M., 1993. Dispersal biology of the ophiostomatoid fungi. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*:

taxonomy, ecology and pathogenicity. American Phytopathological Society Press, St. Paul, MN, pp. 195-206.

Menard, R.D., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. MS Thesis, Louisiana State University, Baton Rouge, LA.

Nevill, R.J., Alexander, S.A., 1992. Root- and stem colonizing insects recovered from eastern white pines with procerum root disease. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 22, 1712-1716.

Nord, J.C., Ragenovich, I., Doggett, C.A., 1984. Pales weevil. U.S.D.A . Forest Service, Southeastern Region State & Private Forestry, Forest Insect and Disease Leaflet No. 104. Asheville, NC.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant and Soil 217, 145-150.

Reay, S.D., Walsh, P.J., Ram, A., Farrell, R.L., 2002. The invasion of *Pinus radiata* seedlings by sapstain fungi, following attack by the Black Pine Bark Beetle, *Hylastes ater* (Coleoptera : Scolytidae). Forest Ecology and Management 165, 47-56.

SAS Institute, 2003. SAS Version 9.1. Cary, NC.

Seifert, K.A., 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. American Phytopathological Society Press, St Paul, MN, pp. 141-151.

Six, D.L., 2003. Bark Beetle-Fungus Symbioses. In: Bourtzis, K., Miller, T.A. (Eds.), Insect Symbiosis. CRC Press, Boca Raton, FL, pp. 99-116.

Sullivan, B.T., Fettig, C.J., Otrosina, W.J., Dalusky, M.J., Berisford, C.W., 2003. Association between severity of prescribed burns and subsequent activity of conifer-infesting beetles in stands of longleaf pine. Forest Ecology and Management 185, 327-340.

Wagener, W.W., Mielke, J.L., 1961. A staining fungus root disease of ponderosa, jeffrey and pinyon pines. Plant Disease Reporter 45, 831-835.

Wingfield, M.J., 1986. Pathogenicity of *Leptographium procerum* and *Leptographium terebrantis* on *Pinus strobus* seedlings and established trees. European Journal of Forest Pathology 16, 299-308.

Witcosky, J.J., Schowalter, T.D., Hansen, E.M., 1986. *Hylastes nigrinus* (Coleoptera, Scolytidae), *Pissodes fasciatus*, and *Steremnius carinatus* (Coleoptera, Curculionidae) as vectors of black-stain root disease of Douglas-fir. Environmental Entomology 15, 1090-1095.

Zhou, X.D., de Beer, W., Wingfield, B.D., Wingfield, M.J., 2002. Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. Fungal Diversity 10, 229-240.

Zhou, X.D., de Beer, Z.W., Ahumada, R., Wingfield, B.D., Wingfield, M.J., 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Diversity 15, 261-274.

Zhou, X.D., de Beer, Z.W., Wingfield, B.D., Wingfield, M.J., 2001. Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. Sydowia 53, 290-300.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Studies in Mycology, 75-97.

CHAPTER IV. CONFIRMATION OF THE PRESENCE OF *GROSMANNIA HUNTII* AND *LEPTOGRAPHIUM SERPENS* IN THE SOUTHEASTERN UNITED STATES.

4.1. Abstract

Grosmannia huntii and *Leptographium serpens* (Ascomycota: Ophiostomataceae) are both known to infect *Pinus* spp. and are associated with bark beetle and weevil vectors. Both fungi have been reported from areas where pine species have been introduced. Their presence in the southeastern U.S. have only recently been documented by their distinctive morphology. Previous reports of *L. serpens* from the U.S. have been challenged, and reports of *G. huntii*, originally described from Canada, have not been confirmed in the southeastern U.S. In this study DNA sequence data from the partial ITS ribosomal DNA operon, β-tubulin and elongation factor 1-α genes were collected from isolates of these two fungi from the southeastern U.S. and other localities and compared to previously published sequences. As these fungi both produce serpentine hyphae potentially leading to confusion, a PCR-RFLP based diagnostic tool was developed using sequence data from the β-tubulin gene. Phylogenetic analysis confirms the presence of *L. serpens* and *G. huntii* in the southeastern U.S.

4.2. Introduction

Ophiostomatoid fungi are commonly known as blue-stain fungi, and include some known pathogens and saprogens (Wingfield *et al.*, 1993). These fungi have been implicated in important tree diseases including Dutch elm disease (causal agents, *Ophiostoma ulmi* [Buismann] Nannf. and *O. novo-ulmi* Brasier) (Brasier, 1991), black stain root disease (causal agent, *Leptographium wagerneri* [Kendrick] Wingfield sensu lato) (Wagener and Mielke, 1961; Kendrick, 1962), as well as in declines in several pine species (contributing agents including *L. procerum* (Kendrick) Wingfield, *L. terebrantis* Barras and Perry, and *L. serpens* (Goid.) Siemaszko) (Wingfield, 1986; Klepzig *et al.*, 1991; Eckhardt *et al.*, 2007). These fungi rely upon insects to vector them, typically bark beetles (Coleoptera: Curculionidae, Scolytinae) and regeneration weevils (Coleoptera: Curculionidae, Molytinae) (Klepzig *et al.*, 1995; Eckhardt *et al.*, 2007).

Leptographium serpens was originally described from Italy as a novel species of the newly circumscribed genus *Grosmannia*, though no direct reference to the range of the species was indicated (Goidanich, 1936). More recent research from Italy showed *L. serpens* to be associated with root disease in *P. pinea* L. near Tombolo (Lorenzini and Gambogi, 1976). The fungus has been recovered from bark beetles in the United Kingdom (Wingfield and Gibbs, 1991) and Poland (Siemaszko, 1939) cited in (Harrington, 1988) and from pines elsewhere in Western Europe including France, Spain, and Portugal (de Ana Magan, 1982; Morelet, 1988; Jacobs and Wingfield, 2001).

A fungus producing serpentine hyphae has also been recovered from *P. radiata* D. Don and *P. pinaster* Aiton plantations in South Africa (Wingfield and Knox-Davies,

1980; Wingfield and Marasas, 1980, 1981; Zhou *et al.*, 2002), with trees exhibiting a similar symptomatology to those found in Italy. This fungus was described as a new species, *Verticicladiella alacris* Wingfield and Marasas, and then synonymized with *L. serpens* (Wingfield and Marasas, 1981) based on the presentation of new data on conidial development from Italian isolates (Gambogi and Lorenzini, 1977). In South Africa, it is reasonable to conclude that *L. serpens* is introduced, as the hosts and the insect vectors are known to have been introduced, and unlike European reports, the teleomorph has not been reported from South Africa. In reports from Europe, the sexual stage of *L. serpens* (*Grosmannia serpens* Goid.) has been observed and described (Goidanich, 1936; de Ana Magan, 1982; Morelet, 1988). In South Africa perithecia have not been observed in *L. serpens* (M.J. Wingfield, personal communication).

The fungus has been reported from the southeastern U.S. (Zambino and Harrington, 1992), although earlier reports from elsewhere in North America are dubious (Harrington, 1988). In contrast with ophiostomatoid species frequently collected from the eastern U.S., including *L. procerum* and *L. terebrantis*, *L. serpens* causes a distinctive symptomatology on loblolly pine, and appears to be more aggressive than the former two species (Eckhardt *et al.*, 2004). Several scolytine beetles were reported to vector *L. serpens*, including some ambrosia beetles (Eckhardt *et al.*, 2007). As in South Africa, perithecia have not been observed in isolates collected in the southeastern U.S. (Eckhardt, personal communication, author's observations).

Grosmannia huntii (Robinson-Jeffrey) Zipfel, de Beer and Wingfield was originally described from lodgepole pine (*P. contorta* Douglas ex. Louden var. *latifolia* Engelmann ex. S. Watson) and associated with mountain pine beetle (*Dendroctonus*

monticolae Hopkins), and was noted as having "sinuous" hyphae (Robinson-Jeffrey and Grinchenko, 1964). Subsequent survey expanded the range in the U.S. to include Arizona, Colorado, and New York (Davidson and Robinson-Jeffrey, 1965). Like *L. serpens, G. huntii* has been reported from several countries including the United Kingdom, (Gibbs and Inman, 1991) Portugal, and Italy (Jacobs *et al.*, 1998), as well as several Pacific nations where it is thought to have been introduced via the exotic bark beetle *Hylastes ater* Paykill (New Zealand, Australia, and Chile), or infected logs (Korea) (Jacobs *et al.*, 1998; Kim *et al.*, 2005; Thwaites *et al.*, 2005).

Both of these species may produce serpentine hyphae in cultures that have been freshly isolated from pine roots, and may be difficult to distinguish. Conidiophore development may be highly variable; *L. serpens* typically produces many robust conidiophores, although this character is not consistent. *Grosmannia huntii*, by contrast, produces conidiophores with short stipes, produced on aerial hyphae. Other researchers have compared *G. huntii* with morphologically similar species, to find that some records of this species were misidentified, leading to a new species descriptions (Jacobs *et al.*, 1998). In that study they note that *G. huntii* is heterothallic, further complicating identification of isolates from single conidia or hyphal tips that do not form perithecia readily in culture.

As previous accounts of these fungi in the U.S. have been questioned (Harrington, 1988) and the present reports expand the previously described range, the purpose of this study was to confirm the identity of these isolates using DNA-sequence based diagnostics. As these fungi share a niche within the region of interest (Chapter II), and given potential differences in their pathogenicity, abundance and distribution, an

inexpensive and reliable assay to differentiate these species is necessary. While *Grosmannia* and *Leptographium* represent a monophyletic lineage within the Ophiostomataceae, the two species under consideration are not closely related congeners (Zipfel *et al.*, 2006), suggesting that a phenetic character such as PCR-RFLP can be used as a robust marker in distinguishing *L. serpens* and *G. huntii*.

4.3. Methods and Materials

4.3.1. *Isolates*

Isolates used in this study are listed in Table 4.1. For isolates from the collection of Dr. Lori Eckhardt, cultures were derived from roots and insects as described in Chapter II (this dissertation) and (Eckhardt *et al.*, 2007). Other isolates were withdrawn from the culture collection of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria (South Africa). All isolates were grown on 2% Malt Extract Agar (MEA, 20 g L^{-1} malt extract, 15 g L^{-1} agar), and a subset also grown on Pine Twig Agar (PTA, 15 g L^{-1} agar with twice autoclaved *Pinus patula* L. twigs embedded) and Oatmeal Agar (OA, 30 g L^{-1} Jungle Brand Oats in dH_2O, decanted and added to 15 g L^{-1} agar).

Table 4.1. *Leptographium serpens* and *Grosmannia huntii* isolates used for sequence data.

Isolate*		Species	Provenance	Region	Host/Vector
CMW	60	*L serpens*	Western Cape, South Africa	Africa	*Pinus pinaster*
CMW	185	*G huntii*	Australia	Pacific	
CMW	289	*L serpens*	Italy	Europe	
CMW	290	*L serpens*	Italy	Europe	
CMW	305	*L serpens*	Italy	Europe	**
CMW	310	*L serpens*	Western Cape, South Africa	Africa	*P. radiata*
CMW	455	*G huntii*	British Columbia, Canada	N. America	*P. contorta* var. *latifolia***
CMW	745	*L serpens*	Spain	Europe	*P. pinaster*
CMW	746	*L serpens*	France	Europe	*P. pinaster*
CMW	748	*L serpens*	France	Europe	*P. pinea*
CMW	1136	*L serpens*	Holly Springs, Mississippi	N. America	*P. taeda*
CMW	1376	*L serpens*	England	Europe	*H. ater (Pinus)*
CMW	1790	*G huntii*	United Kingdom	Europe	*Tomicus piniperda*
CMW	2320	*L serpens*	Dominican Republic	N. America	*P. occidentalis*

* Isolates prefixed by: CMW are from the culture collection of the Forestry and Agricultural Biotechnology Institute, CLE are from the culture collection of Dr. Lori Eckhardt at The School of Forestry and Wildlife Sciences, Auburn University, AL, CJZ are from the culture collection of James Zanzot, graduate student of Dr. Lori Eckhardt.

** denotes ex-type isolate.

Table 4.1. Continued

Isolate		Species	Provenance	Region	Host/Vector
CMW	6187	*L serpens*	Mpumalanga, South Africa	Africa	*P. patula*
CMW	10767	*G huntii*	Valdivia, Chile	Pacific	*P. radiata/H. ater*
CMW	25936	*L serpens*	Spain	Europe	
CLE	46	*L serpens*	Oakmulgee Ranger District, Alabama	N. America	*P. taeda roots*
CLE	60	*L serpens*	Oakmulgee Ranger District, Alabama	N. America	*P. taeda roots*
CLE	88	*L serpens*	Holly Springs Ranger District, Mississippi	N. America	*P. taeda roots*
CLE	93	*L serpens*	Holly Springs Ranger District, Mississippi	N. America	*P. taeda roots*
CLE	114	*L serpens*	Holly Springs Ranger District, Mississippi	N. America	*P. taeda roots*
CLE	120	*G huntii*	Oakmulgee Ranger District, Alabama	N. America	*P. taeda roots*
CLE	232	*G huntii*	Oakmulgee Ranger District, Alabama	N. America	*P. taeda roots*
CLE	235	*G huntii*	Oakmulgee Ranger District, Alabama	N. America	*P. taeda roots*
CLE	302	*L serpens*	Fort Benning Military Reservation, Georgia	N. America	*P. taeda roots*
CLE	349	*L serpens*	Fort Benning Military Reservation, Georgia	N. America	*P. taeda roots*
CLE	352	*L serpens*	Fort Benning Military Reservation, Georgia	N. America	*P. taeda roots*
CJZ	170	*G huntii*	Fort Benning Military Reservation, Georgia	N. America	*H. tenuis*
CJZ	178	*G huntii*	Fort Benning Military Reservation, Georgia	N. America	*H. tenuis*

4.3.2. *Preparation of genomic DNA, PCR amplification and RFLP analysis*

Axenic cultures of the *Grosmannia* and *Leptographium* isolates noted in Table 3.1 were grown for 2-3 weeks on MEA, and approximately 10 mg of mycelium scraped from the surface with a sterile pipette tip and transferred to a sterile 1.5 mL Eppendorf tube with 100 μL of PrepMan Ultra reagent (Applied Biosystems Inc, Foster City, CA), and ground with an acid-washed, sterilized pestle and a trace of sterilized sand. The resultant slurry was heated to 95 °C for 10 minutes, and centrifuged at 13,000 rpm (22 °C) for 5 minutes. Approximately 50 μL of the supernatant was transferred to a sterile Eppendorf tube, and concentration and quality of DNA measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Typically, this method would yield approximately 100 ng/μL of DNA, with an A260:A280 ratio between 1.7 and 2.0. Genomic DNA preparations were used in PCR reactions to amplify three gene regions: Nuclear ribosomal DNA (SSU, ITS2, and LSU) was amplified using the primers ITS3 and LR3 (White *et al.*, 1990), partial β-tubulin (βt) gene using the forward primer T10 (5′-ACGATAGGTTCACCTCCAGAC-3′) (O'Donnell and Cigelnik 1997) with either BT12 (5′-GTTGTCAATGCAGAAGGTCTCG-3′) (Kim et al. 2003), or BT2b (5'-ACCCTCAGTGTAGTGACCCTTGGC -3') (Glass and Donaldson 1995) as the reverse primer, and partial transcription elongation factor 1-α (EF-1α) using the primers EF1F (5'-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs *et al.*, 2004).

Amplification reactions were performed using the following PCR protocol: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for seven min. Successful amplification was visualized on a 1% agarose gel electrophoresed at 80V for 20 min.

A subset of these amplicons were processed for sequencing using the HighPure PCR CleanUp kit (Roche Pharmaceuticals, Basel, Switzerland). Amplicons were then sequenced using the Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems Inc., Foster City, CA) with the primers noted above, and analyzed on an ABI PRISIM™ 377 or ABI PRISIM™ 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Each amplicon was sequenced using the forward and reverse primers.

