PATHOGENICITY AND TAXONOMY OF FUNGI ASSOCIATED WITH
THE MOUNTAIN PINE BEETLE IN BRITISH COLUMBIA

by

ALEXANDER B. L. PLATTNER

Honours B.Sc., The University of Toronto, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA

February 2008

Abstract

The mountain pine beetle is associated with a diverse array of fungi. Grosmannia clavigera is the most pathogenic of these fungi. A comparison was made between two methods that have been used to assess fungal pathogenicity. Results were similar for older trees inoculated with G. clavigera using either the alternating flap technique or cork borer method. Using the cork borer method, younger lodgepole pine trees were inoculated with five different isolates of G. clavigera. After a 48 week incubation period, isolates ATCC 18086, B5 and H55 had induced stronger pathogenic indicators compared to isolates KW 1407 and B20. After a 7 week incubation period, only isolate ATCC 18086 had induced stronger pathogenic indicators. Usually, this isolate grew faster at lower temperatures and in a low oxygen environment. Isolate KW 1407 consistently produced milder pathogenic indicators during both incubation periods. Among the non-pathogenic fungal associates of the mountain pine beetle, Ceratocystiopsis minuta may be considered the most important because it is the type species for the genus Ceratocystiopsis. The history of this genus is complicated because no physical specimen exists for C. minuta. The phylogeny of the genus Ceratocystiopsis was evaluated. Many isolates of C. minuta were assessed as potential epitypes. Several isolates of C. minuta from previous work were shown to be misidentified. C. minuta isolate CBS 116796 is recommended for future genetic work within the genus Ceratocystiopsis. For morphological work, using measurements from the literature is recommended since CBS 116796 did not produce fruiting bodies.

Table of contents

List of tables

List of figures

Dedication

I would like to thank all the members of my family, especially my mother Mrs. Apollonia Plattner and my deceased father, Dr. Gunther Plattner, and my grandfather Dr. C.K.T. Lee and grandmother Dr. Lillian Hsu, for providing me with all the support and inspiration one could ask for. I would like to thank my siblings, Chris Plattner and Chrissandra Plattner, and E.S. and L. R. for believing in me, even when I did not believe in myself. I would like to thank M.L., K.H., Dr. C.N., M.N., K.P. and S.T. for their friendship and support during my time at UBC.

Co-authorship statement

I would like to acknowledge Dr. Colette Breuil, Dr. Jae-Jin Kim and Dr. Young Woon Lim for their assistance in helping to identify the research topics contained in this thesis.

All the research, data analyses and manuscript preparation were originally prepared by myself, although Dr. Colette Breuil considerably aided in revising all manuscripts in this thesis.

Chapter 1

Introductory chapter

[1]The mountain pine beetle (MPB), *Dendroctonus ponderosae* (Coleoptera, Curculionidae, Scolytidae) (Figure 1.1), is one of the most destructive bark beetles in North America (Farrell et al. 2001). Its northern range extends to British Columbia and along the western edges of Alberta and continues southward from Washington State to the northwestern tip of Mexico (Ono 2003; Li et al. 2005; Stahl et al. 2006).

The MPB infests tree species belonging to the genus *Pinus*, such as lodgepole (*Pinus contorta*), ponderosa (*Pinus ponderosa*), whitebark (*Pinus albicaulis*), Jack (*Pinus banksiana*), eastern white (*Pinus strobus*), limber (*Pinus flexilis*), Scots (*Pinus sylvestris*) and sugar pines (*Pinus lambertiana*) (http://www.barkbeetles.org/mountain/fidl2.htm, Amman et al. 1990; Solheim and Krokene, 1998; Ono 2003). The MPB occupies an important niche in pure and mixed pine forest ecosystems (Pederson 2004; Kim et al. 2005; McFarlane et al. 2006). During endemic phases, other beetles or pathogens usually infest host pines first, followed by MPB that infest weakened or dying trees (Parker et al. 2006), thus removing them from healthy forests. However, during epidemic phases, the MPB primarily targets mature trees, and with its fungal associates can kill healthy individuals, causing widespread tree death (Cole et al. 1976; Amman et al. 1990; Elkin and Reid 2004; Stahl et al. 2006).

The timing of MPB attack is temperature related, with earlier attacks occurring in warmer areas. Typically, the window of attack is from early July to early September (MoFR website 2007). The majority of the MPB one-year life cycle is spent under the bark of trees (Whitney 1971; Paine et al. 1997; Kim et al. 2005). If the beetles successfully overcome host tree defense mechanisms, the adults construct vertical galleries in the phloem under the bark where they lay eggs. During this process, fungal spores are transferred to the phloem and the outer surface of the sapwood (Reid et al. 1967; Shrimpton and Whitney 1968;

---

[1] A version of this chapter has been submitted for publication

Kim et al. 2005). Some of the fungal spores belong to fungi in the Ophiostomatoid group and grow rapidly in the phloem and the sapwood. At the same time, larvae tunnel horizontally in the phloem after they hatch. The combined actions of the fungi and the beetle impede the transportation of nutrients and water within the phloem of the tree, typically resulting in tree death (Amman et al. 1990; Paine et al. 1997). Before emerging, young adult beetles come in contact with fungi that have grown from the spores introduced by the parents and whose hyphae and spores now line the pupal chamber (Six 2003a). After emergence of new adults, the slimy spores of fungi present on the exoskeleton, mycangia and/or gut of new adult beetles will be transported to new trees. Once a tree has been successfully infested by MPB during epidemic phases, it also becomes more vulnerable to infestation by secondary beetles such as *Ips* and ambrosia species (Solheim 1995; Paine et al. 1997; Kim et al. 2005). Secondary beetles carry additional decay fungi; hence, trees infested by primary and secondary beetles are very likely to experience decreases in structural integrity as the white rot and brown rot basidiomycetes spread by beetles degrade lignin, cellulose and hemicellulose within lodgepole pines (Zabel and Morell 1992).

It may be difficult to identify trees that have been attacked during the current year because colour changes in needles do not occur until the following year (Figure 1.2). Recently infested trees may be identified by the appearance of pitch tubes on the lower trunk region and boring dust on the tree and/or on the ground surrounding the tree (Amman et al. 1990) (Figure 1.2D). If successfully attacked, the foliage of a tree changes from green to yellow-green during the first year, then red the year after the emergence of new beetles and finally to grey in the third year, thus making it easy to perform large scale surveying to determine areas that have been recently attacked. The crowns of successfully defended trees do not change colour; some trees can withstand serious MPB challenges and still remain alive (John McLean, pers. comm.). Thus, red trees generally underestimate the amount of MPB attack in a stand. Green, red and grey-phases are terms used to indicate the time since successful MPB colonization of the tree

2

(Amman et al. 1990; Kim et al. 2005). The province of British Columbia contains large areas of red and grey phase attacked trees (Figure 1.3).

Two main factors largely responsible for the beetle's recent success are climate change, where continuously mild winters cause less brood mortality and where summer droughts stress trees, leaving them more susceptible to attack (http://www.for.gov.bc.ca/hfp/mountain_pine_beetle/facts.htm#weather, Aukema et al. 2006), and overly successful use of fire suppression, which has increased the proportion of old, weak and highly susceptible trees in the population to MPB attack (Amman et al. 1990; http://www.for.gov.bc.ca/hfp/mountain_pine_beetle/faq.htm#2).

The impacts of the mountain pine beetle outbreak

Over the last decade, a mountain pine beetle (MPB) epidemic has caused considerable damage to areas of economically important pine forests in Canada and the USA. In Canada, the current epidemic covers an area that stretches northwards to Fort St. James, B.C. (Stahl et al. 2006) and is expanding eastward into Alberta's boreal forests (http://www.for.gov.bc.ca/hfp/mountain_pine_beetle/faq.htm#12; Ono 2003). As of 2006, the MPB had affected 9.2 million hectares out of the ~60 million total hectares of BC forests (Figure 1.3) (BC Ministry of Forest and Range 2006), of which ~25 million hectares are pure or mixed lodgepole pine stands (Eng et al. 2006).

The outbreak is expanding and beetles are moving away from prime hosts as preferred trees become depleted. Lodgepole-jack pine hybrids are already being attacked along the BC-Alberta border. There is serious concern that the MPB, which preferentially inhabits lodgepole pine, may successfully over-winter in jack pine. This would result in an increase in susceptible hosts, allowing for a range expansion eastward. Information gathered during the current epidemic indicates MPB populations do attack jack pine and lodgepole-jack pine hybrids (http://www.for.gov.bc.ca/hfp/mountain_pine_beetle/faq.htm#12), although their ability to mass reproduce in these trees has not yet been fully

assessed. These data suggest that the eastward movement by MPB across Canada's boreal forest may be possible. The current large-scale mortality of mature lodgepole pine stands has caused the government to implement drastic salvage policies that may undermine the long term social stability of small, forestry-based urban population centers (Pederson 2004). As timber salvage increases beyond 100% of the annual allowable cut, the amount of available trees for harvest in the near future becomes greatly reduced.

Trees infested by MPB are worth less than sound trees due to the bluish-black staining caused by the fungi introduced into the sapwood by the MPB (Kim et al. 2005). Even though the stained wood retains its structural strength (Lum et al. 2006), its usage becomes limited due to its altered appearance. It is also likely that the introduction of decay fungi from MPB and secondary beetle infestation of lodgepole pine will give infested wood a short life span before structural properties are severely altered.

Dead lodgepole pine stands also pose various environmental problems such as an increased risk of fire, wind throw, and management problems, and have negative impacts on visual and recreational forest qualities as well as the aforementioned community instability problems (Pederson 2004; Kim et al. 2005; Li et al. 2005; McFarlane et al. 2006; Stahl et al. 2006).

Fungal associates of the MPB
Frequencies of mountain pine beetle-associated fungi
The mountain pine beetle (MPB) is intimately associated with several fungal species, the most abundant of which belong to the Ophiostomatoid group of ascomycetous fungi (Kim et al. 2005; Lee et al. 2006). Ophiostomatoid fungi have been isolated from infested sapwood, MPB body surfaces, mycangia and adult and juvenile galleries (Rumbold 1941; Robinson 1962; Whitney and Farris 1970; Whitney 1971; Six and Paine 1997; Six 2003a,b; Kim et al. 2005; Lee et al. 2006). Historically, the fungi *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Harrington and *Ophiostoma montium* (Rumbold) von Arx and a few yeast species have long been found with the MPB (Rumbold 1941; Robinson

1962; Shrimpton and Whitney 1968; Whitney and Ferris 1970; Solheim 1995; Six 2003a). Typically, *O. montium* is found in greater abundance than *G. clavigera*, but *G. clavigera* is usually found at the leading edge of fungal invasion while *O. montium* trails behind (Solheim 1995). *O. montium* is more commonly isolated from the surface of beetles while *G. clavigera* is more common in the mycangia (Six 2003a). The basidiomycete *Entomocorticium dendroctoni* Whitney Bandoni and Oberwinkler was also found to be associated lodgepole pine infected by the mountain pine beetle (Whitney et al. 1987).