4.3.3. *Phylogenetic analyses*

Electropherograms of the resolved sequences were visualized and ambiguous nucleotides edited, and contigs of forward and reverse sequences compiled using MEGA version 4.0.1 (Tamura *et al.*, 2007). Sequences were subjected to BLAST searches in NCBI GenBank to determine similarity to published sequences. *Leptographium* spp. sequences were downloaded and data sets compiled in MEGA 4.0.1, using the data set from Jacobs *et al.*, (2006) as representative of the genus. Alignments were performed using the E-INS-I strategy in the online version of MAFFT version 6 (Katoh and Toh, 2008). Aligned data sets were analyzed using maximum parsimony (MP), maximum

likelihood (ML), and neighbor joining (NJ) in PAUP* version 4.0beta (Sinauer Associates, Sutherland, MA). Models of nucleotide substitution for ML and NJ analyses were performed using ModelTest (Posada and Crandall, 1998). For MP analyses, the heuristic search option was employed with100 additional sequence replicates. For all datasets, 1000 bootstrap replicates were performed to test branch strength.

4.3.4. *Population genetics statistics*

To examine the relationship between globally distributed isolates of the fungi, summary statistics were performed using the program DnaSP version 5.00.04 (Librado and Rozas, 2009). Sequence sets were delimited for isolates from Europe and the U.S. Summary statistics could not be calculated for South African isolates as these were fewer than 4. The number of haplotypes, number of private alleles, haplotypic diversity (Hd) (Nei, 1987), and nucleotide diversity (π) were determined.

4.3.5. *PCR-RFLP development*

Sequences (confirmed in GenBank) of *L. serpens* and *G. huntii* were then entered into the Webcutter 2.0 web-based application, (available at http://rna.lundberg.gu.se/cutter2/) (Heiman, 1997) with all enzymes in the database selected. Webcutter identified and mapped restriction enzymes predicted to digest the input sequence. Output from Webcutter was compared visually to identify candidate

enzymes that could discriminate between the two species and generate scorable restriction fragments. Selection of potential restriction enzymes was biased towards those that were most readily available from suppliers at the lowest cost per unit.

Amplicons were digested with the restriction enzymes PstI and SacI (Fermentas International, Burlington, Ontario), using a protocol modified slightly from the manufacturer's recommendation for digestion of PCR products directly after amplification. The principal modification was to adjust the amount of PCR product used to optimize visualization of the digestion. Both enzymes used have optimal activity at 37 °C, and digestions were incubated for 15 hours (overnight) to ensure complete digestion, and the resulting reaction visualized on a 1% agarose gel, compared with the undigested amplicon.

4.4. Results

4.4.1. *Isolates*

Older isolates (> 30 years since isolation) of these fungi were often degenerate, having lost pigmentation and the ability to produce conidia or perithecia. None of the isolates of *L. serpens* examined produced perithecia in culture on malt extract agar, pine twig agar, or oatmeal agar. Most *L. serpens* isolates produced typical conidiophores and conidia on pine twig agar.

Fresh isolates of *G. huntii* from the southeastern U.S. produced abundant serpentine hyphae, and typically produced more aerial hyphae than isolates of *L. serpens*. Conidiophores of *G. huntii* were typically shorter and less robust than *L. serpens*, and perithecia were occasionally produced by *G. huntii*.

4.4.2. *DNA sequencing*

The primers ITS3 and LR3 yielded an amplicon approximately 900-950 bp long for isolates of *L. serpens* and *G. huntii*, consisting sequences of the partial 5.8S rDNA, all of ITS2, and partial LSU rDNA. Amplicons derived from the primers T10 and BT2B yielded 700-750 bp for *L. serpens* and *G. huntii*. The EF1F and EF2R primers yielded amplicons 800-900 bp long for both fungi.

4.4.3. *Phylogenetic analyses*

For the rDNA data set, the TIM+I model was selected for the substitution model for ML and NJ analyses. The final alignment consisted of 518 characters, of which 63 characters were parsimony informative. For the β-tubulin data set, the TRN+G model was selected for ML and NJ analyses. The final β-tubulin alignment consisted of 377 characters, of which 11 were parsimony informative. For the ef-1α data set, the GTR+G.

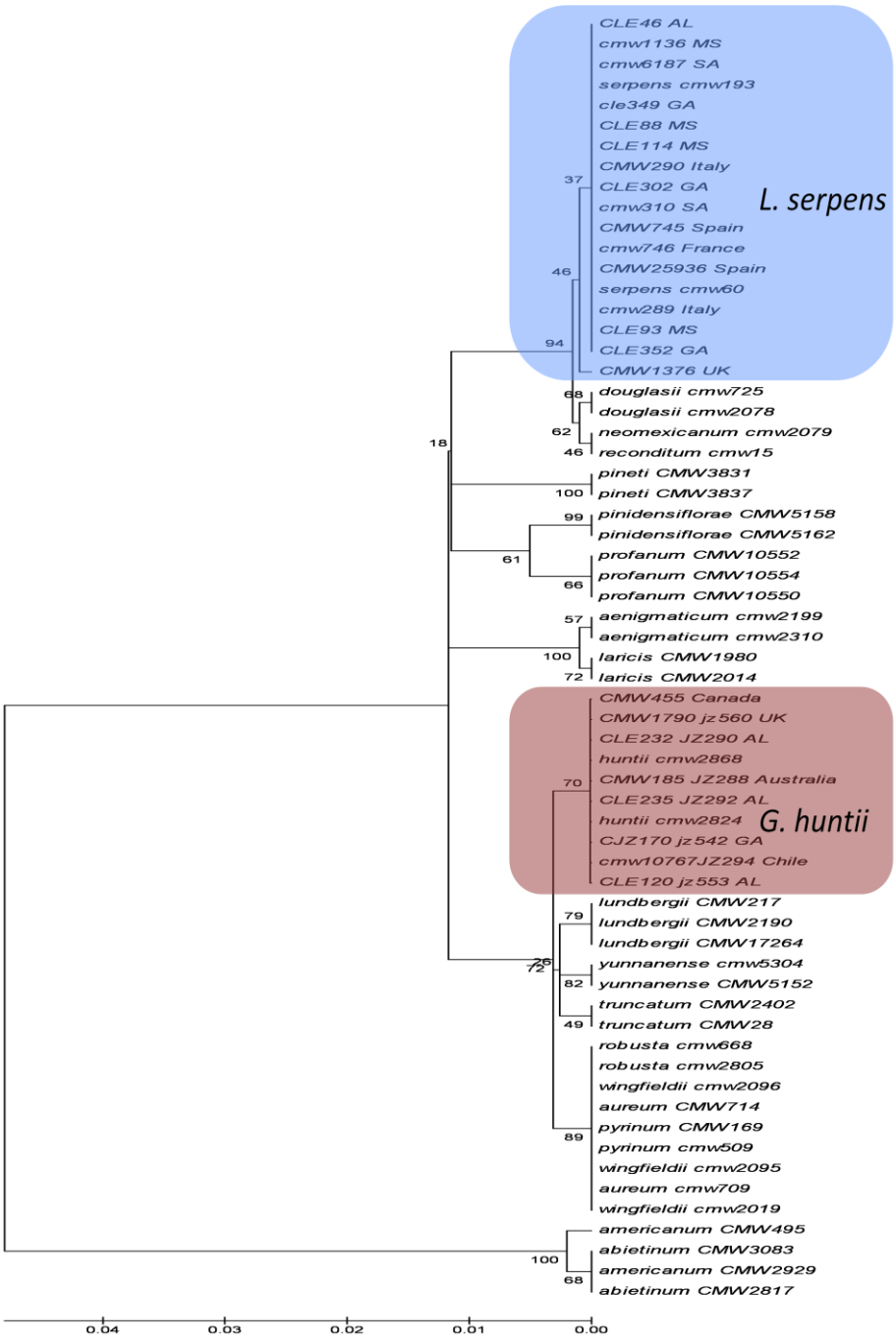

Figure 4.1. Neighbor joining tree of *Leptographium* spp. nrDNA showing conspecifity of *L. serpens* and *G. huntii* isolates collected from the southeastern U.S.

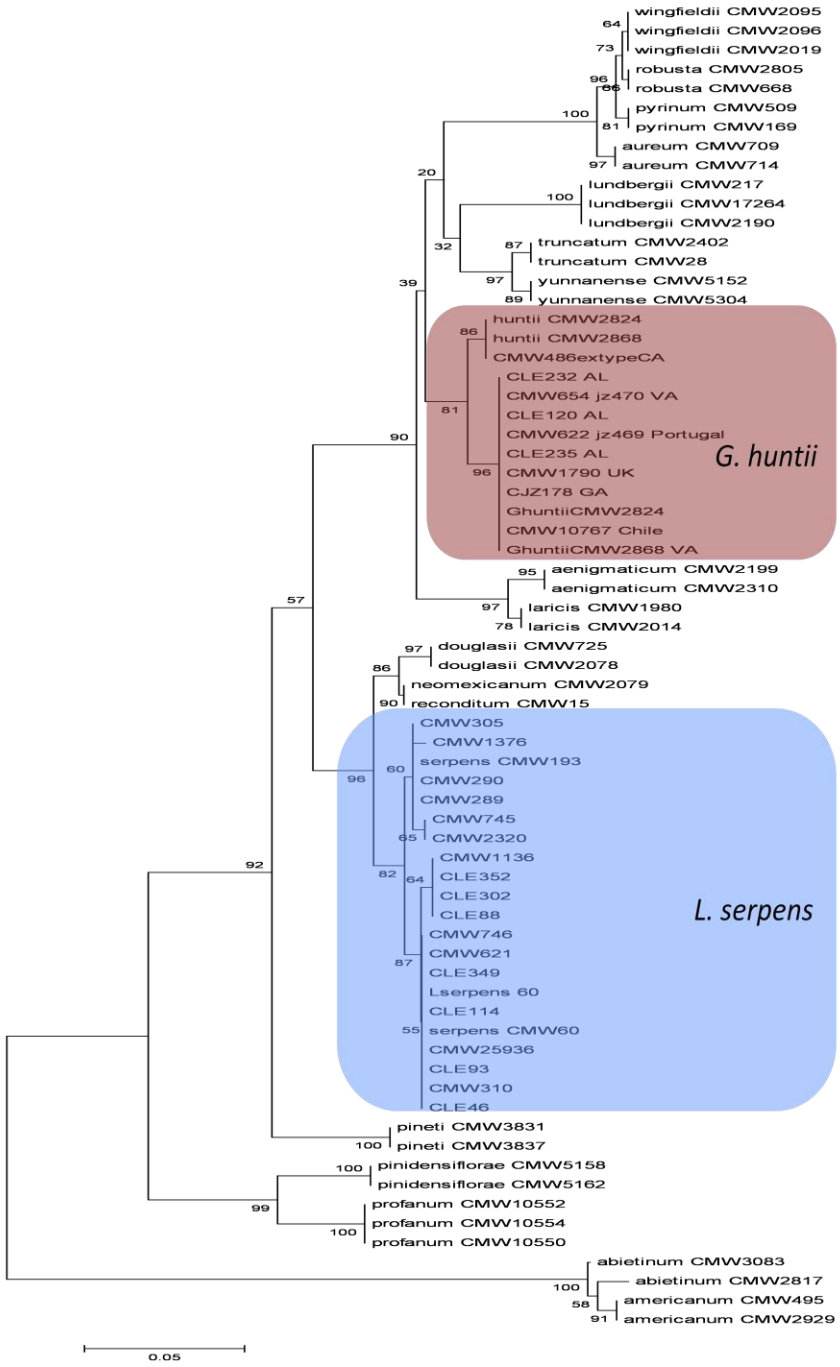

Figure 4.2. Neighbor-joining tree of *Leptographium* spp.β-tubulin showing conspecifity of *L. serpens* and *G. huntii* isolates collected from the southeastern U.S.

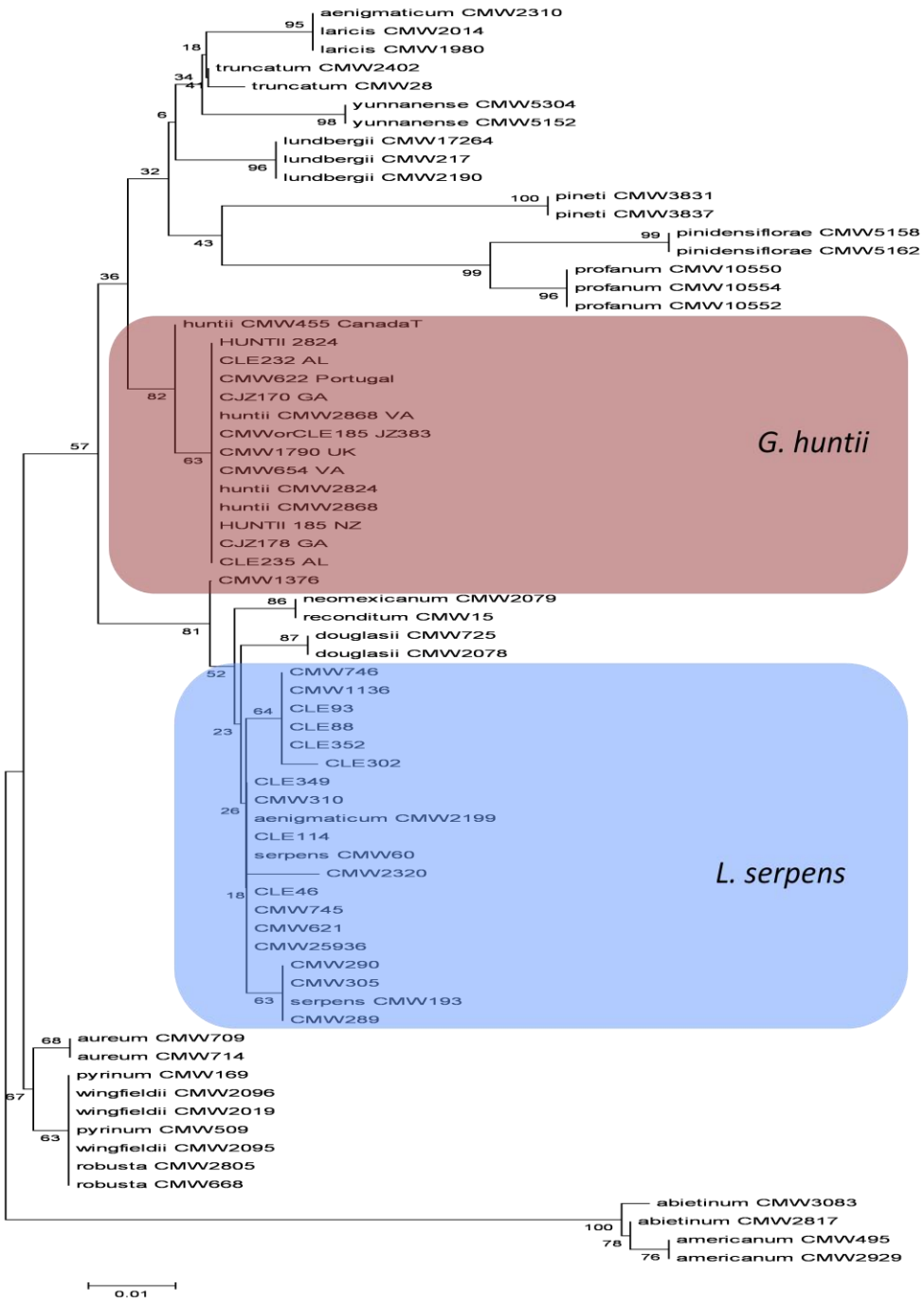

Figure 4.3. Neighbor joining tree of *Leptographium* spp. EF1-α showing conspecificity of *L. serpens* and *G. huntii* isolates collected from the southeastern U.S.

model was selected for ML and NJ analyses. The final ef-1α alignment consisted of 417 characters, of which 11 were parsimony informative. Representative phylogenetic trees are presented in Figures 4.1-4.3

4.4.4. *Population genetics*

Number of haplotypes, private alleles, and haplotypic diversity for *L. serpens* derived from β-tubulin sequence data in DnaSP are presented in Table 4.2. Population level statistics could not be performed for *G. huntii* isolates as only two haplotypes were recovered for β-tubulin and ef1-α, and one from nrDNA, Similarly, only two haplotypes were recovered from nrDNA sequences of *L. serpens*.

4.4.5. *PCR RFLP.*

Resolution of the β-tubulin sequences confirmed the identity of the isolates when compared with the sequences collected from ex-type cultures of the two species. The primer set T10 and BT2B gave the most consistent amplification, and for both species yielded a fragment of approximately 625 base pairs. The ex-type sequences were used to develop the PCR-RFLP as well as other isolates. A total of 6 candidate restriction endonucleases were selected based on the ability to cleave the β-tubulin amplicon of only

Table 4.2. Population genetics statistics for *L. serpens* sequence data.

Gene		No. Haplotypes (Private alleles)	Haplotypic diversity (variance)		Nucleotide Diversity(π)
β-tubulin	Total	6	0.775	(0.00484)	0.00821
	Europe	5 (3)	0.861	(0.0076)	0.01126
	United States	2 (1)	0.509	(0.01015)	0.00136
	South Africa	2 (0)	1	(NA)	0.00267
EF-1α	Total	6	0.688	(0.00715)	0.00464
	Europe	5 (1)	0.833	(0.0096)	0.00764
	United States	2 (1)	0.405	(0.001122)	0.00113
	South Africa	1 (0)	NA		NA

one species to the exclusion of the other, and two endonucleases tested; PstI, predicted by WebCutter to cleave the amplicon of *G. huntii*, and SacI, predicted to cleave the amplicon of *L. serpens*.

Restriction digestion of the two fragments confirmed the efficacy of the assay. In each predicted reaction, two well resolved bands of approximately 375 and 225 bp for *G. huntii* when cleaved with PstI, and 100 and 500 bp for *L. serpens* when cleaved by SacI (Figure 4.4). Reciprocal digestions did not cleave the amplicons.

4.5. Discussion

This study has demonstrated that ophiostomatoid isolates producing serpentine hyphae in the southeastern U.S. represent two species, *L. serpens* and *G. huntii*. This study supports and expands upon previous reports of the presence of *L. serpens* in the region (Zambino and Harrington, 1992; Eckhardt *et al.*, 2007), and expands the range of *G.huntii* in North America (Robinson-Jeffrey and Grinchenko, 1964; Davidson and Robinson-Jeffrey, 1965). In addition, these results have demonstrated the need for a simple diagnostic test to distinguish these species, as the overlap in their morphology has led to ambiguity in identification. To this end, a PCR-RFLP was developed.

In the eastern U.S., *L. procerum* and *L. terebrantis* have been reported from several species of pines including eastern white pine, red pine, loblolly pine and longleaf pine. These fungi have also been associated with a number of insects, *L. terebrantis* was

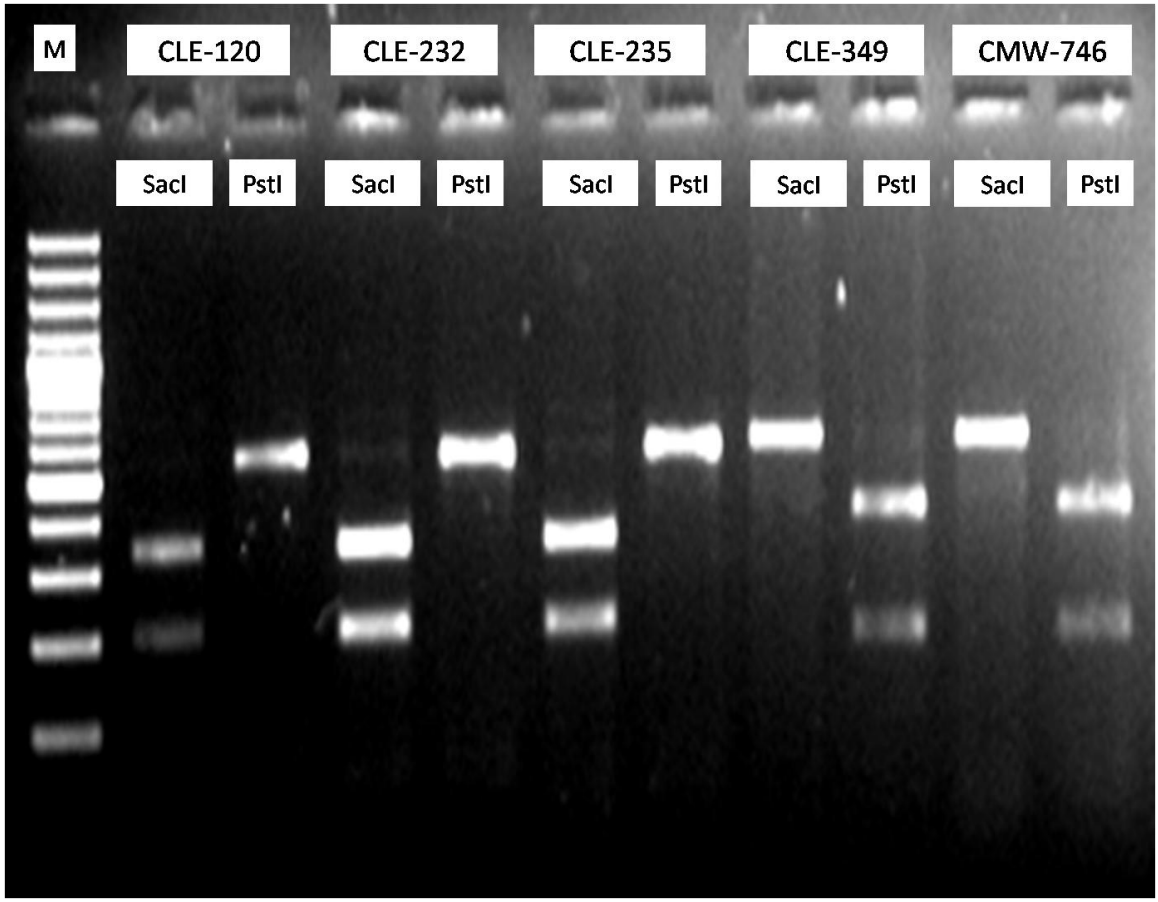

Figure 4.4. Agarose gel of digested PCR products of *L. serpens* and *G. huntii*. M=100 bp marker, *G. huntii* (CLE-120, AL), *G. huntii* (CLE-232, AL), *G. huntii* (CLE-235, AL), *L. serpens* (CLE-349, GA), *L. serpens* (CMW-746, France)

described from southeastern U.S. material, *P. taeda* and *D. terebrans*, and is known from several frequently captured scolytine and molytine insects throughout the region, including members of *Dendroctonus*, *Hylastes*, *Hylobius*, *Pachylobius*, and *Pissodes*.

The ranges of *L. serpens* and *G. huntii* in the eastern U.S. are not as well characterized. Isolates of *L. serpens* have previously been collected in Mississippi, Virginia, (Zambino and Harrington, 1992) Alabama (Eckhardt *et al.*, 2007), Georgia (Menard, 2007), Texas, Louisiana, and South Carolina (Eckhardt, unpublished), and *G. huntii* previously was known from as far east as New York and as far south as Arizona (Davidson and Robinson-Jeffrey, 1965). All of these isolates were identified morphologically, with isozyme data grouping Zambino and Harrington's (1992) isolates with the ex-holotype isolate of *L. serpens*. Some isolates recovered from loblolly pine in Alabama and originally identified as *L. serpens* (Eckhardt *et al.*, 2007) are here shown to be *G. huntii*. Thus both species are present from sites in Alabama and Georgia.

While some vectors of *L. serpens* and *G. huntii* have been reported, it is likely that these data are incomplete, as *Grosmannia* and *Leptographium* spp. frequently exhibit facultative relationships with their vectors, in that several insects may transport the same fungus. In a report on the mycota of insects captured in areas of loblolly pine decline, several insect species were noted as vectoring *L. serpens*, including several ambrosia beetles which did not vector *L. procerum* or *L. terebrantis* (Eckhardt *et al.*, 2007). *Leptographium* spp. are rarely associated with ambrosia beetles, although *Trypodendron* spp. Stephens are an exception (Harrington, 1988).

While *H. ater* Payk. and *H. angustatus*, associated with *G. huntii* and *L. serpens*, respectively, have not been found in North America, *H. opacus* Erichson has been found in the U.S. (Haack, 2006). Thought to be native to palearctic region extending from Korea to France and from Norway to Greece (Wood and Bright, 1992), this bark beetle is sympatric with a beetle known to vector *L. serpens* in the United Kingdom, *H. ater* Payk., (Wingfield and Gibbs, 1991). The mycota of *H. opacus* has not been assessed in the U.S., and may be carrying *L. serpens* and *G. huntii*.

In Chapter III, *G. huntii* is reported from *Hylastes tenuis* Eichhoff, *H. salebrosus* Eichhoff, *Hylobius pales* (Herbst), and *Pachylobius picivorus* (Germar), although the former two insect species yielded the fungus much more frequently than the latter two insect species. Similarly, this fungus was the most frequently isolated fungus from *H. ater* associated with dying *Pinus* spp. in Chile (Zhou *et al.*, 2004). Phylogenetic analysis of *G. huntii* divides the species into two closely related but well supported clades, which, given the congruence in β-tubulin and EF-1α sequence data, suggests that these isolates represent distinct or insipient species. The ex-type and other Canadian isolates group together (as *G. huntii* sensu stricto), while isolates from Europe, Australia, Chile and the southeastern U.S. group in a closely related but distinct and well supported branch (*G. huntii* sensu lato). The two-species hypothesis is supported by the association of *G. huntii* (sensu lato) and *H. ater* in the southern hemisphere. Further studies are necessary to determine whether these lineages bear morphological differences and are distinct species.

The range of these species is of acute interest in the southeastern U.S., as these fungi are associated with declining pines (Eckhardt *et al.*, 2007, Chapter II). To more fully assess the recent evolutionary history of these fungi and determine the ancestral locus of origin, development of more sensitive markers, e.g. short tandem repeats (microsatellites) would be illuminating as they have been in other pathosystems. Also, while the teleomorph of *L. serpens* has rarely been observed in Europe, and not at all in South Africa or the U.S., mating type assays should be performed to determine the mating system of this species. While Europe appears to be a nexus of diversity for *L. serpens*, more complete sampling of the range of *L. serpens* in the U.S. and Europe is necessary to support this hypothesis.

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4.7. Literature Cited

Brasier, C.M., 1991. *Ophiostoma novo-ulmi* sp nov, causative agent of current Dutch elm disease pandemics. Mycopathologia 115, 151-161.

Davidson, R.W., Robinson-Jeffrey, R.C., 1965. New records of *Ceratocystis europhioides* and *C. huntii* with *Verticicladiella* imperfect stages from conifers. Mycologia 57, 488-490.

de Ana Magan, F.J.F., 1982. Las hogueras en el monte y el ataque del hongo *Leptographium gallaeciae* sp. nv. sobre *P. pinaster* Ait. Bol. Serv. Plagas 8, 69-82.

Eckhardt, L.G., Jones, J.P., Klepzig, K.D., 2004. Pathogenicity of *Leptographium* species associated with loblolly pine decline. Plant Disease 88, 1174-1178.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

Gambogi, P., Lorenzini, G., 1977. Conidiophore morphology in *Verticicladiella serpens*. Transactions of the British Mycological Society 69, 217-223.

Gibbs, J.N., Inman, A., 1991. The pine shoot beetle *Tomicus piniperda* as a vector of blue-stain fungi to windblown pine. Forestry 64, 239-249.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61, 1323-1330.

Goidanich, G., 1936. Il genere di Ascomiceti *Grosmannia* G. Goid. Bollettino della Stazione di Patologia Vegetale di Roma 16, 26-60.

Haack, R.A., 2006. Exotic bark- and wood-boring Coleoptera in the United States: recent establishments and interceptions. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 36, 269-288.

Harrington, T.C., 1988. *Leptographium* species, their distributions, hosts and insect vectors. In: Harrington, T.C., Cobb, F.W. (Eds.), *Leptographium* root diseases on conifers. American Phytopathological Society Press, St. Paul, MN, pp. 1-39.

Heiman, M, 1997. Webcutter 2.0. Available from http://rna.lundberg.gu.se/cutter2/ [cited 2007-04-23].

Jacobs, K., Bergdahl, D.R., Wingfield, M.J., Halik, S., Seifert, K.A., Bright, D.E., Wingfield, B.D., 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycological Research 108, 411-418.

Jacobs, K., Eckhardt, L.G., Wingfield, M.J., 2006. *Leptographium profanum* sp.nov., a new species from hardwood roots in North America. Canadian Journal of Botany-Revue Canadienne De Botanique 84, 759-766.

Jacobs, K., Wingfield, M.J., 2001. *Leptographium* species : tree pathogens, insect associates, and agents of blue-stain. APS Press, St. Paul, Minn.

Jacobs, K., Wingfield, M.J., Wingfield, B.D., Yamaoka, Y., 1998. Comparison of *Ophiostoma huntii* and *O. europhioides* and description of *O. aenigmaticum* sp. nov. Mycological Research 102, 289-294.

Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9, 286-298.

Kendrick, W.B., 1962. The *Leptographium* complex: *Verticicladiella*. Canadian Journal of Botany 40, 771-797.

Kim, J.J., Kim, S.H., Lee, S., Breuil, C., 2003. Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated sapstain fungi. FEMS Microbiology Letters 222, 187-192.

Kim, G.H., Kim, J.J., Lim, Y.W., Breuil, C., 2005. Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. Canadian Journal of Botany-Revue Canadienne De Botanique 83, 272-278.

Klepzig, K.D., Raffa, K.F., Smalley, E.B., 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Science 37, 1119-1139.

Klepzig, K.D., Smalley, E.B., Raffa, K.F., 1995. *Dendroctonus valens* and *Hylastes porculus* (Coleoptera, Scolytidae) - vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. Great Lakes Entomologist 28, 81-87.

Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 1451-1452.

Lorenzini, G., Gambogi, P., 1976. Un caso di "moria" di *Pinus pinea* associata alla presenza di *Verticicladiella* sp. (nota preliminare). Informatore Fitopatologico 5, 5-8.

Menard, R.D., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. MS Thesis, Louisiana State University, Baton Rouge, LA.

Morelet, M., 1988. Observations sur trois Deuteromycetes infeodes aux pins [Observations of three deuteromycetes from pines]. Annales de la Societe des Sciences Naturelles et d'Archeologie de Toulon et du Var 40, 41-45.

Nei, M., 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7, 103-116.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Robinson-Jeffrey, R.C., Grinchenko, A.H.H., 1964. A new fungus in the genus *Ceratocystis* occurring on blue-stained lodgepole pine attacked by bark beetles. Canadian Journal of Botany 42, 527-532.

Siemaszko, W., 1939. Zespoly grzybów towarzyszacych kornikom polskim [Fungi associated with bark beetles in Poland]. Planta Polonica 7, 1-54.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C., McNew, D., 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. New Zealand Journal of Botany 43, 653-663.

Wagener, W.W., Mielke, J.L., 1961. A staining fungus root disease of ponderosa, jeffrey and pinyon pines. Plant Disease Reporter 45, 831-835.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., J., W.T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc, New York, NY, pp. 315-322.

Wingfield, M.J., 1986. Pathogenicity of *Leptographium procerum* and *Leptographium terebrantis* on *Pinus strobus* seedlings and established trees. European Journal of Forest Pathology 16, 299-308.

Wingfield, M.J., Gibbs, J.N., 1991. *Leptographium* and *Graphium* species associated with pine-infesting bark beetles in England. Mycological Research 95, 1257-1260.

Wingfield, M.J., Knox-Davies, P.S., 1980. Root disease, associated with *Verticicladiella alacris*, of pines in South Africa. Plant Disease 64, 569-571.

Wingfield, M.J., Marasas, W.F.O., 1980. *Verticicladiella alacris* sp. nov. associated with a root disease of pines in South Africa. Transactions of the British Mycological Society 75, 21-28.

Wingfield, M.J., Marasas, W.F.O., 1981. *Verticicladiella alacris*, a synonym of *V. serpens*. Transactions of the British Mycological Society 76, 508-510.

Wingfield, M.J., Seifert, K.A., Webber, J.F., 1993. *Ceratocystis* and *Ophiostoma* : taxonomy, ecology, and pathogenicity. American Phytopathological Society, St.Paul, Minn.

Wood, S.L., Bright, D.E., 1992. A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic Index. Great Basin Naturalist Memoirs, pp. 835-1553.

Zambino, P.J., Harrington, T.C., 1992. Correspondence of isozyme characterization with morphology in the asexual genus *Leptographium* and taxonomic implications. Mycologia 84, 12-25.

Zhou, X.D., De Beer, W., Wingfield, B.D., Wingfield, M.J., 2002. Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. Fungal Diversity 10, 229-240.

Zhou, X.D., de Beer, Z.W., Ahumada, R., Wingfield, B.D., Wingfield, M.J., 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Diversity 15, 261-274.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Studies in Mycology, 75-97.