The fungal associates of the MPB were extensively sampled in BC from 2001-2003 to investigate further possible associations (Kim et al. 2005; Lee et al. 2006). Results from four sites have been previously published (Kim et al. 2005). However, results from the six most recently sampled sites have not been published. In this chapter, we report additional findings from Little Fort, Robson Park, Monte Lake, Burns Lake, Prince George and Quesnel (see Table 1.2). As expected, the fungal species diversity was similar among sites, with most isolates belonging to the ophiostomatoid group. At five sites, for pines in the green and red phase of attack, *G. clavigera* was the dominant species, while *O. montium* was the second most dominant. For pines in the grey phase, both species were present, but their isolation frequencies were lower than in pines in the green and red phase. *Leptographium longiclavatum* and *Ceratocystiopsis* sp. 1 were also commonly isolated. Both *L. terebrantis* and *O. abietinum*, which was originally described as *O. nigrocarpum* by Kim et al. (2005), were present at a low frequency. It is important to note that our initial identification (Kim et al., 2005) was mistaken, because we used an isolate labeled as *O. nigrocarpum* type (CMW 1468) that we received from a culture collection. We corrected the identification by showing that the DNA sequences of our MPB isolates grouped closely to *O. abietinum* and not to *O. nigrocarpum*.

Fungi were isolated from beetle exoskeletons, beetle galleries and lodgepole pine sapwood at several sites in BC (Kim et al. 2005; Lee et al. 2007). While *G. clavigera* (Figure 1.4) and *O. montium* (Figure 1.5) were frequently isolated from the MPB in accordance with earlier studies (Robinson 1962;

Whitney and Farris 1970; Six 2003b), *Leptographium longiclavatum* (Figure 1.6), *Ceratocystiopsis* sp. 1 (Figure 1.7), an *Entomocorticium* sp. (Figure 1.8) and *Entomocorticium dendroctoni* were also commonly isolated from MPB (See Table 1.1, 1.2). The findings indicate that the MPB is associated with a much larger fungal flora than previously reported. In this chapter, the species referred to as *Ceratocystiopsis* sp. 1 was previously reported as *Ophiostoma minutum* (*Ceratocystiopsis minuta*) (Robinson 1962; Upadhyay 1981; Kim et al. 2005). This is discussed in further detail in the *Ceratocystiopsis* sp. 1 section.

Ophiostoma montium was isolated more frequently than *G. clavigera* from beetle exoskeletons (Robinson 1962; Six 2003a; Lee et al. 2006). Unexpectedly, the frequency of *Ceratocystiopsis* sp. 1 on the MPB exoskeletons was also high (Lee et al. 2006). It was isolated more frequently than *G. clavigera* in many cases (Table 1.1). Both *Ceratocystiopsis* sp. 1 and *E. dendroctoni* were isolated from MPB exoskeletons, while in previous work these species have only been reported in beetle galleries (Whitney et al. 1987). The dominant species on MPB exoskeletons differed from those dominant in galleries and sapwood (Lee et al. 2006). *G. clavigera* and *O. montium* grow optimally under different conditions (Solheim 1995). However, a species' dominance is likely influenced by the timing of isolation and may change throughout the year.

Identification among MPB-associated Ophiostomatoid fungi has been difficult. *Leptographium longiclavatum* and *Grosmannia clavigera*, and *Ophiostoma ips and Ophiostoma montium*, all resemble one another and have only recently been described as separate species (Kim et al. 2003; Lee et al. 2005). The *Ceratocystiopsis minuta* species complex is problematic. *Ceratocystiopsis* sp. 1 is difficult to differentiate from *C. minuta*, while other isolates of *C. minuta* in culture collections appear misidentified. It is only with the use of genetic tools that we have been able to recognize these problems, which are discussed in further detail in chapter 4. Specific details about the more common fungal species reported on the MPB, their ecology, their historic names and their phylogenetic relationships are discussed below.

Grosmannia clavigera

During a MPB outbreak in the 1960's, Robinson reported a new *Leptographium* sp. isolated from the MPB and MPB-attacked lodgepole pine (Robinson 1962). It was first described *Europhium clavigerum* (Robinson-Jeffery and Davidson 1968), then transferred to *Ophiostoma clavigerum*, and most recently to *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield [= Ophiostoma clavigerum (Robinson-Jeffrey and Davidson) Harrington] (Figure 1.4) (Zipfel et al. 2006).

The clavate conidia of *G. clavigera* form in solitary or short acropetal chains, are hyaline, smooth, broadly fusiform, and measure 12.5-62.5 x 4-6 μm. Ramo-conidia bear numerous single-celled hyaline conidia measuring 2-4 x 1-2.5 μm (Upadhyay 1981). In general, conidia may be ovoid to subglobose, globose, ovoid or peanut-shaped with truncated ends. The broad range of sizes and shapes of conidia helps to distinguish this species from *L. longiclavatum,* whose two conidia forms are more homogenous in shape (Lee et al. 2005). Until recently, it was the only known Ophiostomatoid species with a *Leptographium* anamorph to have clavate conidia (Lee et al. 2005). The species is pleoanamorphic, possessing several types of synnematous anamorphs including *Leptographium*, *Verticicladiella*, and *Hyalorhinocladiella* (Upadhyay 1981; Tsuneda and Hiratsuka 1984; Lee et al. 2003). On the other hand, *L. longiclavatum* only has one anamorphic state. Mononematous conidiophores of *G. clavigera* are 100-300 μm long in total. In comparison, stipes of *L. longiclavatum* alone may be up to 1200 μm long and average 750 μm in length. The synnematous form radiates mostly from mycelium and is composed of usually appressed hyphae, becoming brush or broomshaped towards the apex, and measures 500-1150 μm including conidiogenous apparatus (Upadhyay 1981).

Ascocarps appear superficially on wood or embedded in inner bark, sometimes aggregating at the bottom of trees growing in wet environments or appearing in insect galleries, but rarely or sparsely developing on autoclaved wood chips and never appearing in agar media (Upadhyay 1981). Necks, if

present, are short (Upadhyay 1981;Lee et al. 2003). When found, ascocarps are angiocarpous, globose, sometimes black, and 247-650 μm in diameter. Upadhyay (1981) did not observe any asci, but Robinson (1968) described them as eight-spored, evanescent, clavate, becoming globose, 5.5-9 μm in diameter. Ascospores are hyaline, 1-celled, reniform in side view, ellipsoid to oblong in face view, ovate or oval in end view, surrounded by a hyaline, gelatinous sheath that appears cucullate in side view, quadrangular in face view, triangular in end view, 3.5-5.5 x 2.2-4.5 μm including sheath (Upadhyay 1981).

Colonies grew to 65 mm in diameter in 12 days at 22 C°, and are appressed to effuse, colourless at first, becoming pale brown, gray brown to grayish black, reverse black or dark greenish black. Mycelia are superficial and immersed (Upadhyay 1981). Colonies of *L. longiclavatum* appear more 'wet' and show less variation in colony colour compared to colonies of *G. clavigera*, which appear more 'dry' and have a broader range of colony colours (personal observation).

G. clavigera is reported as the most pathogenic fungal associate of the MPB (Reid et al. 1967; Owen et al. 1987; Yamaoka et al. 1990; Yamaoka et al. 1995; Lee et al. 2007). It is capable of killing mature or young lodgepole pines in the absence of the MPB when inoculated at a density similar to that of a mass MPB attack (Yamaoka et al. 1995; Lee et al. 2006). Population studies on *G. clavigera* using allozymes indicated that gene diversity is low, averaging only 1.4% across 10 populations with only 2 of 19 genes being polymorphic (Six and Paine 1999). AFLP analysis found the species to possess an average heterozygosity of 0.053 (Lee et al. 2007).

The blue-sapstaining fungus grows inside sapwood (Figure 1.4-F). *G. clavigera* usually grows at the leading edge of fungal invasion zones in fresh sapwood, whereas *O. montium* is usually found trailing *G. clavigera* (Solheim 1995), suggesting that *G. clavigera* alters its environment as it grows in the sapwood to such a state that it is no longer the favoured species in that environment. It grows well in oxygen deficient conditions, but can be out-competed by other sapwood-inhabiting fungi as oxygen levels increase (Solheim

and Krokene 1998, personal observation). Structural properties of wood are hardly altered by the presence of *G. clavigera* (Lum et al. 2006), but it does reduce trade value by discolouring the wood. Like other sapstaining fungi, *G. clavigera* does not grow in the heartwood of trees (Whitney 1971; Solheim 1995; Figure 1.4F).

Phylogenetically, it is closely related to other bark beetle-vectored pathogenic species possessing a *Leptographium* anamorph such as, *L. pyrinum* and *L. terebrantis* (Lee et al. 2003; Lim et al. 2004; Lee et al. 2005). Morphologically, these species appear very similar, although PCR-based RFLP markers exist to distinguish *G. clavigera* from closely related phylogenetic and morphological species (Lee et al. 2003; Lee et al. 2005).

Ophiostoma montium

The first fungus isolated from the MPB, *Ophiostoma montium* (Rumbold) von Arx (= *Ceratocystis montia*) (Figure 1.3) was described by Rumbold in 1941. The species has synnematous anamorph types including *Hyalorhinocladiella* and *Acremonium* and is morphologically and genetically similar to *Ophiostoma ips* (Rumbold 1941; Upadhyay 1981; Kim et al. 2003). The *Hyalorhinocladiella* state conidiophores are mononematous, hyaline, pale yellowish or pale brown with sympodially proliferating conidiogenous cells between successive conidia. Conidia are holoblastic, hyaline, smooth, clavate to ellipsoidal, ovoid, 3-10 x 1-3 μm.

Perithecia form in bark or insect galleries, are mid to dark brown, globose, 140-380 μm in diameter and have black-brown bases that are usually submerged in the substratum. Necks are slender and long, between 200-1200 μm long, brown to black at the base becoming brown at the apex. Ostiolar hyphae are absent. Ascospores are unicellular, rectangular with square ends, appearing pillow-shaped in side view due to their surrounding hyaline sheaths, measuring 3-5.5 x 1.2-3 μm including sheaths (Upadhyay 1981).

These morphological characteristics of *O. montium* and *Ophiostoma ips* are so similar that the two species have been synonymized with each other

(Upadhyay 1981). However, the two species have been separated recently based on temperature tolerance differences and sequence comparison (Kim et al. 2003). Lodgepole pine's isolates of *O. montium* do not grow at 35 C, whereas isolates of *O. ips* from scots, red and southern pines are able to grow at 35 C. PCR amplicons of the β-tubulin and rDNA gene regions were different in size in the two species (Kim et al. 2003).