CHAPTER V. A NEW OPHIOSTOMA SPECIES FROM PINE FORESTS IN THE SOUTHEASTERN UNITED STATES

5.1. Abstract

During a survey of ophiostomatoid fungi in pine forests at Fort Benning Military Reservation, Georgia, USA, a species of *Ophiostoma* morphologically similar to *O. pluriannulatum* was isolated. These isolates produced perithecia with unusually long necks similar to those of *O. pluriannulatum* but had few or no annuli. To identify the fungus, morphological and DNA sequence analyses for the internal transcribed spacer (ITS) regions of the ribosomal DNA operon and partial β-tubulin gene were employed. Sequences from the ITS region were identical to those of *O. pluriannulatum sensu stricto*. Sequence data from the β-tubulin gene region revealed the absence of intron 4 and presence of intron 5, which distinguished the isolates from *O. pluriannulatum*, which has intron 4 and not intron 5, and *O. multiannulatum* and *O. subannulatum*, which both have introns 4 and 5. Phylogenetic analyses of the β-tubulin sequences showed isolates from loblolly pine roots group together in a lineage distinct from *O. pluriannulatum sensu stricto*. The fungus is consequently described as *O. sparsiannulatum* sp. nov., a novel taxon in the *O. pluriannulatum* complex.

5.2. Introduction

Ophiostomatoid fungi are generally known as blue-stain fungi for the cosmetic effects that they impart on lumber (Seifert, 1993; Schirp *et al.*, 2003) and in sapwood of trees infested by either scolytine bark beetles or molytine weevils (Coleoptera: Curculionidae) which vector these fungi (Six, 2003). Some of these fungi have been demonstrated to be serious pathogens of trees, e.g. *Ophiostoma ulmi* (Buism.) Nannf. and *O. novo-ulmi* Brasier causing Dutch elm disease (Brasier, 1991).

In recent years DNA sequence-based studies have clarified many of the taxonomic uncertainties in this group (Hausner *et al.*, 1993; Spatafora and Blackwell, 1994; Zipfel *et al.*, 2006). The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA) operon are routinely used to address species level questions in fungi, including the Ophiostomatales (Uzunovic *et al.*, 2000; Harrington *et al.*, 2001; de Beer *et al.*, 2003). However, in certain cases the ITS regions fail to resolve closely related species within this group (Harrington *et al.*, 2001; Chung *et al.*, 2006; Roets *et al.*, 2006). Thus, partial β-tubulin sequences have provided ancillary data, as this region has been shown to have improved resolution over nrDNA in examining closely related species (Gorton *et al.*, 2004; Roets *et al.*, 2006; Alamouti *et al.*, 2007).

The *O. pluriannulatum* complex (OPC) is one of the poorly known complexes in the genus *Ophiostoma* (Zipfel *et al.*, 2006). Nuclear ribosomal large subunit (LSU) data have shown that *O. pluriannulatum* and seven other species form a well-supported lineage in *Ophiostoma* (Hausner *et al.*, 1993; Hausner and Reid, 2003). Based on mating compatibility and ribosomal internal transcribed spacer (ITS) region sequences, Thwaites

et al. (2005) suggested that at least three more species are members of the complex. Thwaites *et al.* (2005) stated that *O. californicum* (DeVay, R.W. Davidson & W.J. Moller) Georg Hausner, J. Reid & Klassen and *O. populinum* (T.E. Hinds & R.W. Davidson) de Hoog & R.J. Scheff. are likely members, and the third, an undescribed species referred to as *Ophiostoma* sp. E. (Thwaites *et al.*, 2005), but this needed confirmation. In an ITS phylogeny by Villarreal *et al.* (2005), the ex-type strains of *O. multiannulatum* (Hedgc. & R.W. Davidson) Hendr. and *O. conicola* Marm. & Butin also grouped in the OPC complex. Zipfel *et al.* (2006) added *O. carpenteri* J. Reid & Hausner to the complex based on LSU and partial β-tubulin sequences. The most recent addition to the OPC is a new species from native hardwood trees in South Africa, *O. longiconidiatum* Kamgan, K. Jacobs & J. Roux (Kamgan *et al.*, 2008).

During investigations into loblolly pine decline (*Pinus taeda* L.) and longleaf pine decline (*P. palustris* Mill.) in the southeastern U.S., isolates of an *Ophiostoma* species were collected from Fort Benning Military Reservation, Georgia (FB). Superficially these isolates resembled *O. pluriannulatum* (Hedgc.) H. & P. Sydow (Hedgcock, 1906) and similar related species.

The aim of this study was to identify isolates resembling *O. pluriannulatum* from pine roots and associated insects at FB. Certain morphological anomalies led to the hypothesis that these isolates represent a novel species. This was to be tested by DNA phylogenetic analysis and morphological comparisons with described species.

5.3. Materials and Methods

5.3.1. *Isolation of fungi*

Loblolly and longleaf pine roots were excavated at FB, using a two-lateral root sampling as described by Otrosina *et al.* (1999) and modified by Eckhardt *et al.* (2007). Roots were surface sterilized in a solution of ethanol, commercial bleach, and distilled water (80:10:10, v:v:v), rinsed in tap water and then plated on malt extract agar (MEA, 20 g malt extract, 15 g agar/L) and MEA amended with cycloheximide and streptomycin (CSMA) (Hicks *et al.*, 1980). Ophiostomatoid fungi were identified by their characteristic perithecia or conidiophores and transferred to fresh CSMA or MEA. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Type specimens were deposited in National Collection of Fungi (PREM), Pretoria, South Africa and ex-type cultures in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Isolations from insects associated with longleaf pine are described in Chapter II. Isolates growing on cycloheximide-amended media, and producing typical ophiostomatoid ascomata were archived at the Auburn University School of Forestry and Wildlife Sciences Forest Health Dynamics Laboratory and were compared with loblolly pine isolates cited above.

5.3.2. *Morphological studies*

To assess mycelial, perithecial, and conidial characters, isolates were grown on oatmeal agar (OA, 30 g Jungle brand oats/L with 15 g/L agar), 2% MEA and pine twig agar (PTA, water agar 15 g/L with twice-autoclaved *P. patula* L. twigs embedded). Fungal characteristics were measured using a Zeiss Axioskop2 microscope using brightfield and phase contrast microscopy, and data collected using the software package AxioVision Release 4.4.1.0. (Carl Zeiss Microimaging GmbH). Fifty measurements were made of each structure, and maxima, minima, and means determined.

Agar plugs (5 mm diameter) taken from actively growing cultures were transferred to fresh MEA plates, incubated at temperatures ranging from 5 to 35 °C at five degree intervals using three plates for each temperature. Radial growth was measured every two days until the colony reached the plate edge. Ten single-ascospore isolates were obtained by streaking mature spore droplets on MEA plates and were crossed in all pairwise combinations to determine whether the fungus was homo- or heterothallic.

5.3.3 DNA extraction, PCR and sequencing

DNA was prepared for PCR from fresh mycelium taken from cultures on MEA using PrepMan Ultra Reagent (Applied Biosystems Inc., Foster City, CA), following the protocol described by Aghayeva *et al.* (2004). Portions of the ribosomal DNA were amplified using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*,

1990) and the β-tubulin gene region using the forward primer T10 (O'Donnell and Cigelnik, 1997) and the reverse primer Bt2b (Glass and Donaldson, 1995).

Amplifications were performed in an Eppendorf MasterCycler Personal thermal cycler under the following conditions: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 sec, 50 °C annealing for 30 sec, and 72 °C extension for 60 sec, with a final extension period at 72 °C for 8 min. Reaction mixtures, 50 μL total volume, consisted of 5 μL 10 × PCR reaction buffer (JMR Holdings, UK), 2.5 mM $MgCl_2$, 10 mM dNTPs, 10 μM of each primer, 2.5 U Taq polymerase (SuperTherm, JMR Holdings, UK) and 4 μL of genomic DNA. Amplification success was assessed on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light. PCR products were purified with the High Pure PCR Purification Kit (Roche Pharmaceuticals, Basel, Switzerland) according to the manufacturer's protocol. Amplicons were then cycle sequenced using the Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems Inc., Foster City, CA) with the primers noted above, and analyzed on an ABI PRISIM™ 377 or ABI PRISIM™ 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

Electropherograms of the resolved sequences were visualized, and contigs of forward and reverse sequences compiled using MEGA 4.0.1 (Tamura *et al.*, 2007). Sequences were subjected to BLAST searches in NCBI GenBank to determine similarity to published sequences. Appropriate sequences were downloaded and data sets compiled in MEGA 4.0.1 (Table 4.1). Alignments were done using the E-INS-I strategy in the online version of MAFFT 6 (Katoh and Toh, 2008)

Table 5.1. List of isolates used for phylogenetic comparison and NCBI GenBank numbers.

Species	Country of Origin	Isolate number	ITS	β–Tubulin
Ophiostoma aurorae	South Africa	CMW 19362*	DQ396796	
O.canum	Sweden	C 114	AF198229	
O.carpenteri	USA	CMW 13793		DQ296083
O.conicolum	Mexico	CBS 127.89	AY924384	
O.ips	USA	CMW 7075	AY546704	
O.longiconidiatum	South Africa	CMW 17574*	EF408558	
O.longiconidiatum	South Africa	CMW 14265		
O.montium	USA	CMW 13221	AY546711	
O.multiannulatum	USA	CBS 357.77		
O.multiannulatum	USA	MUCL 19062	AY934512	
O.multiannulatum	USA	CMW 2567		DQ296086
O.novoulmi	Russia	C 1185	AF198235	
O.perfectum	USA	CBS 636.66 = C 1104	DQ062970	
O.piceae	United Kingdom	CMW 7648	AF493249	
O.piliferum	Netherlands	CBS 129.32	AF221070	
O.piliferum	Canada	AU 55-4		AF221631
O.pluriannulatum	USA	MUCL 18372	AY934517	
O.pluriannulatum	New Zealand	C 1033	DQ062971	
O.pluriannulatum	New Zealand	C 1567	DQ062972	
O.pluriannulatum	South Africa	CMW 19360	DQ396793	
O.pluriannulatum	USA	C 1300	DQ062973	
O.pluriannulatum	USA	CMW 75	DQ294365	DQ296085
O.pulvinisporum	Mexico	CMW 9022*	AY546714	
O.quercus	South Africa	CMW 2520	AF493241	
O.setosum	USA	AU 160-53	AF128927	
O.stenoceras	Norway	CBS 237.32	AF484462	
O.subannulatum	USA	CBS 188 .86	AY934522	
O.subannulatum	Unknown	CBS118 =CMW 518		DQ296084
Ophiostoma sp.	Spain	CCMA 27	AY934508	
Ophiostoma sp.	New Zealand	C 1097	DQ062974	
Ophiostoma sp.	Canada	C 1626	DQ062975	
O.ulmi	Netherlands	CBS 102.63	AF198232	
S.schenckii	South Africa	CMW 7132	AF484467	
O. sparsiannulatum	USA	CMW 17229	FJ906816^	FJ907176^
O. sparsiannulatum	USA	CMW 17231 =CBS122815*	FJ906817^	FJ907177^
O. sparsiannulatum	USA	CMW 17233 =CBS122816**	FJ906818^	FJ907178^

* = Ex holotype isolate, ** =Ex-paratype isolate, ^=from this study,

(http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). For the β-tubulin gene region, manual adjustments of the alignment were performed in MEGA 4.0.1 using the translation option to ensure appropriate alignment of exons based on amino acid sequences.

5.3.4. *Phylogenetic analyses*

Aligned sequences of the ITS and β-tubulin gene sequences were subjected to phylogenetic modeling using maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) using PAUP* version 4.0 beta (Sinauer Associates, Sunderland, MA) and Bayesian analysis using MrBayes (Huelsenback and Ronquist, 2001). For ML and NJ analyses of the two gene regions, the evolutionary model was selected using ModelTest version 3.7 (Posada and Crandall, 1998), and for Bayesian analysis the evolutionary model was selected using MrModelTest version 2 (Nylander, 2004). For each method, 1000 bootstrap replicates were performed to test branch stability.

5.4 Results

5.4.1. *Isolation of fungi*

Three isolates of an unidentified *Ophiostoma* sp. were recovered from *P. taeda* roots (Table 4.1). All isolates were cycloheximide tolerant, and were found in association with other cycloheximide-tolerant taxa including *Leptographium* spp. and *Penicillium* spp. In addition, ten isolates from insects were also found, which match the morphological description of the isolates from *P. taeda* (Table 5.2).

Table 5.2. *Ophiostoma sparsiannulatum* isolates collected from root-feeding insects at Fort Benning, GA.

Isolate number	Isolate Source	Date of Collection
CJZ-I-360	*Hylastes tenuis*	3/31/2006
CJZ-I-568	*Hylastes salebrosus*	4/2/2006
CJZ-I-826	*H. tenuis*	3/13/2007
CJZ-I-846	*Pachylobius picivorus*	3/13/2007
CJZ-I-1011	*Hylobius pales*	4/1/2007
CJZ-I-1013	*Dendroctonus terebrans*	3/20/2007
CJZ-I-1014	*Hb. pales*	3/20/2007
CJZ-I-1067	*H. tenuis*	4/15/2007
CJZ-I-1086	*P. picivorus*	5/16/2007
CJZ-I-1092	*H. salebrosus*	5/16/2007

5.4.2. *Morphological studies*

Isolates produced abundant aerial hyphae bearing dry, micronematous conidia typical of the anamorph genus *Sporothrix*. Long-necked, darkly pigmented ascomata formed abundantly in culture within 8 days. The ascomata were variable in size, with large and small ascomata producing droplets with viable ascospores, suggesting that all were mature. Colonies tended to darken with age. Morphological characteristics were consistent with those of the OPC (Table 5.3), although annuli on the ascomatal necks were few in number or lacking. The ascospores were slightly smaller than those reported for the other species most closely related to isolates from loblolly pine roots (Table 5.3).

5.4.3. *PCR and sequencing*

Amplicons of the ITS regions yielded 618 bp of readable sequence data. These sequences were identical to those for *O. pluriannulatum* published in NCBI GenBank, and similar to several other *Ophiostoma* spp. Amplicons of the β-tubulin gene yielded 575 bp of readable sequence data, which shared 93% homology with *O. subannulatum*, the closest current match in GenBank. The partial β-tubulin sequences of these isolates included 14 bp of exon 3, no intron 3, exon 4 (27 bp), no intron 4, exon 5 (42 bp), intron 5 (49 bp), and 443 bp of the 5’ end of exon 6.

Table 5.3. Comparison of characters of *O. sparsiannulatum* and morphologically similar species. Bolded cells indicate key characteristics for diagnosis.

Species		*O. sparsiannulatum*	*O. pluriannulatum*	*O. multiannulatum*	*O. subannulatum*
Perithecia:	Neck length	1.1 (up to 8) mm	1.5 (0.9-2.0) mm	4.0-8.5 mm	0.2-1.2 (up to 2.2) mm
	Annuli	**0-2 (4)**	3-8	6-9	1-2 (4)
	Diameter of base	135 (54-201) μm	120 (90-200) μm	170-275 μm	75-200 μm
Ascospores:	Shape	Allantoid	Reniform	Reniform	Allantoid to broadly lunate
	Length	3.0 (2.2-4.0) μm	4.5 (4.0-5.0) μm	2.8-3.8 μm	3.5-5.0 (6.0) μm
	Width	1.0 (0.7-1.6) μm	1.5 (1.5-1.7) μm	1.2-1.4 μm	1-2.5 μm
Anamorph:	Form	***Sporothrix* (with dry, floccose spores)**	*Sporothrix*	*Sporothrix*	*Sporothrix* (with sticky spores)
Conidia:	Length	2.7-9.2 μm	4.6-18.0 μm	6.0-25.0 μm	2.0-6.0 μm
	Width	0.75-2.3 μm	1.5-3.0 μm	2.0-3.2 μm	1.0-4.0 μm
Hosts:		*Pinus taeda, P. palustris*.	*Quercus* spp., *Pinus* spp.	*Pinus* spp.	*Abies* spp.
Distribution:		Georgia, USA	Southeastern USA, New Zealand, Chile, South Africa	Southeastern USA	Idaho, USA

NA = not available

Table 5.3. Continued.