Phylogenetically, *O. montium* is closely related to *O. ips* and other species within the *O. ips* complex (Kim et al. 2003).

O. montium is able to kill a several different pine species in the absence of the beetle following heavy artificial inoculation (Mathre 1964; Basham 1970; Strobel and Sugawara 1986; Owen et al. 1987), but whether the host range would be equally susceptible under natural conditions remains to be seen. (Solheim 1995; Yamaoka et al. 1995). Overall, *O. montium* is considered a weaker pathogen than *G. clavigera* (Strobel and Sugawara 1986; Yamaoka et al. 1995; Lee et al. 2003).

Leptographium longiclavatum

Leptographium longiclavatum (Lee, Kim and Breuil) (Figure 1.6) is a blue-sapstaining fungus that was not described during previous epidemics, likely because it bears a close morphological resemblance to *G. clavigera*. It is found on MPB exoskeletons. It grows in sapwood and is more often isolated from lodgepole pine trees in the early green phase of attack than late green phase (Lee et al. 2005).

Conidiophores stipes are simple and 420-1080 µm long, which is much longer those of *G. clavigera*. Conidia accumulate in light cream-coloured mucilaginous masses at the apices of conidiophores; large conidia are hyaline, clavate to obclavate, 10-46 x 2.5-5.5 µm; smaller conidia are hyaline, aseptate, ovoid to clavate, 2.5-7.5 x 1.5-4.5 µm, and are produced on smaller conidiophores on aerial mycelium or occasionally on large clavate conidia (Lee et al. 2005). Conidia are more uniform in shape compared to *G. clavigera* conidia. *L. longiclavatum* possesses only a *Leptographium* anamorph while *G. clavigera* has

various anamorphic states. The optimal growth temperature, 25 C, is similar to *G. clavigera*'s optimal temperature of 22.5-25 C (Solheim and Krokene 1998; Lee et al. 2005). Phylogenetically, *L. longiclavatum* is closely related to other pine infesting species with a *Leptographium* anamorph such as *O. robustum*, *L. terebrantis* and *L. pyrinum* (Lee et al. 2005).

      L. longiclavatum is pathogenic to lodgepole pines and is capable of killing mature trees in the absence of the beetle; however, symptoms are less severe than those of *G. clavigera*, indicating it may be less pathogenic (Lee et al. 2006).

Ceratocystiopsis sp. 1

      Recent surveys in British Columbia uncovered the presence of a slow-growing, white fungus with a *Hyalorhinocladiella* anamorph with oblong conidia and teleomorph (perithecia) not found in culture but occasionally found in nature that we refer to as *Ceratocystiopsis* sp. 1 (Figure 1.7). This species has been identified as *Ceratocystiopsis minuta* (= *Ophiostoma minutum*) in other studies (Robinson 1962; Upadhyay 1981). This fungus was frequently isolated from beetle and beetle galleries but rarely from sapwood.

      Genetic analysis by the Breuil lab at UBC indicates that MPB-associated isolates of "*C. minuta*" are genetically distinct from other isolates of *C. minuta* isolated from hosts other than MPB-infested lodgepole pine. The MPB fungal-associate was not the same as *Ceratocystiopsis* Tax. sp. 1 described by Hausner et al. in 1993 and later described as *Ophiostoma carpenteri* (Hausner et al. 2003). *Ceratocystiopsis* sp. 1 closely resembles other *Ceratocystiopsis minuta* from MPB-infested lodgepole pine in 1960 (Robinson 1962; Upadhyay 1981). *C. minuta* is a slow-growing white fungus found on wood in three continents, nine beetles and five tree hosts (Siemaszko 1939; Davidson 1942; Mathiesen 1951; Hunt 1956; Mathiesen-Kaarik 1960; Wright and Cain 1961; Robinson 1962; Griffin 1966; Olchowecki and Reid 1974; Upadhyay and Kendrick 1975; Solheim 1986; Yamaoka et al. 1997; Jankowiak 2005). However, differences between isolates have been noted (Davidson 1942; Mathiesen-Kaarik 1960; Hsiau and Harrington 1997; Hausner et al. 2003) and more than one author has questioned

whether all these isolates truly represent the same species (Hausner et al. 2003). The "*Ceratocystiopsis minuta*" isolated by Robinson 1962 is probably *Ceratocystiopsis* sp. 1 because of observed morphological differences between isolates of *Ceratocystiopsis minuta* and because genetic relationships show that the group *Ceratocystiopsis* sp. 1 is separated from the other isolates of *C. minuta* with strong bootstrap support (data unpublished; see Chapter 3).

Siemaszko originally described *C. minuta* as having a fairly large perithecial base, 84-140 x 74-125 µm and a long ostiole, 98 – 140 µm (Siemaszko 1939). The uniquely shaped lunate ascospores measured 8-10 x 1-1.5 µm. However, samples from the USA, Sweden, Canada and Japan have had a smaller base size. Samples from Japan showed the most variation in base size, 48 – 87 µm (Davidson 1942; Mathiesen 1951; Hunt 1956; Upadhyay 1981; Yamaoka et al. 1997). Ascospore size varied widely, although the lunate shape is consistent. *Ceratocystiopsis* sp. 1 has short truncated oblong conidia, 0.6-1.2 x 1.3 – 2.0 µm with *Hyalorhinocladiella* anamorphs (personal observation). Anamorphs are mononematous or macronematous, but no semimacronematous conidiophores were observed. Yamaoka et al. (1997), however, did observe *C. minuta* with semimacronematous conidiophores or with macronematous conidiophores that were structurally different from those of *C.* sp. 1 (Fig 1.7D). Conidia illustrated by Yamaoka et al. tended to be more pyriform than those of *Ceratocystiopsis* sp. 1 (Figs. 1.7A and 1.7B). These observations are supported by phylogenetic evidence indicating that *Ceratocystiopsis* sp. 1 is not *C. minuta*. The teleomorph of *Ceratocystiopsis* sp. 1 has apparently only been photographed once (Figure 1.7E,F) and not enough measurements exist to properly assess the sizes of perithecia and ascospores. Repeated attempts to produce the teleomorph in culture have been unsuccessful.

Ceratocystiopsis sp. 1 is sometimes more frequent than *G. clavigera* (Table 1.1) in infested lodgepole pine, although this difference may be dependent upon the time of year isolations occurred. It does not produce melanin and does not seem to be pathogenic to the tree. Because of its high association with the beetle, its slow growth on artificial media, beetle galleries and wood and its lack

of melanin production, it is likely that *Ceratocystiopsis* sp. 1 may be a source of nutrients for the beetle, but no testing has been done with this species.

Ceratocystiopsis* sp. 1 is phylogenetically within the *Ceratocystiopsis minuta* species complex (Hausner and Reid 2003). Genetic analysis indicates it is not *C. minuta* (discussed further in chapter 4). It is more closely related to *C. ranaculosa* and *C. manitobensis* than to any isolate of *C. minuta* taken from the USA, Europe or Japan.

Entomocorticium dendroctoni

Entomocorticium dendroctoni* (Whitney, Bandoni and Oberwinkler) was the first basidiomycete isolated from the MPB. Unlike other fungi that were often found in sapwood, *D. dendroctoni* was only isolated from pupal chambers and on the walls of larval galleries of MPB broods on lodgepole pine. It has not been reported as an aggressive sapwood decayer, growing only 2 mm into sapwood after four weeks of growth. MPB reared on *E. dendroctoni* produced 19% more eggs than adults reared on mixtures of blue-staining fungi, but MPB reared on fresh phloem alone produced significantly more eggs than any of the MPB reared with fungi. However, due to its location and its non-detrimental effects on beetle brood, it is possible this species may be a nutritional symbiont of the MPB (Whitney et al. 1987).

E. dendroctoni* has clamp connections and fruits readily in culture on autoclaved inner bark and sapwood segments. Basidiocarps are white to pale cream or buff. Cystidia are 22-60 x 5-10 μm, thick-walled above, mostly becoming incrusted, thin-walled and sometimes collapsing below the incrustation. Basidia are 24-27 x 5.5-6.5 μm, clavate or suburniform, frequently secondarily septate, collapsing after spore production, mostly with 4 spores but sometimes with 1,2 or 6 spores. Basidiospores accumulate to form a fragile crust on the hymenium, with individual spores being mostly ellipsoid, 8-10 x 4-6 μm and frequently narrowed near the middle (Whitney et al. 1987). The basidia of this species have short, flattened sterigmata and an apparent lack of a forcible discharge mechanism for basidiospores, possibly allowing for more effective

grazing by MPB and adherence of the basidiospore to the exoskeleton of beetles for dispersal (Hsiau and Harrington 2003).

Other species of *Entomocorticium* (Figure 1.6) found on the MPB (Tsuneda et al. 1992, Lee et al. 2007) have yet to be formally described.

Other species

In contrast to all the above fungal species, which appear to be specifically associated with the MPB, *L. terebrantis*, the *Ambrosiella*-like *Ophiostoma* sp. 1 and 2 and the unknown *Graphium* species seem to be incidental associates (Table 1.2). The presence of these fungi on MPB is likely due to cross-contamination with fungal associates of the other beetles (e.g. *Ips* and ambrosia beetles) that frequently co-habit trees (Kim et al. 2005; Lee et al. 2006). The group of isolates identified as *Ambrosiella* sp. by Kim et al. (2005) was re-examined; one cluster from these isolates did not produce monilioid conidiophores and confluent sporodochia, which are characteristic of the genus *Ambrosiella*. While the rDNA of some isolates showed high sequence identity with that from *Ambrosiella macrospora* (Kim et al. 2005), these sequences formed a monophyletic clade that was separate from the *Ambrosiella* associates of both bark and ambrosia beetles (data not shown). A second cluster of isolates showed some similarity with an *Ambrosiella* associate of the bark beetle *Ambrosiella ips*. This isolate group lacked defined morphological characteristics and appeared to be genetically related to *A. ips*, the sister taxon of *O. montium*.

Several yeast species (Whitney and Farris 1970; Kim et al. 2005) such as *Pichia scolytii* (Figure 1.9) and *Pichia capsulata* (Figure 1.10) are also found in close association with the MPB, but this area has been poorly studied.