Species	Gene Region	*O. sparsiannulatum* sp. nov.	*O. pluriannulatum*	*O. multiannulatum*	*O. subannulatum*
Descriptions:		Present study	Hedgcock 1906; Upadhyay 1981	Davidson 1935	Livingston and Davidson 1987
Sequence data:	LSU	Present study	Hausner & Reid 2003; Zipfel et al. 2006	Zipfel et al. 2006	Hausner & Reid 2003; Zipfel et al. 2006
	ITS	Present study	Thwaites et al. 2005; Villarreal et al. 2005	Villarreal et al. 2005	Villarreal et al. 2005
	β-tubulin	Present study	Zipfel et al. 2006	Zipfel et al. 2006	Zipfel et al. 2006

5.4.4. *Phylogenetic analyses*

For the ML and NJ analyses of the ITS data set, the TrN+G substitution model was selected. For the Bayesian analysis, GTR+I+G was selected. The aligned ITS data set consisted of 742 characters and confirmed the placement of the isolates from loblolly pine root in the genus *Ophiostoma* and in the OPC (Fig. 5.1). However, they could not be unambiguously distinguished from *O. pluriannulatum* sensu stricto based on ITS in any of the analyses and grouped in a single clade with that species

The aligned β-tubulin data used for phylogenetic analyses consisted of 354 characters, including exon 4, 5 and 6, interspersed by introns 4 and 5. In the isolates under consideration, intron 4 was lacking and intron 5 was present, as was the case for *O. multiannulatum* and *O. subannulatum*. *Ophiostoma pluriannulatum, O. carpenteri* and *O. piliferum* had intron 4 and lacked intron 5, thus separate phylogenetic analyses were conducted for data sets including and excluding species with different intron arrangements (Figs 5.1-5.3).

For ML and NJ analyses, the TrN+I model was selected for the β-tubulin data set including *O. pluriannulatum*, *O. carpenteri* and *O. piliferum*, and for the β-tubulin data set excluding these three taxa, the HKY+I model was selected. For the Bayesian analysis, GTR+I+G was selected for the inclusive data set, and HKY+I for the exclusive dataset. All models were selected using the Akaike Information Criterion, instead of the Likelihood Ratio Test.

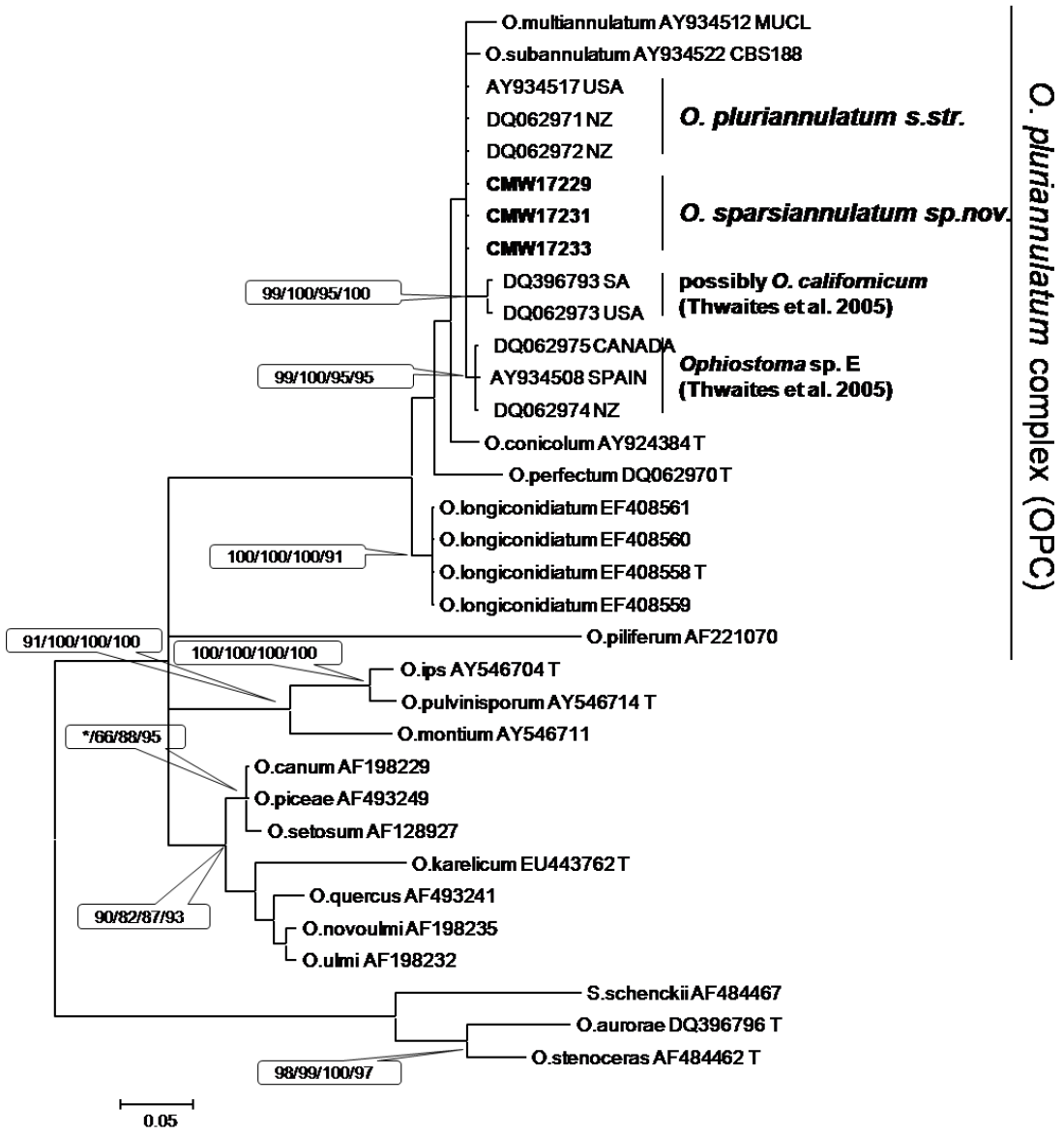

Figure 5.1. Bayesian tree of ITS sequences of selected *Ophiostoma* isolates. Branch support is indicated by bootstrap analyses (1000 replicates) for maximum parsimony, maximum likelihood, and neighbor joining analyses, and posterior probabilities for Bayesian analysis, respectively.

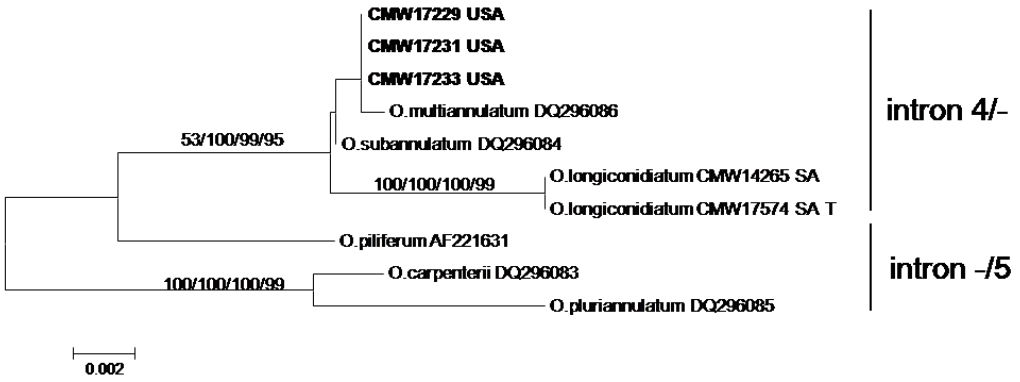

Figure 5.2. Bayesian tree of β-tubulin sequences of *Ophiostoma* spp. from the OPC. Branch support is indicated by bootstrap analyses (1000 replicates) for maximum parsimony, maximum likelihood, and neighbor joining analyses, and posterior probabilities for Bayesian analysis, respectively.

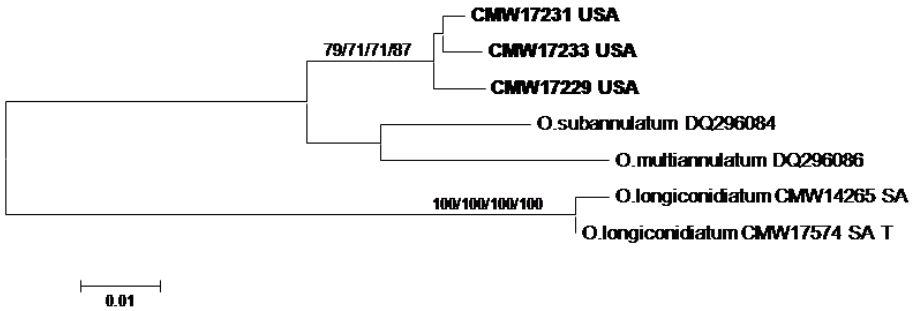

Figure 5.3. Bayesian tree of β-tubulin sequences of *Ophiostoma* spp. from the OPC with intron 4 present. Branch support is indicated by bootstrap analyses (1000 replicates) for maximum parsimony, maximum likelihood, and neighbor joining analyses, and posterior probabilities for Bayesian analysis, respectively.

Phylogenetic trees generated from β-tubulin sequence data showed that the isolates under investigation were distinct from *O. pluriannulatum* sensu stricto. The pattern of exons and introns was similar to that of *O. multiannulatum* and *O. subannulatum* (Figures 5.2 and 5.3), although the sequences were distinct from the latter two species, forming a lineage with good statistical support in the analyses that only included species with intron 4 (Figure 5.3).

5.4.5. *Taxonomy*

The *Ophiostoma* sp. from pine roots bears gross similarity to *O. pluriannulatum* and related species. However, based on close morphological examination and DNA sequence comparisons, the isolates clearly represent a novel species, which is described as follows:

Ophiostoma sparsiannulatum Zanzot, Z.W. de Beer and M.J. Wingf. sp. nov. Figs 5.4-5.10.

Etymology: having few annuli, a propos of the characteristic of the perithecium in comparison to related annulate *Ophiostoma* species

Colony hyaline at first, darkening with age and with copious white floccose aerial growth of the anamorph with growth typically zonate. *Mycelium* aerial and submerged. *Optimal temperature* for growth 25 °C, no growth at 35 °C. *Hyphae* smooth, not constricted at

septa. *Ascomata.* typically produced within 8 days, and only in crosses between single spore isolates, suggesting heterothallism. *Ascomatal bases* dark brown-black, globose, bases (54-) 166 (-201) μm in diameter. *Ascomatal necks* dark brown-black, (500–) 800-1400 (-1850) μm long with annuli absent or occasionally 1-4 present. Some submerged perithecia observed on PTA have much longer necks, as long as 8600 μm, diameter of the neck (7.1-) 10.0-16.6 (-21.0) μm at the apex and (13.0-) 26.5-47.9 (-65.0) μm at the base, *Ostiolar hyphae* divergent, hyaline, (17.6-) 31.6-62.2 (-80.1) μm long. *Asci* not observed. *Ascospores* hyaline, allantoid, (2.2-) 2.8-3.4 (-4.0) μm long, by (0.7-) 0.9-1.1 (-1.6) μm wide. Ascospores accumulating in a clear-white mucilaginous mass at the ascomatal apex, turning buff-yellow with age.

Sporothrix anamorph: growing abundantly amongst the perithecia, in white fluffy masses. *Conidiophores* of variable length, (8.6-) 12.5-52.0 (-150.3) μm, with denticles (0.68-) 0.76-1.28 (-1.64) μm long. *Conidia* typically ovoid-obovoid with a basal abscission collar evident, (2.68-) 3.77-6.73 (-9.20) μm long by (0.75-) 1.02-1.78 (-2.33) μm wide, with secondary conidia occasionally forming acropetally.

Specimens examined: USA, Fort Benning, Georgia, from roots of *Pinus taeda*, July 2004. *Collector: L.G. Eckhardt*. Holotype PREM 59847, ex-holotype culture CBS 122815 = CMW17231, Paratype: PREM 59848, Ex-paratype culture CBS 122816 = CMW17233, CMW 17229.

Figure 5.4. Large mature perithecium of *Ophiostoma sparsiannulatum*.

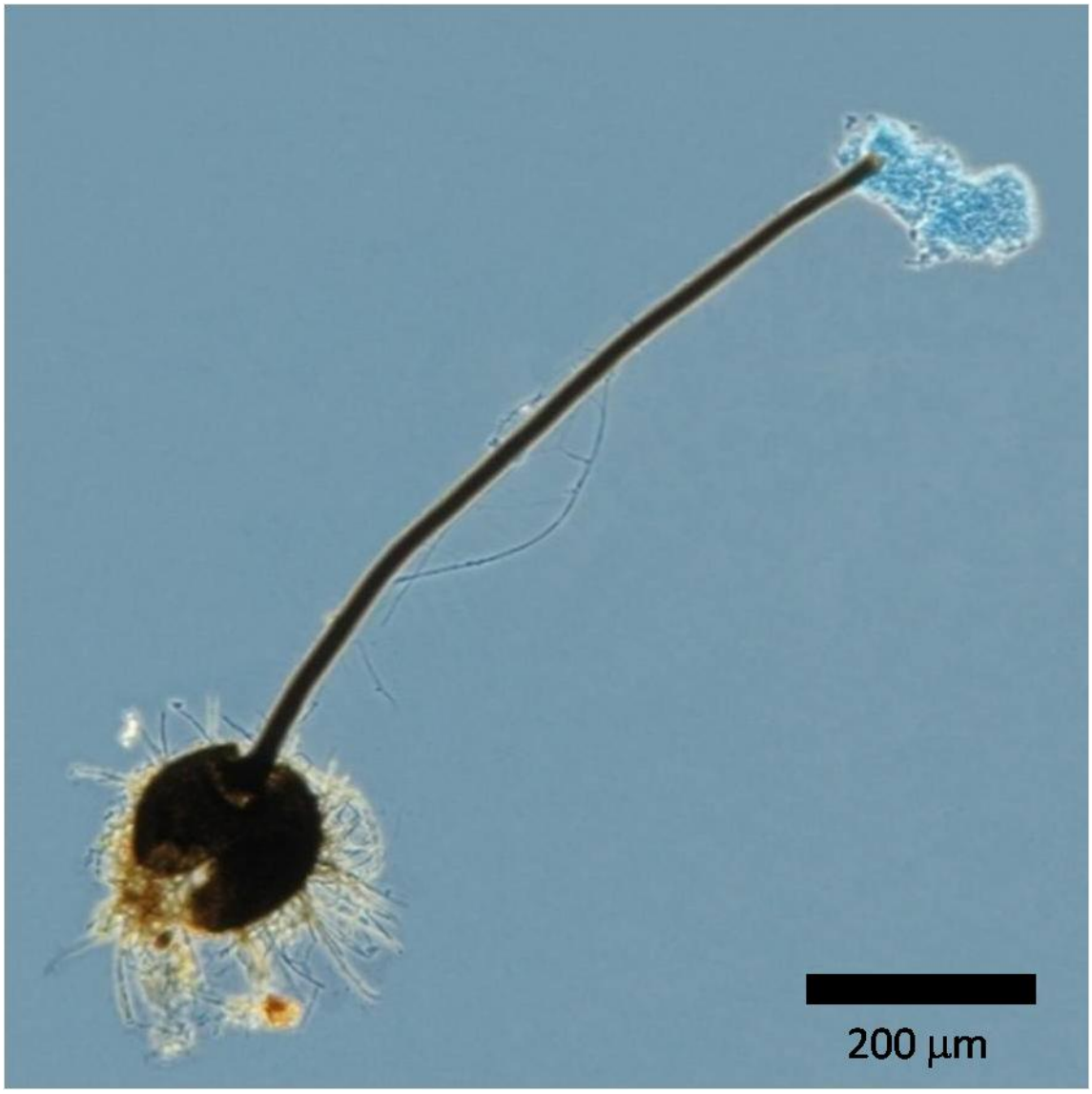

Figure 5.5. Small mature perithecium of *Ophiostoma sparsiannulatum.*

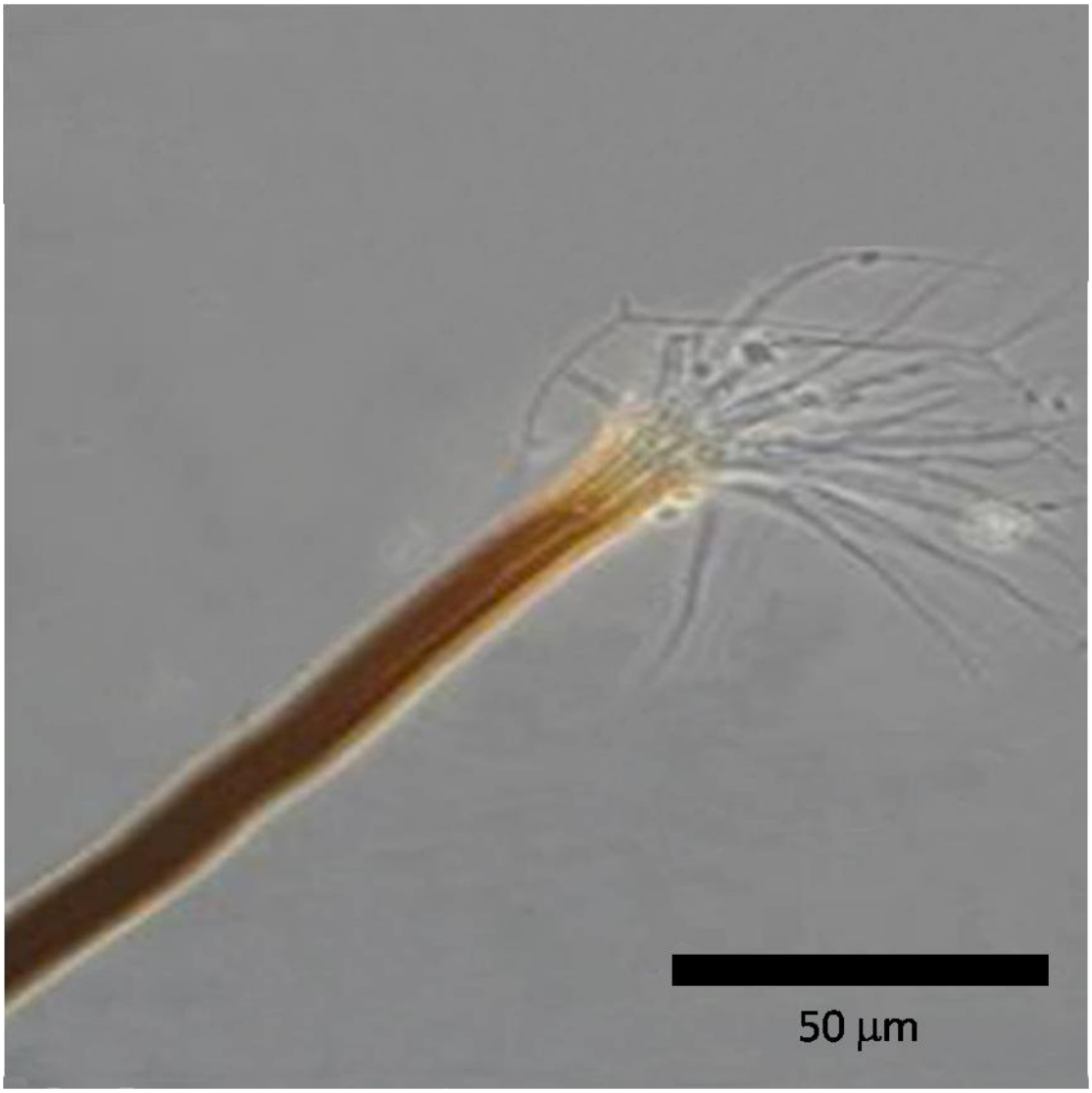

Figure 5.6. Detail of ostiolar hyphae of *Ophiostoma sparsiannulatum*.

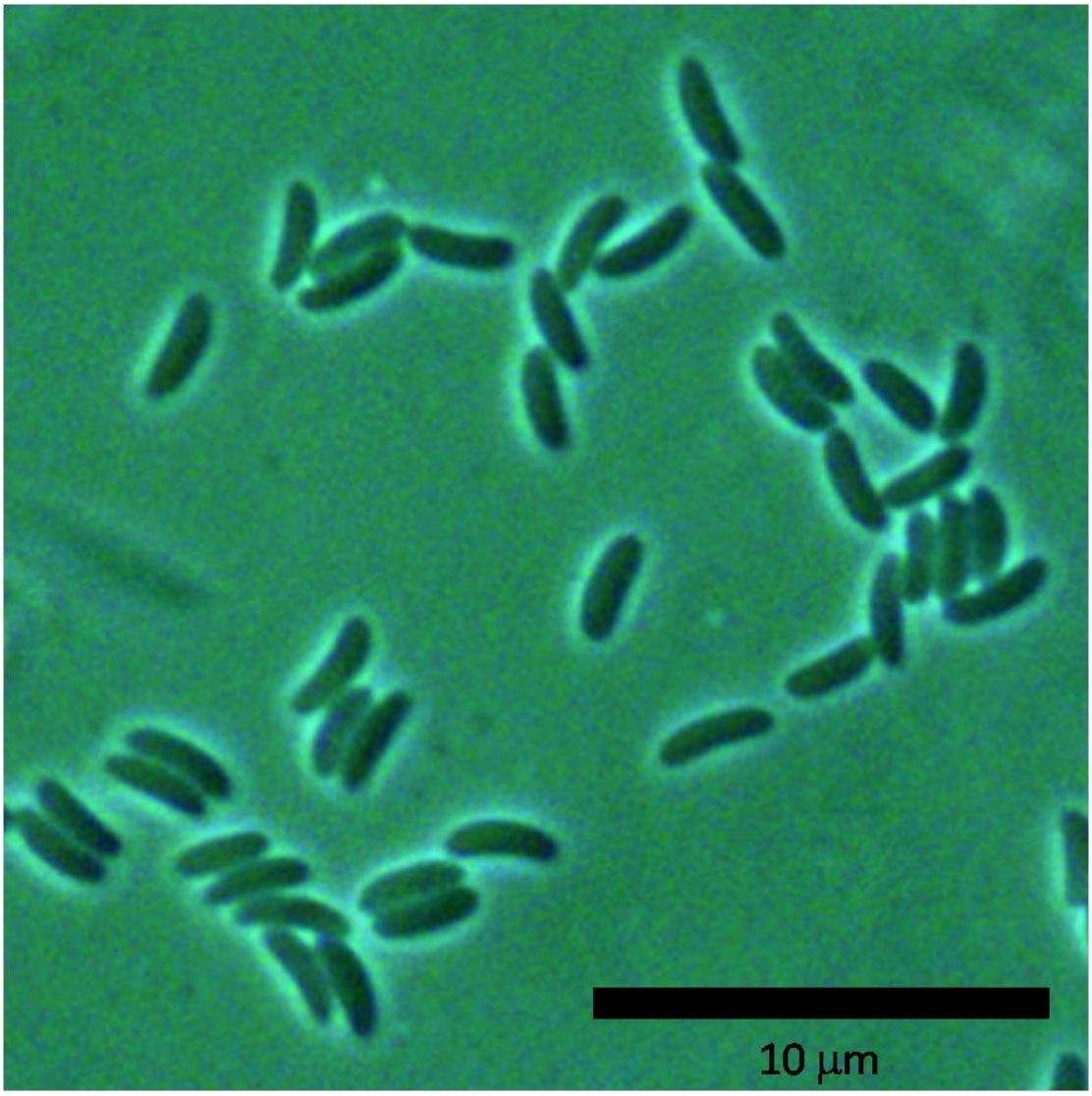

Figure 5.7. Ascospores of *Ophiostoma sparsiannulatum*.

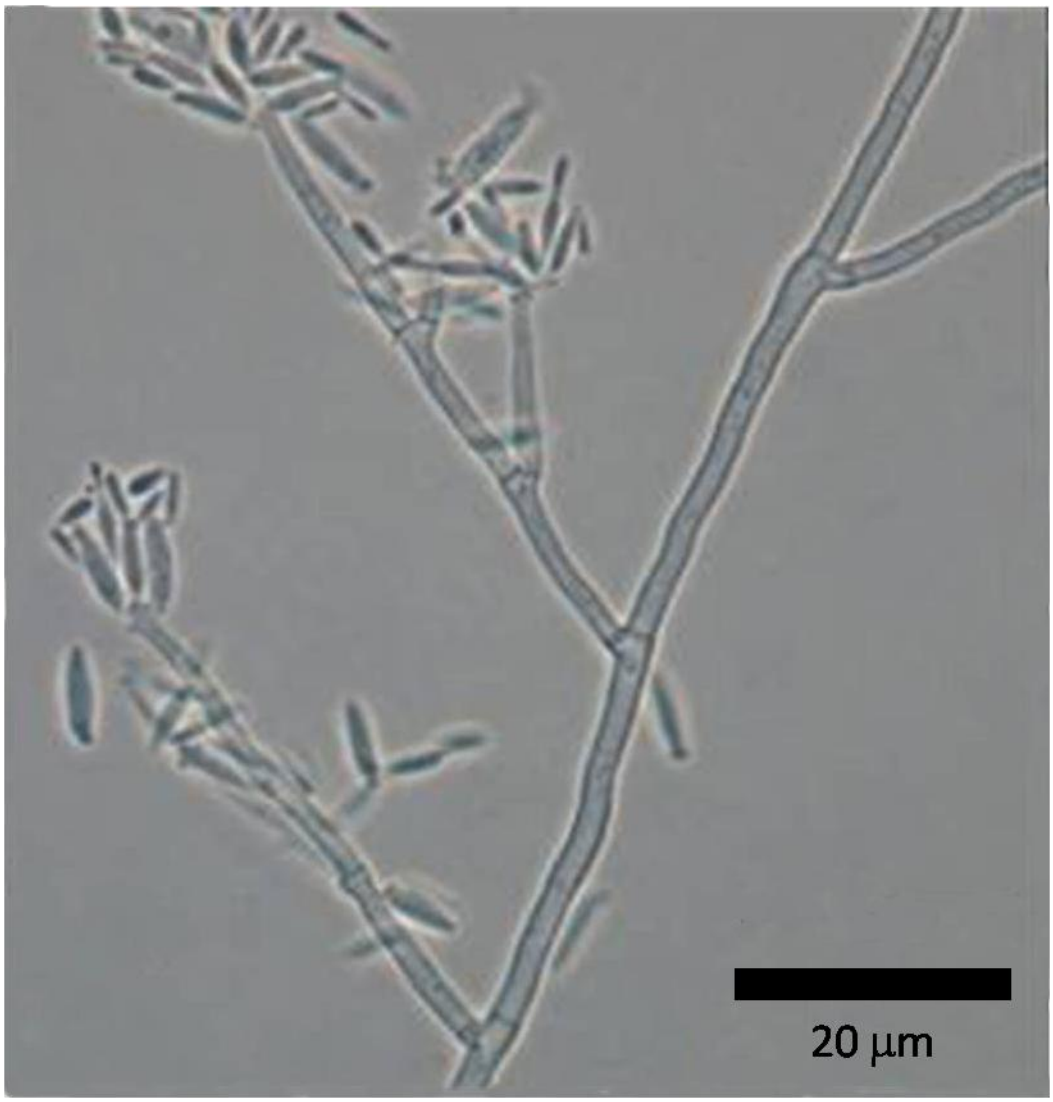

Figure 5.8. *Sporothrix* anamorph of *Ophiostoma sparsiannulatum*. Secondary conidia may be seen at apices of primary conidia.

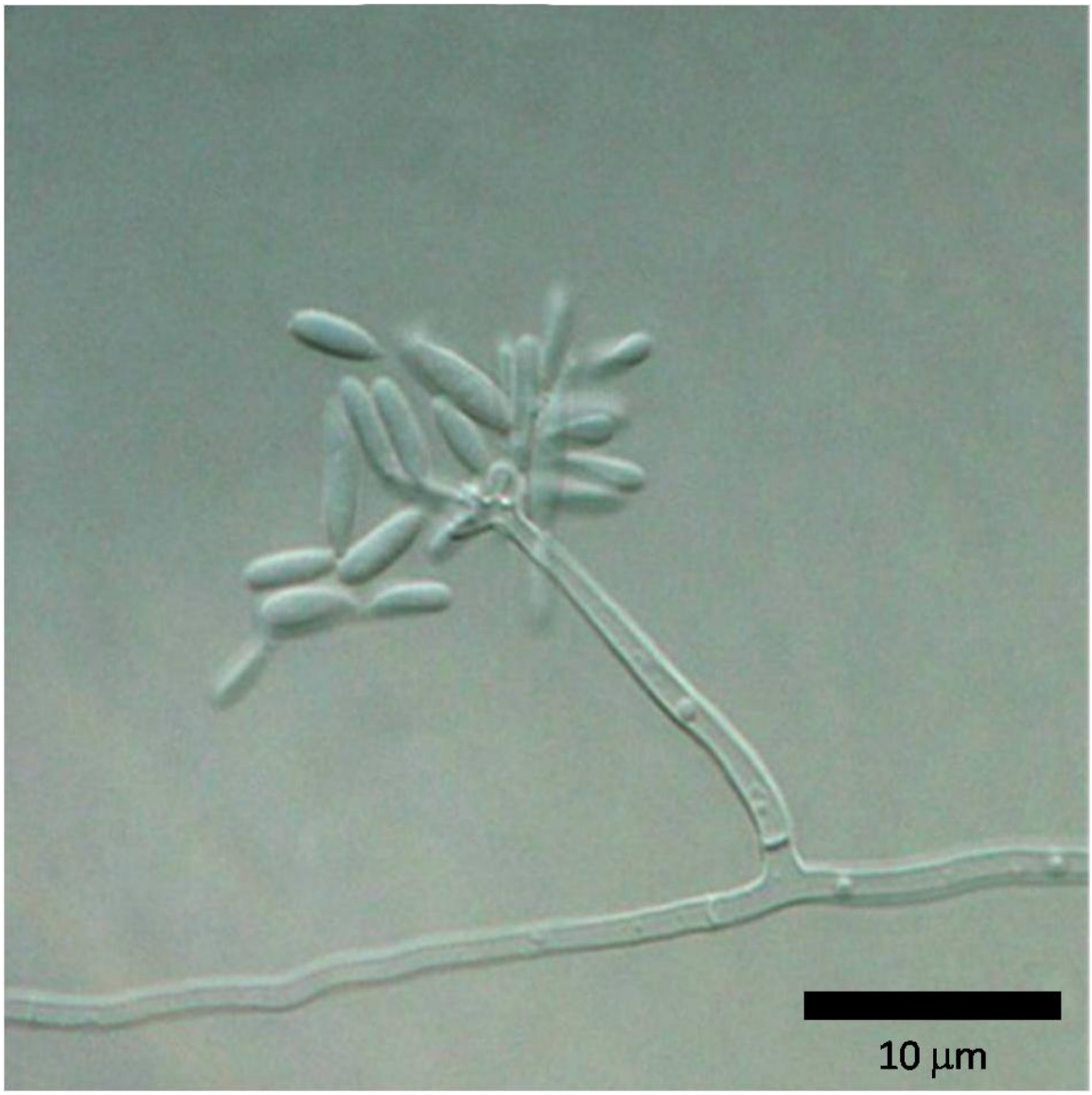

Figure 5.9. Detail of *Sporothrix* anamorph.