Interactive associations: benefits to MPB and to fungi

The relationship between mountain pine beetles and their associated fungi is mostly mutualistic (Paine et al. 1997; Kim et al. 2005; Lee et al. 2005 but see Harrington 1993 for opposing view). The mountain pine beetle benefits from its association with fungi in several ways. First of all, the blue-staining fungi *G.*

clavigera, O. montium, and *L. longiclavatum* are pathogenic (Mathre 1964; Reid et al. 1967; Basham 1970; Strobel and Sugawara 1986; Owen et al. 1987; Yamaoka et al. 1990; Yamaoka et al. 1995; Lee et al. 2006) and aid the beetle in successfully colonizing and reproducing in host trees (Whitney 1971; Six and Paine 1998). By helping to exhaust the trees' preformed defenses, by blocking water flow and by consuming carbon reserves stored in parenchyma rays, the fungi reduce the host's defensive capabilities against beetle invasion (Craighead 1928; Nelson 1934; Caird 1935; Whitey and Cobb 1972; Wagner et al. 1979; Paine 1984; Paine et al. 1997) although water blockage took several weeks to develop (Ballard et al. 1982). Moisture content decreased one or two month after the infestation (Kim et al. 2005). Second, once inside the tree, beetles probably feed on fungal mycelium and spores (Whitney 1971; Bridges 1981; Whitney et al. 1987; Paine et al. 1997) since this also occurs in similar systems (Hodges et al. 1968; Barras and Hodges 1969; Whitney and Cobb 1972; Paine et al. 1997; Hsiau and Harrington 2003). Six and Paine (1998) showed that successful brood development required either *G. clavigera* or *O. montium*, but the production of progeny was significantly higher and emergence occurred earlier for brood developing on *G. clavigera* compared with *O. montium* (Six 2003a). Bentz and Six (2006) demonstrated significant increases in ergosterol concentration in trees infested with MPB and their fungal symbionts, specifically *O. montium* and *G. clavigera*. Sterols are necessary for normal growth, reproduction and metamorphosis of MPB; however, they are found in low amounts in tree phloem. Thus, it has been suggested that fungal sterols could be advantageous for the development of MPB (Bentz and Six 2006). In similar systems, adult beetles were more fecund and have higher fitness in the presence of associated fungi (Barras 1973; Bridges 1983; Coppedge et al. 1985; Goldhammer et al. 1990). Ayres (2000) suggested that beetle associated fungi are higher in nutrients such as nitrogen and potassium and that by feeding on fungi in addition to phloem, the amount of material ingested and the period of time needed for maturation is reduced. Some MPB-associated fungi, like *Entomorcorticium* species and *Ceratocystiopsis* sp. 1, are found almost exclusively in larval galleries or on the

beetle, suggesting that these species may be preferentially grazed by the mountain pine beetle (e.g. Whitney et al. 1987), although the nutritional content of these species has not been tested. The fact that these species do not melanize, and are thus not likely pathogenic, strengthen this possibility, since melanization generally offers some form of protection against environmental stress but does not contribute to the nutritional value of a fungus (Butler and Day 1998). Furthermore, the presence of several yeast species found on or within the gut of the mountain pine beetle suggests that yeasts may also serve as an additional nutritional source for mountain pine beetles (Whitney et al. 1987). However, these hypotheses have not been rigorously tested; future experiments may shed more light on the exact role and nature of various fungi as nutritional sources for the MPB.

The main benefit to fungi from the mountain pine beetle is transportation to new hosts (Paine et al. 1997; Six and Paine 1999). Without the MPB to serve as vector, it would be virtually impossible for these fungal species to reach new hosts. As such, without MPB, it would not be possible for the fungal species to continue their life cycle, just as without the fungi, the mountain pine beetle would have an extremely difficult time colonizing new trees. While this association benefits both the MPB and its associated fungi, trees are negatively impacted by the presence of the beetle and the fungi. The pathogenic effects of the beetle-fungi complex usually result in a decrease in tree health, typically resulting ultimately in tree death.

Pathogenicity of fungi associated with the mountain pine beetle

The ability of a fungus to induce a disease or disease-like symptoms in trees mass-attacked by the MPB has been the topic of several previous studies. Reid et al. (1967) demonstrated resistant lodgepole pine trees produced more secondary resins compared to successfully attacked trees. Shrimpton et al. (1967) found that these secondary resins had inhibitory effects on the growth rate of *G. clavigera* and *O. montium*. Raffa and Berryman (1982) monitored the effects of natural beetle attack and artificial inoculation of *G. clavigera* on

lodgepole pine and in both cases, resistant trees produced greater quantities of secondary resins which lead to a greater chance of survival. Thus it was established that the trees natural defenses against the mountain pine beetle had a negative effect on the beetles' associated fungi, that trees that succumbed to beetle attacked produced lower levels of defensive compounds in comparison to their successfully defended counterparts and that the results from artificial inoculations of *G. clavigera* were similar to results from natural beetle attack.

The pathogenicity of fungal associates of the mountain pine beetle has been tested using either the alternating flap technique (AFT) first proposed by Strobel and Sugawara (1986) or by the cork borer method, as developed by Christiansen (e.g. Christiansen 1985a,b; Christiansen et al. 1987). The AFT involved inoculating fungi into larger areas after removing strips of bark from trees, while the cork borer method involved inoculating fungi into individual points on the tree at a controlled density. These two methods have never been compared directly. Seven of eight trees inoculated with *O. montium* using the AFT method developed pathogenic symptoms and three of these eight trees died after 2 years (Strobel and Sugawara 1986). Owen et al. (1987) found that artificial inoculations of *G. clavigera* into 2 year old ponderosa pines were sufficient to kill a large proportion of the trees, while Yamaoka et al. (1995), using a modified form of the AFT, found that *G. clavigera* or *G. clavigera* in combination with *O. montium* killed lodgepole pine trees after one year, whereas trees inoculated with *O. montium* did not die. Lee et al. (2006) used the cork borer method to inoculate trees at a low (200 holes/m^2) and high (800 holes/m^2) density with both *G. clavigera* and *Leptographium longiclavatum*. At the high inoculation density, both fungi induced tree foliage discolouration in 5 of 6 trees after one year, whereas trees inoculated at the low density maintained a green crown. Foliar discolouration is a symptom of successful mountain pine beetle attack (Amman et al. 1990). Rice et al. (2007a, b) examined lesion development in lodgepole pines, jack pines and lodgepole pine-jack pine hybrids inoculated with either *O. montium* or *G. clavigera*. *G. clavigera* induced longer lesions than

O. montium in all cases, but both fungi were capable of inducing longer lesions than agar controls in all three tree types (Rice et al. 2007a, b).

The ability of mountain pine beetle associated fungi to induce pathogenic symptoms and to kill both young and mature pine trees has been previously demonstrated. However, in most studies, only one isolate of each fungal species has been tested. In similar bark beetle-fungal systems, Krokene and Solheim (2001) demonstrated that serial subculturing of the same fungal isolate of *Ceratocystis polinica* resulted in varying levels of pathogenicity ranging from non-virulent to virulent. Lieutier et al. (2004) demonstrated significant variation in the level of virulence of 15 different isolates of *Leptographium wingfieldii* from the same geographic region. Salle et al. (2005) found significant variation for in vitro growth in both *Ophiostoma piceaperdum* and *O. bicolor* when they were exposed to varying levels of phenolic compounds. Rice et al. (2007a,b) did use three isolates of *G. clavigera* when measuring lesion length development in lodgepole pine but these differences were not significantly different. The variation was, however, significant in jackpine and lodgepine-jackpine hybrids. The aims of her test were not to specifically examine intraspecific fungal variation, but rather to monitor lesion development in lodgepole pine, jack pine and lodgepole-jack pine hybrids. No studies have demonstrated intraspecific variation in lodgepole pine symptom development for mountain pine beetle associated fungi, even though studies in closely related complexes have shown intraspecific pathogenicity variation to be present.

Thesis objectives

The MPB is associated with several fungal species whose exact roles have yet to be fully clarified. *Grosmannia clavigera*, *Ophiostoma montium* and *Leptographium longiclavatum* are pathogenic and capable of killing trees, but the pathogenicity tests performed using different techniques had never been directly compared to one another. Variation has yet to be characterized within a fungal species in regard to that species' ability to kill healthy trees. In similar bark beetle-fungi-tree complexes, intraspecific variation in pathogenic symptom

development had been observed for only a couple of species. No studies had demonstrated pathogenic variability in MPB-associated fungi. Specific roles of non-pathogenic fungi associated with the MPB were even less well understood. Species history and ecology suffered from a lack of investigation. *Ceratocystiopsis minuta*, the type species of the genus *Ceratocystiopsis*, does not have a type specimen, resulting in confusion and misidentification in the literature. *Ceratocystiopsis* sp. 1, a MPB-associated fungi, previously referred to as *Ceratocystiopsis*, is one such example.

Thus, the goals of this thesis are to a) compare the AFT and cork borer methods directly when assessing the pathogenicity of *G. clavigera* in mature lodgepole pine trees, to b) compare pathogenic symptom development in young lodgepole pines inoculated with five separate isolates of *G. clavigera* to examine intraspecific variation within the species and to c) examine different isolates of *C. minuta* for taxonomic incongruencies in order to suggest a candidate epitype strain for future phylogenetic studies and to better define the species in general.

References

Aghayeva DN, Wingfield MJ, De Beer ZW, Kirisits T (2004). Two new O*phiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia **96**: 866-878.

Amman GD, McGregor MD, Dolph Jr. RE (1990). Mountain pine beetle forest insect and disease leaflet 2. US dept. of agriculture. Last updated July 2002. Available online at http://www.barkbeetles.org/mountain/fidl2.htm.

Aukema BH, Carroll AL, Zhu J, Raffa KF, Sickly TA, Taylor SW (2006). Landscape level analysis of mountain pine beetle in British Columbia, Canada: spatiotemporal development and spatial synchrony within the present outbreak. Ecograph **29**: 427-441.

Ayres MP, Wilkens RT, Ruel JJ, Lombardero MJ, Vallery E (2000). Nitrogen budges of phloem-feeding bark beetles with and without symbiotic fungi. Ecology **81**(8): 2198-2210.

Ballard RG, Walsh MA, Cole WE (1982). Blue-stain fungi in xylem of lodgepole pine: a light-microscopy study on extent of hyphal distribution. Canadian Journal of Botany **60**: 2334–41.

Barras SJ, Hodges JD (1969). Carbohydrates in inner bark of *Pinus taeda* as affected by *Dendroctonus frontalis* and associated microorganisms. Canadian Entomologist **101**: 489–93.

Barras SJ (1973). Reduction of progeny and development in southern pine beetle following removal of symbiotic fungi. Canadian Entomologist **105**(10): 1295-1299.

Basham HG (1970). Wilt of loblolly pine inoculated with blue-stain fungi of the genus *Ceratocystis*. Phytopathology **60**: 750–54.

Bentz BJ and Six DL (2006). Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). Annals of the Entomological Society of America **99**(2):189-194.

Bridges JR (1981). Nitrogen-fixing bacteria associated with bark beetles. Microb. Ecology **7**:131–37.

Bridges JR (1983). Mycangial fungi of *dendroctonus frontalis* (*Coleoptera, Scolytidae*) and their relationship to beetle population trends. Environmental Entomology **12**(3): 858-861.

Butler MJ, Day AW (1998). Fungal melanins: a review. Canadian Journal of Microbiology **44**: 1115-1136.

Caird RW (1935). Physiology of pines infested with bark beetles. Bot. Gaz. **96**: 709–733.

Christiansen E (1985a). *Ips/Ceratocystis* infection of Norway spruce: what is a deadly dosage? Journal of Applied Entomology **99**: 6-11.

Christiansen, E. (1985b). *Ceratocystis polonica* inoculated in Norway spruce: blue-staining in relation to inoculum density, resinosis and tree growth. European Journal of Forest Pathology **15**: 160-167.

Craighead FC (1928). Interrelation of tree killing bark beetles (*Dendroctonus*) and blue stain. Journal of Forestry **26**: 886–887.

Cole WE, Amman GD, Jensen CE (1976). Mathematical models for the mountain pine beetle-lodgepole pine interaction. Environmental Entomology **5**: 11-19.

Coppedge BR, Stephen FM, Felton GW (1995). Variation in female southern pine beetle size and lipid content in relation to fungal associates. Canadian Entomologist **127**:145–54

Davidson RW (1942). Some additional species of *Ceratostomella* in the United States. Mycologia **34**: 650-662.

De Beer ZW, Harrington TC, Vismer HG, Wingfield BD, Wingfield MJ (2003). Phylogeny of the *Ophiostoma stenoceras – Sporothrix schenckii* complex. Mycologia **95**: 434-441.

Elkin CM, Reid ML (2004). Attack and reproductive success of mountain pine beetles in fire-damaged lodgepole pines. Environmental Entomology **33**(4): 1070-1080.

Eng M, Fall A, Hughes J, Shore T, Riel B, Walton A, Hall P (2006). Provincial-level projection of the current mountain pine beetle outbreak: Update of the infestation projection based on the 2005 provincial aerial overview of forest health and revisions to 'the model' (BCMPB.v3). Available at http://www.for.gov.bc.ca/hre/bcmpb/BCMPB.v3.ReferenceScenario.Update.pdf.

Farrell BD, Sequeira AS, O'Meara BC, Normark BB, Chung JH, Jordal BH (2001). The evolution of agriculture in beetles. Evolution **55**(10): 2011-2027.

Goldhammer DS, Stephen FM, Paine TD (1990). The effect of the fungi *Ceratocystis minor* (Hedgecock) Hunt, *Ceratocystis minor* (Hedgecock) Hunt var barrasii Taylor, and SJB 122 on reproduction of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae). Canadian Entomologist **122**: 407–18.

Griffin HD (1966). The genus *Ceratocystis* in Ontario. Canadian Journal of Botany **46**: 689-718.

Harrington TC (1993). Diseases of conifers caused by species of *Ophiostoma* and *Leptographium*. In *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity, ed. MJ Wingfield, KA Seifert, JF Webber, pp. 161–72. St Paul, MN: American Phytopathological Society.

Hausner G, Reid J, Klassen GR (1993). Grouping of isolates and species of *Ceratocystis* sensu lato on the basis of molecular and morphological characters. In: *Ceratocystis* and *Ophiostoma* Taxonomy, Ecology and Pathogenicity (Wingfield, M.J., Seifert, K.A. and Webber, J.F., Eds.), pp. 93-104.The American Phytopathological Society Press, St. Paul, MN.

Hausner G, Eyjolfsdottir GG, Reid J (2003). Three new species of *Ophiostoma* and notes on *Cornuvesica falcata*. Canadian Journal of Botany **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 **81**: 865-876.

Hodges JD, Barras SJ, Mauldin JK (1968). Amino acids in inner bark of loblolly pine as affected by the southern pine beetle and associated microorganisms. Canadian Journal of Botany **46**: 1467–72.

Hsiau PT-W, Harrington TC (1997). *Ceratocystiopsis brevicomi* sp. nov., a mycangial fungus from *Dendroctonus brevicomis*. Mycologia **89**(4): 661-669.

Hsiau PT-W, Harrington TC (2003). Phylogenetics and adaptations of basidiomycetous fungi fed upon by bark beetles (Coleoptera: Scolytidae). Symbiosis **34**: 111–131.

Hunt J (1956). Taxonomy of the genus *Ceratocystis*. Lloydia **19**: 1-59.

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. Forest Pathology **35**: 37-55.

Kim JJ, Kim SH, Lee S, Breuil C. (2003). Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated sapstain fungi. FEMS Micribiology Letters **222**: 187-192.

Kim JJ, Allen EA, Humble LM, Breuil C (2005). Ophiostomatoid and basidiomycetous fungi associated with green, red, and grey lodgepole pines after mountain pine beetle infestation. Canadian Journal of Forest Research **35**: 274-284.

Krokene P, Solheim H (2001). Loss of pathogenicity in the blue-stain fungus *Ceratocystis polonica*. Plant Pathology **50**: 497-502.

Lee S, Kim JJ, Fung S, Breuil C (2003). A PCR-RFLP marker distinguishing *Ophistoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Canadian Journal of Botany **81**: 1104-1112.

Lee S, Kim JJ, Breuil C (2005). *Leptographium longiclavatum* sp. nov., a new species associated with the mountain pine beetle, *Dendroctonus ponderosae*. Mycological Research **109**(10): 1162-1170.

Lee S, Kim JJ, Breuil C (2006). Pathogenicity of *Leptographium* longiclavatum associated with *Dendroctonus ponderosae* to *Pinus contorta*. Canadian Journal of Forest Research **36**: 2864-2872.

Lee S, Kim JJ, Breuil C (2007). Fungal diversity associated with the mountain pine beetle and infested lodgepole pines in British Columbia. In press.

Li C, Barclay HJ, Hawkes BC, Taylor SW (2005). Lodgepole pine forest age class dynamics and susceptibility to mountain pine beetle attack. Ecological Complexity **2**: 232-239.

Lieutier F, Yart A, Ye H, Sauvard D, Gallois V (2004). Variations in growth and virulence of *Leptographium wingfieldii* Morelet, a fungus associated with the bark beetle *Tomicus piniperda* L. Annals of Forest Science **61**:45-53.

Lim YW, Alamouti SM, Kim JJ Lee S, Breuil C (2004). Multigene phylogenies of *Ophiostoma clavigerm* and closely related species from bark beetle-attacked *Pinus* in North America. FEMS Microbiology Letters **237**: 89-96.

Lum C, Byrne T, Casilla R (2006). Mechanical properties of lodgepole pine containing beetle-transmitted blue stain. Forest Products Journal **56**(6): 45-50.

Mathre DE (1964). Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa* pine. Contributions of the Boyce Thomson Institute **22**: 363-388.

Mathiesen A (1951). Einge neue Ophiostoma-arten in Schweden. Svensk Botanisk Tidskrift. BD **45**: 204-235.

Mathiesen-Kaarik A (1960). Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis.* Oikos **11**: 13-25.

McFarlane BL, Stumpf-Allen RC, Watson DO (2006). Public perceptions of natural disturbance in Canada's national parks: the case of the mountain pine beetle. Biological Conservation **130**: 340-348.

Ministry of Forests and Ranges (2007). Beetle facts. http://www.for.gov.bc.ca/hfp/mountain%5Fpine%5Fbeetle/facts.htm. Last updated March 2007.

Nelson RM (1934). Effect of bluestain fungi on southern pines attacked by bark beetles. Phytopathology. Z. **7**: 327–353.

Olchowecki A, Reid J (1974). Taxonomy of the genus *Ceratocystis* in Manitoba. Canadian Journal of Botany **52**: 1675-1711.

Ono H (2003). The mountain pine beetle: scope of the problem and key issues in Alberta. In: Shore TL, Brooks JE, Stone JE (eds), Mountain Pine Beetle Symposium: Challenges and Solutions 30-31 October 2003, Kelowna, BC. Canadian Forest Service Pacific Forestry Centre Information Report BC-X-399, Victoria, BC, pp. 62–66.

Owen DR, Lindahl KQ Jr, Wood DL, Parmeter JR Jr (1987). Pathogenicity of fungi isolated from *Dendroctonus valens*, *D. brevicomis* and *D. ponderosae* to ponderosa pine seedlings. Phytopathology **77**: 631.636.

Paine TD (1984). Influence of the mycangial fungi of the western pine beetle on water conduction through ponderosa pine seedlings. Canadian Journal of Botany **62**: 556–558.

Paine TD, Raffa KF, Harrington TC (1997). Interactions among scolytid bark beetles, their associated fungi and live host conifers. Annual Review of Entomology **42**: 179-206.

Parker TJ, Clancy, KM, Mathiasen RL (2006). Interactions among fire, insects and pathogens in coniferous forests of the interior western United States and Canada. Agricultural and Forest Entomology **8**: 167-189.

Pederson L (2004). Expedited timber supply review for the Lakes, Prince George and Quesnel timber supply. Public discussion paper.

Pfister RD, Daubenmire R (1975). Ecology of lodgepole pine *Pinus contorta Dougl.* In: Baumgartner, D.M. (Ed.), Proceedings of the Symposium on Management of Lodgepole Pine Ecosystems, Washington State University, Pullman, 9–11 October 1973, pp. 27–44.

Raffa KF, Berryman AA (1982). Physiological differences between lodgepole pines resistant and susceptible to the mountain pine beetle and associated microorganisms. Environmental Entomology **11**: 486-492.

Reid RW, Whitney HS, Watson JA (1967). Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Canadian Journal of Botany **45**: 1115-1126.

Rice AV, Thormann MN, Langor DW (2007a). Virulence of, and interactions among, mountain pine beetle-associated blue-stain fungi on two pine species and their hybrids in Alberta. Canadian Journal of Botany **85**: 316-323.

Rice AV, Thormann MN, Langor DW (2007b). Mountain pine beetle-associated blue-stain fungi cause lesions on jack pine, lodgepole pine, and lodgepole-jack pine hybrids in Alberta. Canadian Journal of Botany **85**: 307-315.

Robinson RC (1962). Blue stain fungi in lodgepole pine (*Pinus contorta* Dougl. var *latifolia* Engelm.) infested by the mountain pine beetle (*Dendroctonus monticolae* Hopk.). Canadian Journal of Botany **40**: 609.614.

Robinson-Jeffrey RC, Davidson RW (1968). Three new *Europhium* species with *Verticicladiella* imperfect states on bluestained pine. Canadian Journal of Botany **46**: 1523–1527.

Rumbold CT (1931). Two blue-staining fungi associated with bark beetle infestation of pines. Journal of Agricultural Research **43:** 847-873.

Rumbold, C.T. (1941). A blue stain fungus, *Ceratostomella montium* n. sp., and some yeasts associated with two species of *Dendroctonus.* Ibid. **62**: 589-601.

Salle A, Monclus R, Yart A, Lieutier F (2005). Effect of phenolic compounds on the in vitro growth of two fungi associated with Ips typographus. Forest Pathology **35**:298-304.

Siemaszko W (1939). "Zespoly grzybow towarzyszacych kornikom polskim."Fungi associated with bark beetles in Poland. Planta Polonica **7**(3): 1-52.