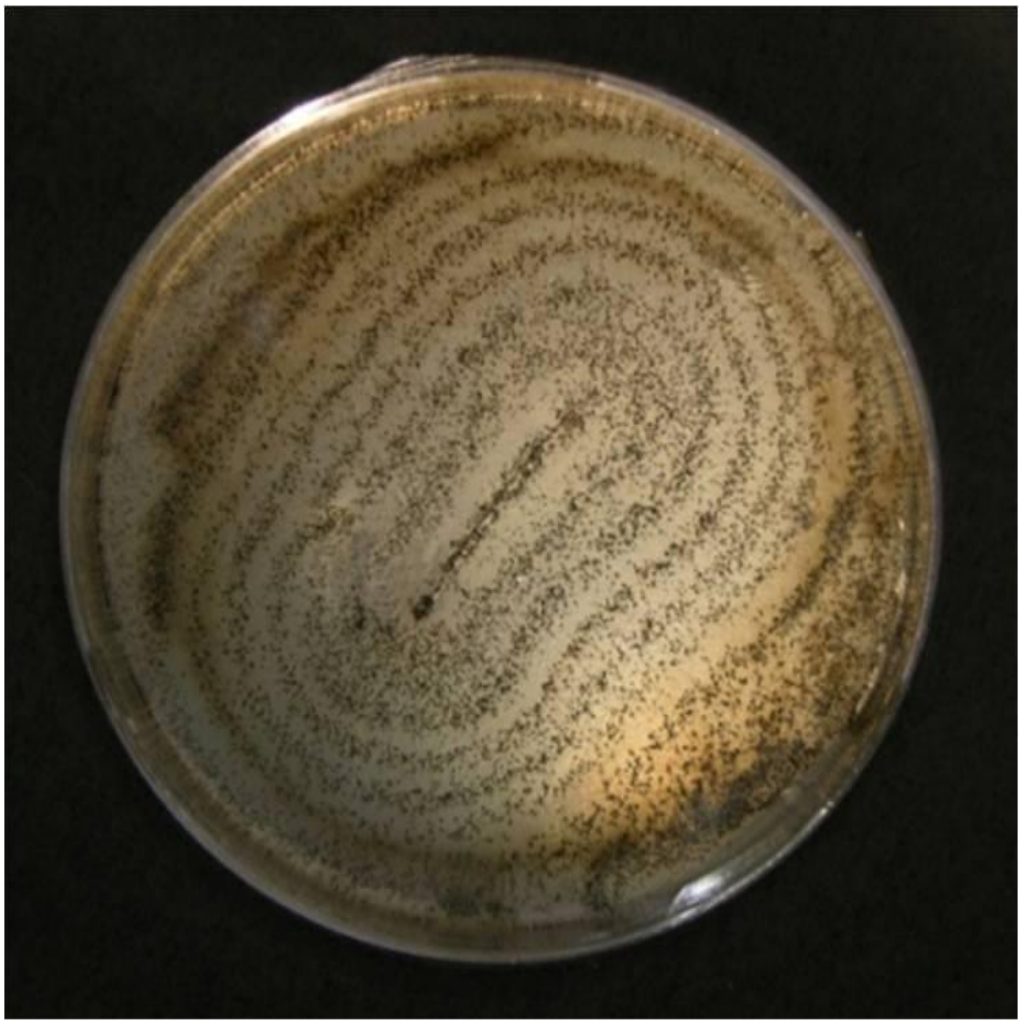

Figure 5.10. Culture plate of *Ophiostoma sparsiannulatum* on oatmeal agar, showing zonate growth

5.5. Discussion

This investigation revealed a new species, *Ophiostoma sparsiannulatum*, isolated from roots of pines and associated insects in the southeastern U.S. This species is morphologically similar and closely related to *O. pluriannulatum*. Recognition of the new species initially rested strongly on DNA sequence comparisons with *O. pluriannulatum* and related species in the OPC. Having recognized that it represented a novel taxon, it was possible to also recognize *O. sparsiannulatum* based on a unique ecology and various morphological characteristics, including a lack of annuli on mature ascomata, and the presence of the *Sporothrix* anamorph born in dry, floccose clusters.

Most members of the OPC have long perithecial necks with annuli. These annuli are likely vestiges of ostiolar hyphae through which the necks have proliferated. Both *O. pluriannulatum* and *O. multiannulatum* have perithecia with several annuli. While *O. sparsiannulatum* has perithecial necks similar in length to these species, it seldom produces annuli. In this regard, *O. sparsiannulatum* is more similar to *O. subannulatum*. The latter species is reported to produce its conidia in gelatinous masses, and thus *O. sparsiannulatum* can be distinguished by its dry conidia.

The importance of annuli as a diagnostic character for *Ophiostoma* spp. has been debated, with their importance either discounted (de Hoog, 1974) or emphasized (Upadhyay, 1981). Phylogenetic inference based on DNA sequences support the OPC as a monophyletic clade accommodating *Ophiostoma* spp. with annulate perithecial necks (Zipfel *et al.*, 2006) and this study supports that view. The sequence data presented also reiterate a point previously mentioned; a lack of species level resolution in the nrDNA

sequences. Analysis of the β-tubulin sequence data demonstrated improved species level resolution, and is recommended for further study in much of the ophiostomatoid fungi. A substantial dataset current exists for β-tubulin in NCBI GenBank, although caution must be exercised in sequence alignment as the variation in intron arrangement may cause problems in automated alignment algorithms such as ClustalX or MAFFT. Examination of the translated amino acid sequences can aid in manual alignment of these regions.

Gene trees are used to infer phylogenetic relationships in all ranks of taxonomy, including resolution of species. However, several processes may cause gene trees to be incongruent with species trees, i.e. a hypothesized phylogeny may misrepresent the actual phylogeny. Horizontal gene transfer (HGT) is prevalent in prokaryotes but also occurs in eukaryotes. In fungi, HGT has been invoked in many cases, but rarely proven (Rosewich and Kistler, 2000). Even so, evidence of eukaryotic HGT has more recently been show to occur between eukaryotic kingdoms (fungus-like Chromista and true Fungi, Richards *et al*., 2006), and between prokaryotes and eukaryotes (Fitzpatrick *et al*., 2008).

Hybridization has been observed in *Ophiostoma* spp. but is also apparently infrequent (Brasier *et al*., 1998). Hybridization can mask reticulation events, as trees only show divergence and not convergence. Paralogy (gene duplication) can lead to erroneous conclusions if sequences compared between taxa are not orthologous, as paralogous sequences do not reflect common ancestry. Insufficient data or sampling effects may also skew observed phylogenies. Of all the potential sources of phylogenetic

incongruities between genes and presented in this study, this is the most likely to have played a role, due to the limited number of isolates not only of *O. sparsiannulatum*, but of other members of the OPC as well.

In the present study, nrDNA sequence data lumped previously described species with *O. pluriannulatum*. β-Tubulin sequence data showed isolates here described as *O. sparsiannulatum* possess a different intron arrangement in this gene that is distinct from known isolates of *O. pluriannulatum*. In more detailed analysis with only sequences with similar intron arrangement, the isolates presented here as *O. sparsiannulatum* represent a unique lineage and morphology. Intron rearrangements are not common events (L.Goertzen, *personal communication*) but within β-tubulin sequences in *Ophiostoma* sensu lato the arrangement of introns 4 and 5 varies by clade, and arrangement varies within members of the OPC (Zipfel *et al*., 2006). While species trees may only be approximated by using single genes, the sequence data presented here are congruent with other species level phylogenies within the genus (Zipfel *et al*., 2006). Thus within the context of species in the OPC described based on morphological characters and supported by DNA sequence data, *O. sparsiannulatum* represents a novel species.

Within the OPC, *O. carpenteri* J. Reid & Hausner (Hausner *et al.*, 2003) represents an exception morphologically, in having short and wide necks basal constrictions, and conidia which are distinctly more globose or obovoid to clavate. In this regard, it is not likely to be confused with the other species in the OPC. Likewise, several species of *Ophiostoma* with annulate perithecia reside outside the clade accommodating the OPC.

The first species to be described in the OPC was *O. pluriannulatum* from stained sapwood of northern red oak (*Quercus rubra* L.) (Hedgcock, 1906). Several eastern hardwoods are known hosts for *O. pluriannulatum*, including *Liquidambar styraciflua* L., *Liriodendron tulipifera* L., *Aesculus glabra* Willd., and *Carya glabra* (Mill.) Sweet (Upadhyay, 1981), and on hardwood logs and lumber (Davidson, 1935). In Davidson's survey (1935), *O. pluriannulatum* and *O. multiannulatum* were also found on pine logs and lumber, though less frequently than on hardwoods. However, identification of these fungi was based solely on morphology and a clear indication of whether host specificity exists for this group of fungi has yet to emerge. It may be possible that reports of *O. pluriannulatum* from pines, including in New Zealand (Thwaites *et al.*, 2005) and Chile (Zhou *et al.*, 2004), have misidentified the fungus as these studies rely on nrDNA data for determination. For the present, *O. sparsiannulatum* is known from the roots of *P. taeda* and *P. palustris*, and from five associated root-infesting insect species. Given that these insects feed in and inhabit *Pinus* spp., it seems likely that the fungus will share this habit closely.

While *O.sparsiannulatum* has been recovered from the living roots of declining pine trees, its recovery is infrequent. This suggests that the fungus is not an important contributing factor in southern pine decline (Eckhardt *et al.*, 2007; Menard, 2007). Pathogenicity data are not available for all members of the OPC but these fungi are not considered to be serious pathogens (Hedgcock, 1906; Davidson, 1935). Nonetheless, further studies should include root inoculations with *O. sparsiannulatum* to evaluate 1.)

its capacity to infect roots, 2.) whether it can be transmitted to lumber, 3.) its capacity to produce blue-stain, 4.) further examination of how *O. sparsiannulatum* is vectored, and 5.) its range beyond FB.

5.6. Acknowledgments

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5.7. Literature cited

Aghayeva, D.N., Wingfield, M.J., de Beer, Z.W., Kirisits, T., 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia 96, 866-878.

Alamouti, S.M., Kim, J.J., Humble, L.M., Uzunovic, A., Breuil, C., 2007. Ophiostomatoid fungi associated with the northern spruce engraver, *Ips perturbatus*, in western Canada. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 91, 19-34.

Brasier, C.M., 1991. *Ophiostoma novo-ulmi* sp nov, causative agent of current Dutch elm disease pandemics. Mycopathologia 115, 151-161.

Brasier, C.M., Kirk, S.A., Pipe, N.D., Buck, K.W., 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycological Research 102, 45-57.

Brockway, D., Outcalt, K., Boyer, W., 2006. Longleaf pine regeneration ecology and methods. In: Jose, S., Jokela, E., Miller, D. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 95-133.

Chung, W.H., Kim, J.J., Yamaoka, Y., Uzunovic, A., Masuya, H., Breuil, C., 2006. *Ophiostoma breviusculum* sp nov (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. Mycologia 98, 801-814.

Davidson, R.W., 1935. Fungi causing stain in logs and lumber in the southern states, including five new species. Journal of Agricultural Research 50, 789-807.

de Beer, Z.W., Wingfield, B.D., Wingfield, M.J., 2003. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. Mycological Research 107, 469-476.

de Hoog, G.S., 1974. The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. Studies in Mycology 7, 84p.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

Fitzpatrick, D.A., Logue, M.E., Butler, G., 2008. Evidence of recent interkingdom horizontal gene transfer between bacteria and *Candida parapsilosis*. BMC Immunology 9, 15.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113-118.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61, 1323-1330.

Gorton, C., Kim, S.H., Henricot, B., Webber, J., Breuil, C., 2004. Phylogenetic analysis based on partial ITS of the bluestain fungus *Ophiostoma minus* rDNA and beta-tubulin gene sequences. Mycological Research 108, 759-765.

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D., Farrell, R., 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. Mycologia 93, 111-136.

Hausner, G., Eyjolfsdottir, G.G., Reid, J., 2003. Three new species of *Ophiostoma* and notes on *Cornuvesica falcata*. Canadian Journal of Botany-Revue Canadienne De Botanique 81, 40-48.

Hausner, G., Reid, J., 2003. Notes on *Ceratocystis brunnea* and some other *Ophiostoma* species based on partial ribosomal DNA sequence analysis. Canadian Journal of Botany-Revue Canadienne De Botanique 81, 865-876.

Hausner, G., Reid, J., Klassen, G.R., 1993. On the phylogeny of *Ophiostoma*, *Ceratocystis* ss, and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. Canadian Journal of Botany-Revue Canadienne De Botanique 71, 1249-1265.

Hedgcock, G.G., 1906. Studies upon some chromogenic fungi which discolor wood. Mo. Bot. Gard. Ann. Rept. 17, 59-114.

Hicks, B.R., Cobb, F.W., Gersper, P.L., 1980. Isolation of *Ceratocystis wageneri* from forest soil with a selective medium. Phytopathology 70, 880-883.

Huelsenback, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. In. Bioinformatics, pp. 754-755.

Kamgan, N.G., Jacobs, K., de Beer, Z.W., Wingrield, M.J., Roux, J., 2008. *Ceratocystis* and *Ophiostoma* species, including three new taxa, associated with wounds on native South African trees. Fungal Diversity 29, 37-59.

Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9, 286-298.

Menard, R., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. Louisiana State University, Baton Rouge, LA, p. 54.

Nylander, J.A.A., 2004. MrModeltest. Evolutionary Biology Center, Uppsala University, Program distributed by the author.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7, 103-116.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant and Soil 217, 145-150.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. In. Bioinformatics, pp. 817-818.

Richards, T.A., Dacks, J.B., Jenkinson, J.M., Thornton, C.R., Talbot, N.J., 2006. Evolution of filamentous plant pathogens: Gene exchange across eukaryotic kingdoms. Current Biology 16, 1857-1864.

Roets, F., de Beer, Z.W., Dreyer, L.L., Zipfel, R., Crous, P.W., Wingfield, M.J., 2006. Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Studies in Mycology, 199-212.

Rosewich, U.L., Kistler, H.C., 2000. Role of horizontal gene transfer in the evolution of fungi. Annual Review of Phytopathology 38, 325-363.

Schirp, A., Farrell, R.L., Kreber, B., 2003. Effects of New Zealand sapstaining fungi on structural integrity of unseasoned radiata pine. Holz Als Roh-Und Werkstoff 61, 369-376.

Seifert, K.A., 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield, M.J., Seifert, K.A., Webber, J.F. (Eds.), *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. American Phytopathological Society Press, St Paul, MN, pp. 141-151.

Six, D.L., 2003. Bark Beetle-Fungus Symbioses. In: Bourtzis, K., Miller, T.A. (Eds.), Insect Symbiosis. CRC Press, Boca Raton, FL, pp. 99-116.

Spatafora, J.W., Blackwell, M., 1994. The polyphyletic origins of Ophiostomatoid fungi. Mycological Research 98, 1-9.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C., McNew, D., 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. New Zealand Journal of Botany 43, 653-663.

Upadhyay, H.P., 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, GA.

Uzunovic, A., Seifert, K.A., Kim, S.H., Breuil, C., 2000. *Ophiostoma setosum*, a common sapwood staining fungus from western North America, a new species of the *Ophiostoma piceae* complex. Mycological Research 104, 486-494.

Villarreal, M., Rubio, V., De Troya, M.T., Arenal, F., 2005. A new *Ophiostoma* species isolated from *Pinus pinaster* in the Iberian Peninsula. Mycotaxon 92, 259-268.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., J., W.T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc, New York, NY, pp. 315-322.

Zhou, X.D., de Beer, Z.W., Ahumada, R., Wingfield, B.D., Wingfield, M.J., 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Diversity 15, 261-274.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Studies in Mycology, 75-97.

CHAPTER VI. SUMMARY AND GENERAL CONCLUSIONS

6.1. Decline and longleaf pine.

A previous study of longleaf pine (*Pinus palustris* Mill.) decline has shown (Otrosina *et al.*, 1999) and study of loblolly pine (*P. taeda* L.) decline suggests (Eckhardt *et al.*, 2007) a potential role of ophiostomatoid fungi and their insect vectors in longleaf pine decline. However, it is important to clearly state that the results obtained in this dissertation do not address the role of these fungi in a direct way.

The first objective of this research was to apply the loblolly pine decline risk model (LPDRM) developed in Alabama (Eckhardt, 2003) to longleaf pine stands at Fort Benning Military Reservation, GA (FB). The LPDRM has been previously applied to loblolly pine stands at several sites in Alabama (Eckhardt and Menard, 2008), Louisiana, Mississippi, South Carolina, Texas, (Eckhardt and Menard, unpublished), and at FB (Menard, 2007). Declines are thought to result from a constellation of predisposing, inciting, and contributing factors (Manion, 1981), which can also be considered in terms of their respective "corners" of the Disease Triangle. The Disease Triangle is a basic

principle of plant pathology in which disease only results when three elements co-occur; host, pathogen, and environment. Here, the definition of "pathogen" is expanded to include pestiferous insects and other biotic agents (Table 6.1.).

6.2. Host factors

In testing the applicability of the LPDRM to longleaf pine, an array of host variables was observed; tree height and diameter growth, crown condition, preformed resin reserves, and age. Few differences were observed between the predicted symptomatic and predicted asymptomatic plots in crown data, growth data, standing resin reserve mass, and or class, with the exception of some differences in the 20-40 year age class. Previous studies of longleaf pine decline have focused on trees in this 20-40 year age class (Otrosina *et al.*, 1999; Sullivan *et al.*, 2003). It should be noted that the delineation of age classes was intended to serve as a surrogate for size classes (Menard, 2007), which is not always possible in longleaf pine due to the vagaries associated with height growth initiation after the grass stage. As the observed differences in decline class predictions did not extend into the >40 age class, it is reasonable to focus on questions of how this age class may have differed from younger and older trees.

Table 6.1. Examples of predisposing, inciting and contributing factors leading to decline, adapted from Manion, 1981. Each factor is also designated as environmental (E), host (H), and pathogen/pest (P) from the Disease Triangle concept.