Six DL, Paine TD (1997). *Ophiostoma clavigerum* is the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi.* Mycologia **89**: 858.866.

Six DL, Paine TD (1998). Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Environmental Entomology **27**: 1393–1401.

Six DL, Paine TD (1999). Allozyme diversity and gene flow in *Ophiostoma clavigerum*, the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi.* Canadian Journal of Forest Research **29**: 324-331.

Six DL (2003a). A comparison of mycangial and phoretic fungi of individual mountain pine beetles. Canadian Journal of Forest Research **33**: 1331-1334.

Six DL (2003b). Bark beetle-fungus symbiosis, chapter 7. In Contemporary topics in entomology series: Insect Symbiosis. CRC Press, New York, New York.

Shrimpton DM, Whitney HS (1968). Inhibition of growth of blue stain fungi by wood extractives. Canadian Journal of Botany **46**: 757-761.

Solheim H (1986). Species of *Ophiostomataceae* isolated from *Picea abies* infested by the bark beetle *Ips typographus.* Nordic Journal of Botany **6**(2): 199-207.

Solheim H (1995). Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. Canadian Journal of Botany **73**: 70-74.

Solheim H, Krokene P (1998). Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium.* Canadian Journal of Botany **76**: 561-566.

Stahl K, Moore RD, McKendry IG (2006). Climatology of winter cold spells in relation to mountain pine beetle mortality in British Columbia, Canada. Climate Research **32**: 13-23.

Strobel GA Sugawara F (1986). The pathogenicity of *Ceratocystis montia* to lodgepole pine. Canadian Journal of Botany **64**(1): 113-116.

Tsuneda A, Hiratsuka Y (1984). Sympodial and annellidic conidiation in *Ceratocystis clavigera.* Canadian Journal of Botany **62**: 2618-2624.

Tsuneda A, Murakami S, Sigler L, Hiratsuka Y (1992). Schizolysis of dolipore-parenthosome septa in an arthroconidial fungus associated with *Dendroctonus ponderosae* and in similar anamorphic fungi. Canadian Journal of Botany **71**: 1032-1038.

Uphadyay HP, Kendrick WB (1975). Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. Mycologia **67**: 798-805.

Upadhyay HP (1981) A Monograph of Ceratocystis and Ceratocystiopsis. The University of Georgia Press, Athens, GA.

Wagner TL, Gagne JA, Doraiswamy PC, Coulson RN, Brown KW (1979). Development time and mortality of *Dendroctonus frontalis* in relation to changes in tree moisture and xylem water potential. Environmental Entomology **8**: 1129–1138.

Whitney HS, Farris SH (1970). Maxillary mycangium in the mountain pine beetle. Science **167**: 54.55.

Whitney HS (1971). Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. Canadian Entomologist **103**: 1495–1503.

Whitney HS, Cobb FW Jr. (1972). Non-staining fungi associated with the bark beetle *Dendroctonus brevicomis* (Coleoptera: Scolytidae) on *Pinus ponderosae.* Canadian Journal of Botany **50**: 1943–1945.

Whitney HS, Bandoni RJ, Oberwinkler F (1987). *Entomocorticium dendroctoni* gen. et. sp. nov. (Basidiomycotina), a possible nutritional symbiote of the mountain pine beetle in lodgepole pine in British Columbia. Canadian Journal of Botany **65**:95–102

Wright EF, Cain RF (1961). New species of the genus *Ceratocystis.* Canadian Journal of Botany **39**: 1215-1230.

Yamaoka Y, Swanson RH, Hiratsuka Y (1990). Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine beetle, monitored by a heat pulse velocity (HPV) instrument. Canadian Journal of Forest Research **20**: 31-36.

Yamaoka Y, Hiratsuka Y, Maruyama PJ (1995). The ability of *Ophiostoma clavigerum* to kill mature lodgepole pine trees. European Journal of Forest Pathology **25**: 401-404.

Yamaoka Y, Wingfield MJ, Takahashi I, Solheim H (1997). *Ophiostomatoid* fungi associated with the spruce bark beetle *Ips typographus f. japonicus* in Japan. Mycological Research **101**(10): 1215-1227.

Yamaoka Y, Wingfield MJ, Ohsawa M, Ibaraki T (1998). *Ophiostomatoid* fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. Mycoscience **39**: 367-378.

Zabel RA, Morrell JJ. (1992). Changes caused by decay fungi in the strength and physical properties of wood. In Wood Microbiology, Decay and its Prevention. Acad. Press Int. 476 pp.

Zipfel RD, de Beer ZW, Jacobs K (2006). Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Studies in Mycologia **55**: 75-97.

Figure 1.1. The mountain pine beetle
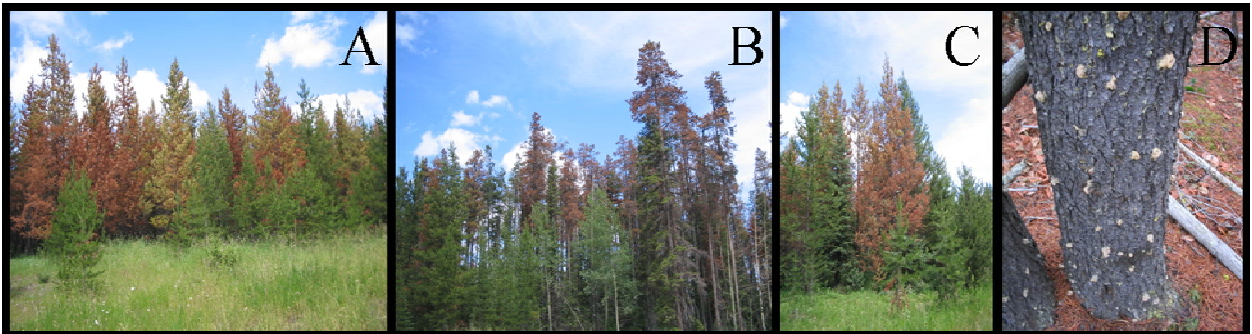
Figure 1.2. A-D**.** Lodgepole pine trees. A-C. Examples of red-attacked trees by the mountain pine beetle. D. Pitch tubes; evidence of recent mountain pine beetle attack.
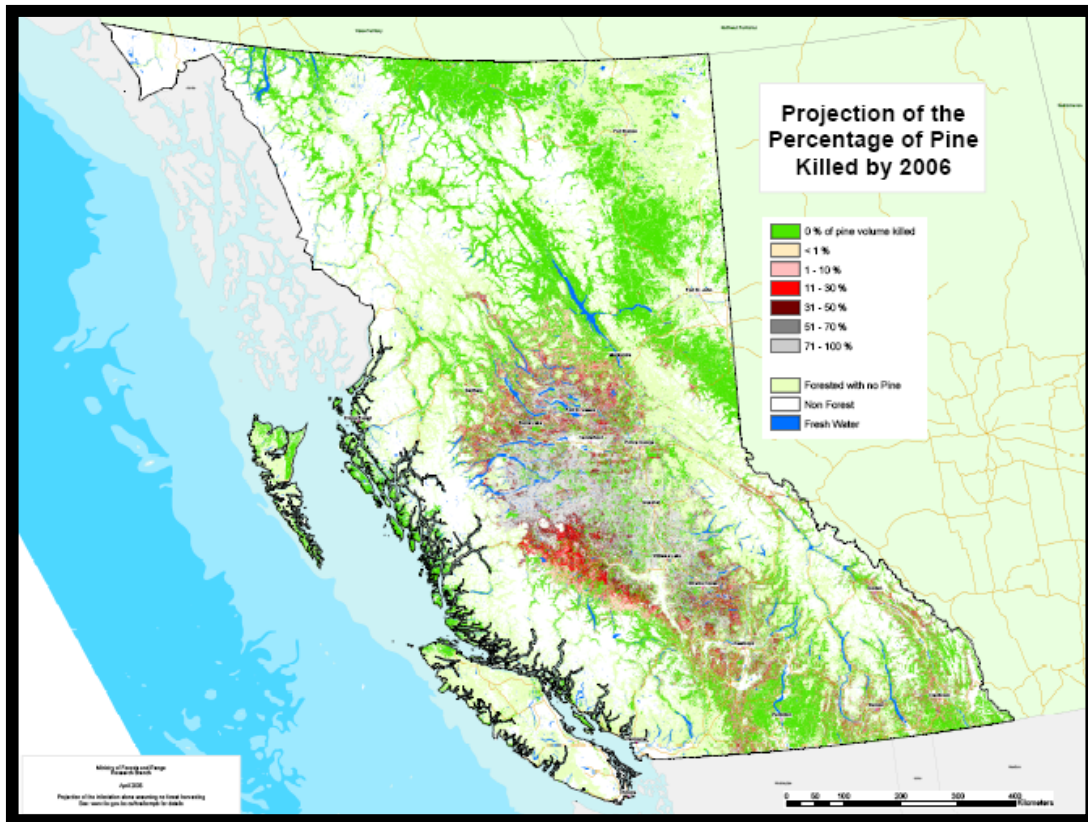
Figure 1.3. 2006 BC provincial projection of forest infested with MPB
(http://www.for.gov.bc.ca).
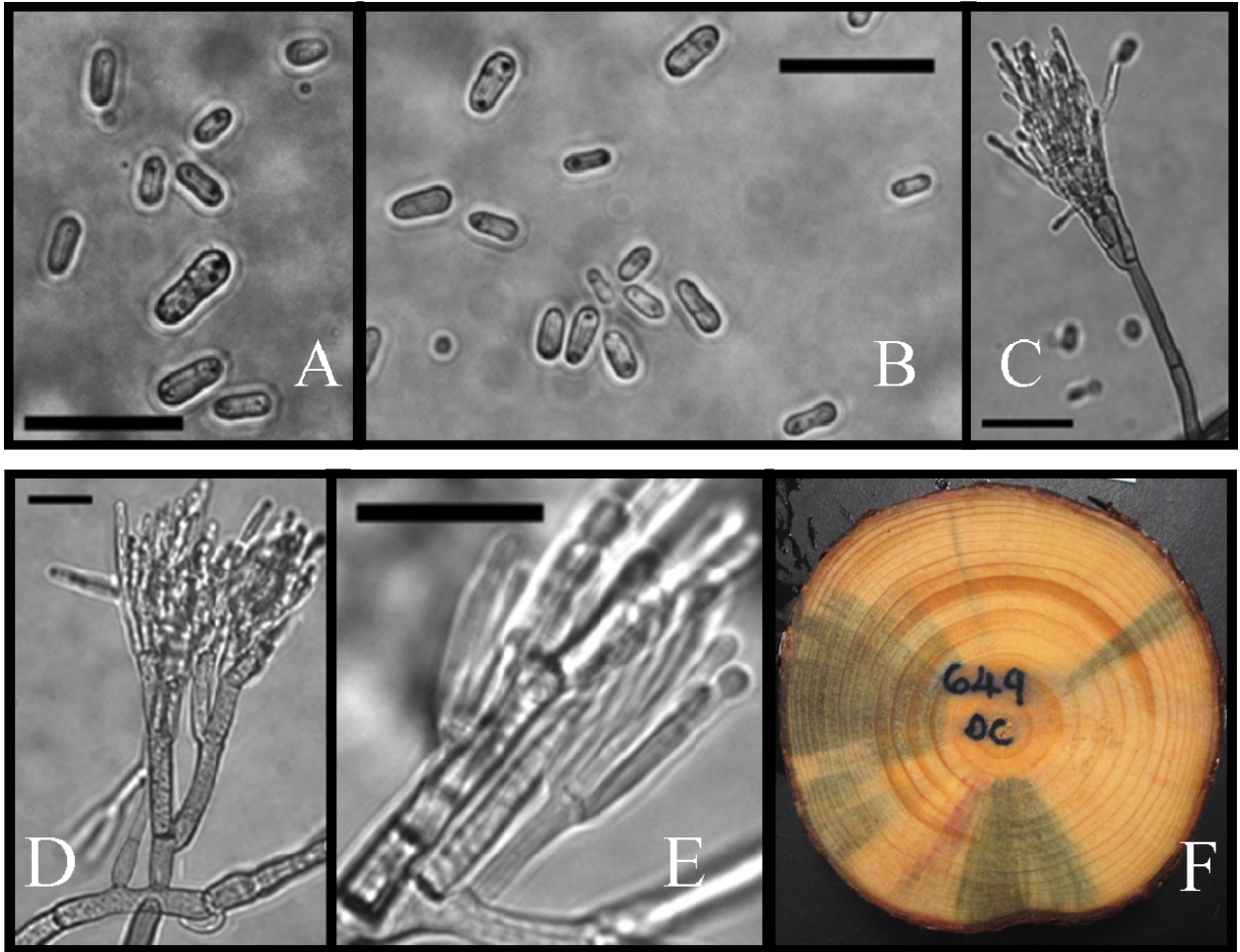
Figure 1.4. A-E**.** *G. clavigera*. A. Conidia; bar = 10 μm. B. Conidia; bar = 10 μm.