Predisposing Factors	Inciting Factors	Contributing Factors
Aspect (E)	Air pollutants, acute (E)	Bark beetles (P)
Air pollutants, chronic (E)	Chemical injury (E)	Canker fungi (P)
Climate (E)	Drought (E)	Defoliating insects (P)
Climate change (E)	Excessive salt (E)	Nematodes (P)
Diseases of site (e.g. *Armillaria* spp.) (P)	Fire (E)	Root-decay fungi (P)
Host genotype (H)	Frost (E)	Saprogenic fungi (P)
"Off site" (E)	Fungal infection (e.g. brown spot needle blight) (P)	Viruses (P)
Salt (E)	Hog damage (P?)	
Slope (E)	Insect defoliation (P)	
Soil moisture (E)	Mechanical injury (E)	
Urban environment (E)	Overmaturity (H)	

The 20-40 year age class began during a period of range-wide reduction in growth and reproduction in longleaf pine (West *et al.*, 1993; Brockway *et al.*, 2006). Since 1986, the growth of longleaf began to increase, and several large cone crops were produced in successive years (Brockway *et al*., 2006). Also the 20-40 year age class is likely to have been planted from bare root stock rather than container grown seedling, though natural stands may also have been present. Improved understanding of how to regenerate longleaf pine naturally has increased this method of establishment (Croker and Boyer, 1975). Exposure to adverse environmental factors as juveniles has been shown to have repercussions that can extend into maturity in longleaf pine (Wakeley, 1970), thus the inclusion of planting method as a variable in predicting decline could be illuminating in future studies as a potential predisposing factor.

6.3. Environmental factors

The majority of predisposing and inciting factors listed in Table 6.1 are environmental. While site factors have been used in developing ecological models, including the LPDRM, the effects of inciting factors are difficult to measure without direct manipulation. Often these types of manipulations are neither possible nor practical, or can only be considered indirectly, in greenhouse assays (Matusick *et al.*, 2008). These observations in longleaf pine stands at FB came after a prolonged drought. Longleaf pine is a drought tolerant species, and one functional explanation of having

a grass stage is allocation of resources into taproot growth, protecting the host from drought. Drought effects also extend to insect populations, again not measurable within the time scale of this dissertation.

In addition to drought, fire may be an important inciting factor. Longleaf pine is dependent on fire in all stages of its life history. Fire return interval varied throughout the historic range (Frost, 2006), and distribution of fire intensity was certainly patchy pre-European settlement. Even in studies where fire has been applied, the effects are often inconsistent, as is seen in comparing the insect capture results obtained by Hanula *et al.* (2002) and Sullivan *et al.* (2003). Whether a plot was burned or not was considered as an explanatory variable for *Leptographium* Lagerb.& Melin incidence, however stepwise logistic regression did not result in burning as a predictor of disease or fungal incidence. Although fire effects are difficult to measure under ideal conditions, fire is considered an important factor in longleaf pine decline (Otrosina *et al.*, 1999; Sullivan *et al.*, 2003; Varner *et al.*, 2007), and future studies should systematically incorporate fire.

6.4. An anecdotal data point

Only one tree in this study can be said to have expressed decline symptoms as described by Otrosina *et al.*, (1999). While anecdotal, some of the circumstances leading to the death of this single tree can offer direction for future research. This tree was over

100 years old which is young considering the potential for longleaf pine. The tree was the only one to yield *G. huntii*, one of the predominant fungi of *Hylastes* spp. Erichson during this investigation.

This tree also yielded other *Leptographium* and *Ophiostoma* Syd. & P. Syd. species (though *O. sparsiannulatum* could not be definitively identified as one of the species infecting this individual). The stand was burned during the dormant season prior to excavation, and prior to burning had dense underbrush. The plot was initially classified as a predicted symptomatic site, although the aspect was in the lowest predicted risk class (339 °), and slope (6.2 %) was only of moderate risk according to LPDRM. The tree was similar to other trees in the stand in all observed crown and size parameters when observed in 2006. The two other excavated trees in the plot did not yield *Leptographium* spp, and were asymptomatic when observed in 2007. The plot did not have a significantly higher number of insect vector captures.

Thus, low numbers of beetles may be sufficient to transfer ophiostomatoid fungi to their host. As suggested above, fire is likely to have had an important inciting role, presuming the tree was uninfected prior to the fire, or possibly infected with *L. procerum* (Kendrick) Wingfield and *L. terebrantis* Barras & Perry which were common in asymptomatic trees in other stands. Loblolly pine, also present within the site, were also symptomatic, with thinning crowns and several dead individuals throughout the stand.

Plot size and sparsity of trees prevented incorporation of these symptomatic loblolly

pines in the data set. In future studies, it is recommended variable area plots be used to empirically examine the distribution of longleaf pine decline at the stand level, and to examine interactions with symptomatic loblolly pines.

6.5. Pathogens and pests

These results present new information relevant to insect and fungi biology in southeastern pine forests. The scolytine fauna of southern pine forests is dynamic, based on seasonal, annual, and interannual periods. In this study the insect alpha diversity was similar to previous studies in the region, although information about the distribution of *H. porculus* Erichson in the southeast suggests that this species may be an important fungal vector during the winter months. *Hylastes tenuis* Eichhoff was the predominant vector insect captured, outnumbering captures of the larger *Hylastes* spp. by almost threefold. This study reports a numerical disparity in favor of *H. tenuis*, a relationship observed infrequently (Cibulsky, 1971). More frequently observed are ratios where *H. salebrosus* predominates by 3:1::*H .salebrosus* :*H. tenuis* (Eckhardt *et al.*, 2007), or higher, e.g. (Hanula *et al.*, 2002; Bauman, 2003; Sullivan *et al.*, 2003).

The timing of peaks observed in other species (*Pachylobius picivorus* [Germar], *Dendroctonus terebrans* [Olivier]) must be interpreted cautiously. While temperature maxima and minima correlated well with insect captures in some species (*P. picivorus* with high temperatures, *D. terebrans* with low temperatures), these weather variables are serially autocorrelated (i.e. cold weeks are temporally near other cold weeks, warm

weeks are temporally near other warm weeks, and the two grade into each other). To remedy this, multiple years of data would be necessary to be able to correct for serial autocorrelation. However, these observations do indicate tolerances of hot and cold extremes for *P. picivorus* and *D. terebrans*, respectively. *Hylobius pales* (Herbst) and *P. picivorus* are known to be sympatric throughout their range, but differ in ratio regionally (Nord *et al.*, 1984). In the southeastern U.S., *Hb. pales* typically exceeds *P. picivorus*, but the ratio increases in the northern part of the range. In the present study, *P. picivorus* outnumbered *Hb pales* by more than 2:1, a result similar to the observations of other studies in the southeastern U.S. (Sullivan *et al.*, 2003; Eckhardt *et al.*, 2007; Menard, 2007). However, Hanula *et al.* (2002) found a ratio of *Hb. pales* : *P. picivorus* as high as 7:1 at a site in Florida. *Hylobius pales*, unlike *P. picivorus*, did not correlate with weather predictors, but was 'noisy' at both the spatial and temporal levels. An extreme number for *Hb. pales* captures in spring 2006 (37, compared with a mean of approximately 3 captures from all plots/week) was found to have extended to 17 plots, for an average 2.12 captures per trap during that week. The presence of infested insects during temperature extremes (excluding freezes) suggests that root infection by ophiostomatoid fungi may occur year round. Study to determine when trees are most likely to be infected merits future research.

In this study a novel fungal species, *O. sparsiannulatum*, is described. Little is known of the ecology of the new species; its pathogenicity, or whether it causes blue stain in logs as do *O. pluriannulatum* (Hedgcock) Syd. & P. Syd. and *O. multiannulatum* (Hedgcock & Davidson) Hendr. (Hedgcock, 1906; Davidson, 1935). Currently, it is

known that *O. sparsiannulatum* may infect loblolly roots, and that it may be vectored by all five of the most frequently captured root-feeding beetles. As this fungus has a *Sporothrix* anamorph, it might be moved by either air or water, but lack of isolation from soil suggests that insects are the predominant mode of movement. As has been shown in other *Ophiostoma* spp. (Harrington *et al.*, 2001; Chung *et al.*, 2006), and elsewhere in this dissertation, multiple gene phylogenies may reveal fungal misidentifications. It is likely that reports of *O. pluriannulatum*, based solely on morphology or nuclear ribosomal DNA phylogenies (Thwaites *et al.*, 2005; Zhou *et al.*, 2006), may actually be either *O. sparsiannulatum* or other novel species.

The ophiostomatoid mycota of FB may be shifting over time, with *G. huntii* (Robinson-Jeffery & Grinchenko) Zipfel, de Beer, & Wingf. becoming an important species on insects and *L. serpens* (Goid.) Siem. becoming much less abundant. It is possible that misidentifications were made in earlier study, but in a survey of 154 isolates previously identified as *L. serpens* from loblolly pine roots and associated insects at FB (Menard and Eckhardt, Unpublished), only one isolate (from *H. salebrosus*) was found to be *G. huntii*. Both *G. huntii* and *L. serpens* produce larger lesions than *L. terebrantis* and *L. procerum* in artificial inoculations (Matusick and Eckhardt, in preparation), which further supports the hypothesis of southern pines being new hosts to the former two species, i.e., *G. huntii* and *L. serpens* are recently introduced. Future studies of the ophiostomatoid mycota of the southeast are necessary to monitor spread of these fungi, and to determine the conditions that may favor or inhibit infection by these fungi. Also,

it is hypothesized that *H. opacus* may have been the original vector to bring these fungi from Europe,but a survey of the mycota of this species in North America is wanting.

The rarity of *G. huntii* in isolations from longleaf pine roots presents a conundrum. Given the abundance of *Hylastes* spp. in the insect collections, and the frequency of *G. huntii* recovered from them, a higher incidence of *G. huntii* from roots was expected. Several hypotheses, not mutually exclusive, may explain these observations. The most straightforward is undersampling of roots; 96 longleaf pine trees were excavated, and two lateral roots sampled per tree. Previous excavations of symptomatic longleaf pine at FB and elsewhere have yielded isolates of *L. serpens*, which was not recovered in this study (L.G. Eckhardt, unpublished data), and supports the undersampling of roots hypothesis. Another possibility is that *Hylastes* spp. prefer other hosts to longleaf pine (e.g. loblolly pine), and attack longleaf pine only occasionally. The hypothesis that *G. huntii* (and *L. serpens*) might trigger a hypersensitive response that *L. procerum* and *L. terebrantis* do not is supported by inoculation experiments (Matusick and Eckhardt, in preparation). If the suggestion that *G. huntii* and *L. serpens* are both recent introductions is correct, then large shifts in fungal abundance and distribution would be expected. In native species, the long coevolution between tree host, fungus, and insect vectors would make shifts in abundance less abrupt in the absence of large disturbance. Continued research of the biology and ecology of *G. huntii* and *L. serpens* are warranted, as the management of diseases caused by putative exotic agents is distinct from the management of diseases caused by native agents.

6.6. Potential for longleaf pine on high risk loblolly pine decline sites

Longleaf and loblolly pine, while probably not overlapping in habitat to a great extent historically, now occur in sympatry throughout most of their mutual range. Thus while the role of ophiostomatoid fungi in longleaf pine decline is still uncertain, knowledge of these fungi and insect relations contribute to the state of knowledge in loblolly pine decline. In this dissertation, slope and aspect were found to have little effect on the prediction of longleaf pine decline. Thus, I hypothesize that longleaf might succeed on high-risk loblolly pine sites, and represent a tangible option for land managers. However, this hypothesis is not tested in this dissertation. Currently land managers faced with losses attributed to loblolly pine decline are opting to replant affected sites with longleaf pine (Denis LeBleu, personal communication), thus the need for rigorous testing of this hypothesis is timely.

Throughout this chapter, I have attempted to place the results from the original research described in this dissertation into context. Future research needs have been described, and technical refinements suggested. It is my hope that the utility of these data is accepted by the agency soliciting them, and my greater hope that future researchers realize the value in this work and improve upon it.

6.7. Literature cited

Bauman, T.A., 2003. Interaction of fire and insects in the restoration and management of longleaf pine. MS Thesis Louisiana State University, Baton Rouge, LA, 81 p.

Brockway, D., Outcalt, K., Boyer, W., 2006. Longleaf pine regeneration ecology and methods. In: Jose, S., Jokela, E., Miller, D. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 95-133.

Chung, W.H., Kim, J.J., Yamaoka, Y., Uzunovic, A., Masuya, H., Breuil, C., 2006. *Ophiostoma breviusculum* sp nov (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. Mycologia 98, 801-814.

Cibulsky, R.J., 1971. Ecology of *Ips* spp. bark beetles (Coleoptera: Scolytidae) and associated insects on felled loblolly pine in Alabama. MS Thesis, Auburn University, 128 p.

Croker, T.C.J., Boyer, W.D., 1975. Regenerating longleaf pine naturally. USDA Forest Service, Southern Forest Experiment Station, Research Paper SO-105. New Orleans, LA, 26 p.

Davidson, R.W., 1935. Fungi causing stain in logs and lumber in the southern states, including five new species. Journal of Agricultural Research 50, 789-807.

Eckhardt, L.G., 2003. Biology and ecology of *Leptographium* species and their vectors as components of loblolly pine decline. In, Louisiana State University, Baton Rouge, LA, 158 p.

Eckhardt, L.G., Menard, R.D., 2008. Topographic features associated with loblolly pine decline in central Alabama. Forest Ecology and Management 255, 1735-1739.

Eckhardt, L.G., Weber, A.M., Menard, R.D., Jones, J.P., Hess, N.J., 2007. Insect-fungal complex associated with loblolly pine decline in central Alabama. Forest Science 53, 84-92.

Frost, C., 2006. History and future of the longleaf pine ecosystem. In: Jose, S., Jokela, E., Miller, D. (Eds.), The longleaf pine ecosystem: ecology, silviculture, and restoration. Springer, New York, NY, pp. 9-48.

Hanula, J.L., Meeker, J.R., Miller, D.R., Barnard, E.L., 2002. Association of wildfire with tree health and numbers of pine bark beetles, reproduction weevils and their associates in Florida. Forest Ecology and Management 170, 233-247.

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D., Farrell, R., 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. Mycologia 93, 111-136.

Hedgcock, G.G., 1906. Studies upon some chromogenic fungi which discolor wood. Mo. Bot. Gard. Ann. Rept. 17, 59-114.

Manion, P.D., 1981. Tree disease concepts. Prentice-Hall, Englewood Cliffs, N.J.

Matusick, G., Eckhardt, L.G., Enebak, S.A., 2008. Virulence of *Leptographium serpens* on longleaf pine seedlings under varying soil moisture regimes. Plant Disease 92, 1574-1576.

Menard, R.D., 2007. An assessment of the risk mapping system for the use of managing loblolly pine decline sites within red-cockaded woodpecker habitat. MS Thesis, Louisiana State University, Baton Rouge, LA, 54 p.

Nord, J.C., Ragenovich, I., Doggett, C.A., 1984. Pales weevil. U.S. Dept. of Agriculture, Forest Service, Southeastern Region State & Private Forestry, Forest Insect and Disease Leaflet No. 104. Asheville, NC.

Otrosina, W.J., Bannwart, D., Roncadori, R.W., 1999. Root-infecting fungi associated with a decline of longleaf pine in the southeastern United States. Plant and Soil 217, 145-150.

Sullivan, B.T., Fettig, C.J., Otrosina, W.J., Dalusky, M.J., Berisford, C.W., 2003. Association between severity of prescribed burns and subsequent activity of conifer-infesting beetles in stands of longleaf pine. Forest Ecology and Management 185, 327-340.

Thwaites, J.M., Farrell, R.L., Duncan, S.M., Reay, S.D., Blanchette, R.A., Hadar, E., Hadar, Y., Harrington, T.C., McNew, D., 2005. Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. New Zealand Journal of Botany 43, 653-663.

Varner, J.M., Hiers, J.K., Ottmar, R.D., Gordon, D.R., Putz, F.E., Wade, D.D., 2007. Overstory tree mortality resulting from reintroducing fire to long-unburned longleaf pine forests: the importance of duff moisture. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 37, 1349-1358.

Wakeley, P.C., 1970. 30-year effects of uncontrolled brown spot on planted longleaf pine. Forest Science 16, 197-202.

West, D.C., Doyle, T.W., Tharp, M.L., Beauchamp, J.J., Platt, W.J., Downing, D.J., 1993. Recent growth increases in old growth longleaf pine. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 23, 846-853.

Zhou, X.D., de Beer, Z.W., Wingfield, M.J., 2006. DNA sequence comparisons of *Ophiostoma* spp, including *Ophiostoma aurorae* sp nov, associated with pine bark beetles in South Africa. Studies in Mycology, 269-277.