C. Single *Leptographium* anamorph; bar = 20 μm. D. Conidiophore; bar = 10 μm.

Conidiogenous cells producing conidia; bar = 10 μm.
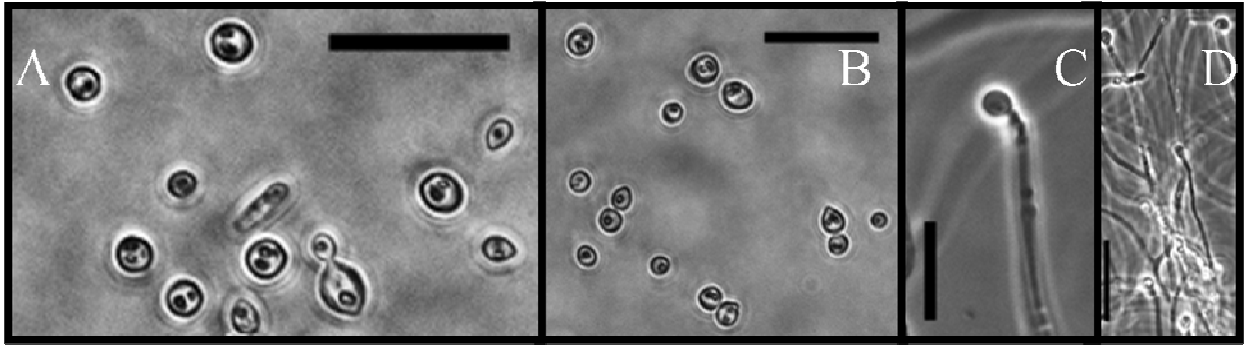
Figure 1.5. A-D. *O. montium*. A. Conidia; bar = 10 μm. B. Conidia; bar = μm. C.

Tip of conidiogenous cell; bar = 5 μm. D. *Hyalorhinocladiella* anamorph; bar =

10 μm.
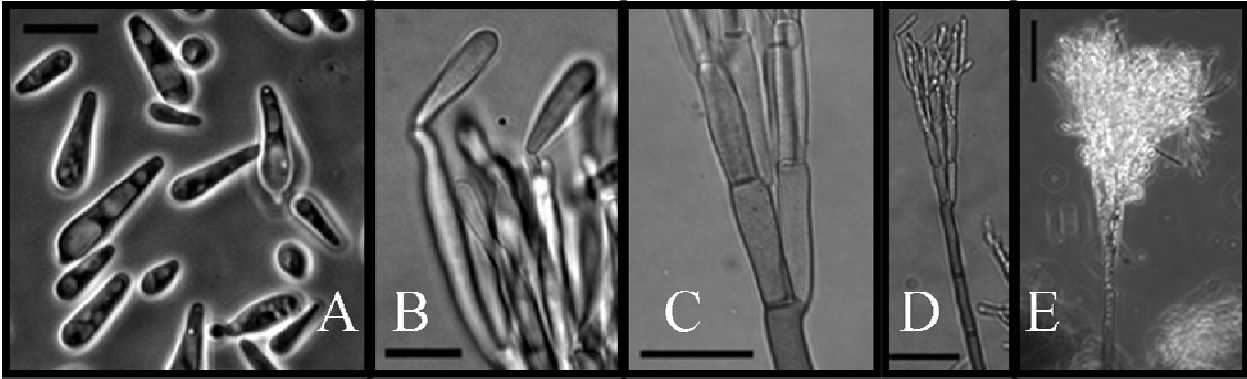
Figure 1.6. A-E. *L. longiclavatum*. A. Conidia; bar = 5 μm. B. Conidiogenous

cells; bar = 5 μm. C. Primary branches; bar = 10 μm. D. Conidiophore; bar = 20

μm. E. Conidiophore; bar = 20 μm.
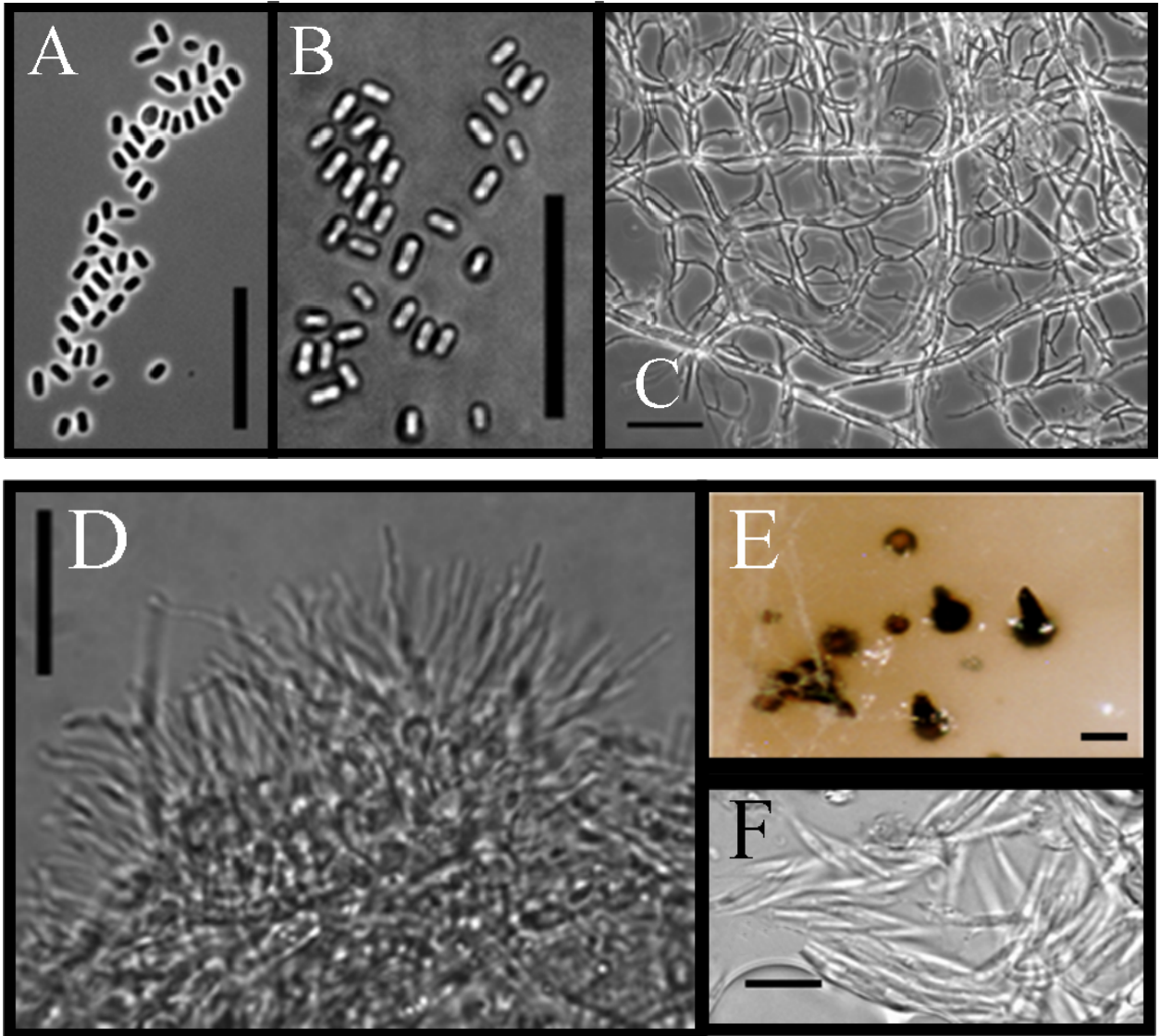
Figure 1.7. A-F. *Ceratocystiopsis* sp. 1. A. Conidia; bar = 10 μm. Conidia; bar = 10 μm. C. Hyphal network; bar = 20 μm. D. Macronematous conidiophore; bar = 20 μm. E. Perithecia embedded in media; bar = 70 μm. F. Ascospores; bar = 5 μm.
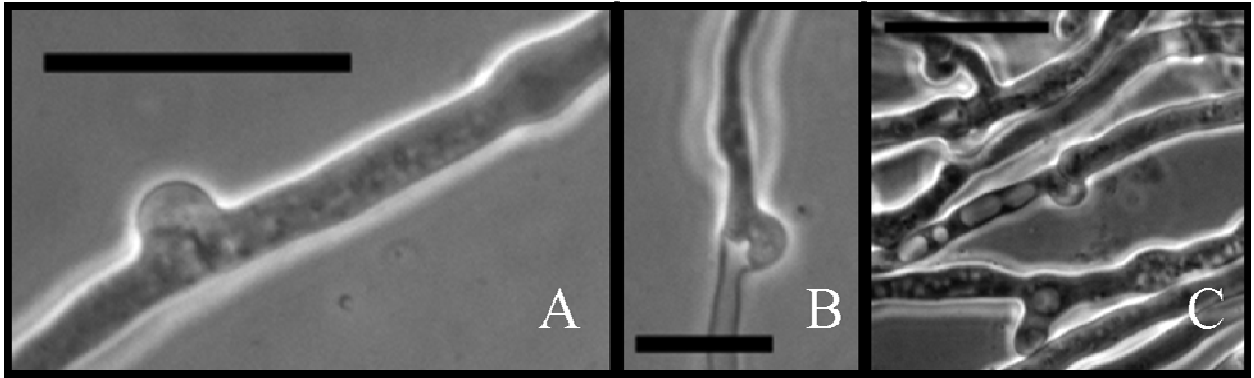
Figure 1.8. A-C. Unidentified *Entomocorticium* sp. A) Clamp connection; bar = 10 µm. B. Clamp connection; bar = 5 µm. C. Hyphal network showing multiple clamp connections; bar = 10 µm.
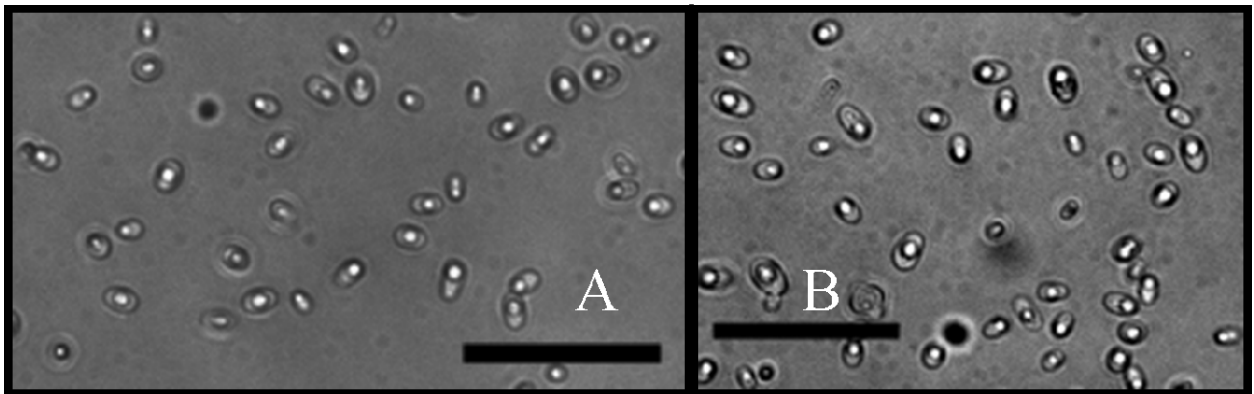
Figure 1.9. A-B. *P. scolytii*. A. Proliferation of yeast cells; bar = 10 µm. Yeast cells; bar = 10 µm.
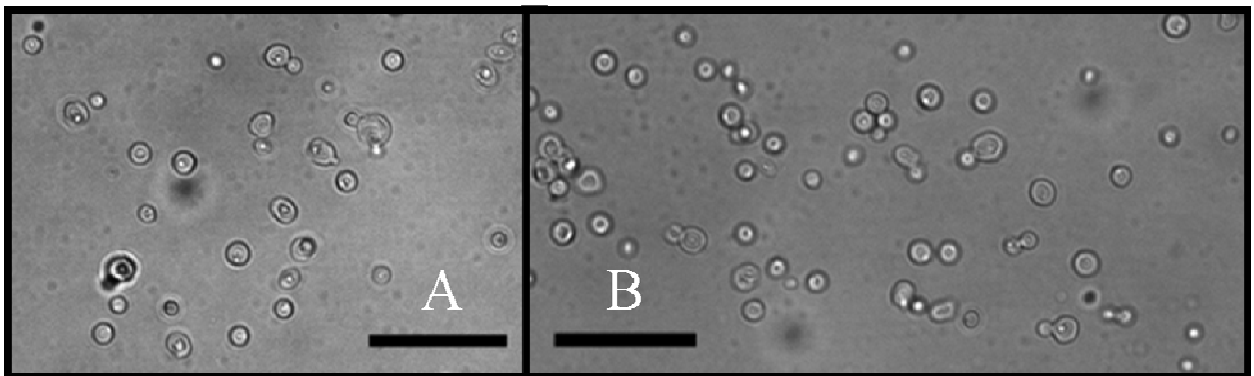
Figure 1.10. A-B. *P. capsulata*. A.. Yeast cells; bar = 10 µm. B. Proliferation of yeast cells; bar = 10 µm.

Taxon	Number of times isolated
Ambrosiella sp.	3
Entomocorticium dendroctoni	20
Entomocorticium sp.	11
Leptographium longiclavatum sp. nov.	2
Leptographium terebrantis	1
Grosmannia clavigera	44
Ceratocystiopsis sp. 1	84
Ophiostoma montium	351
Number of times fungi isolated	**516**

Table 1.1. Total number of fungal isolations from 1042 mountain pine beetles, from Lee *et al*. 2006. Fungi were scored as either present or absent on a petri dish, regardless on the number of colonies of each fungi on the petri dish.

Fungus (Previously named)	Manning Park			Riske creek			Radium			Cranbrook			Little Fort			Robson Park			Monte Lake			Burns Lake			Prince George			Quesnel			Sub Total			Total
	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	G	R	Y	Total
Grosmannia clavigera	49	28	4	36	43		37	28	6	33	31	8	56	20	3	42	34	24	18	32	12	49	46	17	40	29	21	35	8	9	395	299	104	798
Ophiostoma montium	32	19	6	31	36		30	37	6	19	21	7	51	11	1	46	22	15	7	20	5	42	13	9	22	31	25	28	3	5	308	213	79	600
Unknown Ophiostoma sp. 1 (unknown Ambrosiella sp.)	6	21	9	14	37	2	2	25	4		5	5	10	13	4	2	13	5		13	12	1	6	3	6	26	32	6	15	3	47	174	79	300
Leptographium longiclavatum	5	3		5							1		11	4		5	2		2	5	1	5	2		21	18	21	12	2		66	37	22	125
O. abietinum (prev. reported as *Ophiostoma nigrocarpum*)	2	6	5	2	2	7	2	3	2		1			2	4		6	4		2	3	1	1	2		8	9	2	6	5	9	37	41	87
Ceratocystiopsis sp. 1		3	2		2			8	3	1			3	13	4		1	1	1	5	1	1	4	5		3	6	3	2	6	9	41	28	78
Leptographium terebrantis			5		2	1		2					3	5				1		1	2			1	2	1	3	4	1		6	10	18	34
Unknown Graphium sp.	1	2	1	1				2							1		4	3		1	1							1			3	9	6	18
Ophiostoma minus			2			2		3	1		4	2																			0	7	7	14
Unknown Ophiostoma sp. 2 (A.mbrosiella ferruginea-like)																					2			1							0	0	3	3
Pesotum fragrans																					1						1				0	0	2	2
Ophiostoma aureum																				1							1				0	1	1	2
Ophiostoma piceae																														1	0	0	1	1
Ophiostoma olivaceum-like						1																									0	0	1	1
Unidentified fungi														6	3		1	1		4	7	4	1	2	2			9		1	15	12	14	41
Total Isolates	**95**	**82**	**35**	**89**	**122**	**12**	**71**	**108**	**22**	**53**	**63**	**22**	**131**	**72**	**25**	**95**	**84**	**55**	**28**	**83**	**46**	**103**	**73**	**42**	**93**	**116**	**118**	**100**	**37**	**29**	858	840	406	2104
Number of points sampled	80	120	120	60	120	120	64	120	120	48	120	120	72	80	80	80	80	80	80	80	80	80	80	80	76	80	80	68	80	80	700	960	960	2620

Table 1.2. Frequency of fungal isolation from green (G), red (R) and grey (Y) attacked trees in BC. Adapted from Kim *et al*. 2005, and appended with 4 additional and previously unreported sites.

Chapter 2

Two methods to assess fungal virulence

Introduction

[2]British Columbia is experiencing the largest mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (MPB) outbreak in recorded history, with 9.2 million hectares of pure and mixed stands of lodgepole pine (*Pinus contorta* var*. latifolia* Engelm.) already attacked by the MPB as of 2007 (Ministry of Forests and Range 2007). The MPB is found in close association with *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel et al., *Ophiostoma montium* (Rumbold) von Arx and *Leptographium longiclavatum* Lee Kim and Breuil, all of which are pathogenic, sap-staining ophiostomatoid fungi (Mathre 1964; Reid et al. 1967; Basham 1970; Strobel and Sugawara 1986; Owen et al. 1987; Yamaoka et al. 1990; Yamaoka et al. 1995; Lee et al. 2006a, 2006b). *G. clavigera* has been studied the most extensively among all the fungal associates of the MPB because it is considered to be more aggressive than either *O. montium* (Reid et al. 1967; Owen et al. 1987; Yamaoka et al. 1990; Yamaoka et al. 1995; Solheim 1995; Solheim and Krokene 1998) or *L. longiclavatum* (Lee et al. 2006).

Sapwood moisture content, sapwood occlusion area and needle discoloration are pathogenicity indicators that have been used to assess the virulence of sap-staining ophiostomatoid fungi (Yamaoka et al. 1990, 1995;

---

[2] A version of this chapter, entitled "A comparison of two methods to assess the virulence of the mountain pine beetle associate, *Grosmannia clavigera*, to lodgepole pine," by Jae Jin Kim, Alex Plattner, Young Woon Lim and Colette Breuil, is in press in the Scandinavian Journal of Plant Pathology.

Krokene and Solheim 1998; Bois and Lieutier 2000; Solheim et al. 2001; Fernandez et al. 2004; Lieutier et al. 2004; Lee et al. 2006a). Sap-staining fungi grow inside the sapwood and phloem of trees and impede water and nutrient transport (Amman et al. 1990; Langstrom et al. 1993; Paine et al. 1997), leading to a reduction in sapwood moisture content and the occlusion of host cells. As adult beetles construct vertical galleries to lay eggs, they disseminate fungal spores and mycelia, resulting in narrow, vertical inoculations of the fungi at the surface of the sapwood. Further growth of fungi in the tree also results in foliage discoloration, as needles turn from green to yellow and then red due to continued moisture loss (Amman et al. 1990). Since bark beetles carry many fungal species, it is difficult at first to assess which species are pathogenic and thus responsible for participating in tree decline.

Rapidly characterizing the virulence of the many fungal species associated with bark beetles is important at the onset of new epidemics. Most pathogenicity tests involving ophiostomatoid fungi have been conducted using a cork borer method (Christiansen 1985a,b; Horntvedt 1988; Yamaoka et al. 1990; Solheim et al. 1993; Lanstrom et al. 1993; Krokene and Solheim 1998; Yamaoka et al. 1998; Bois and Lieutier 2000; Solheim et al. 2001; Krokene and Solheim 2001; Fernandez et al. 2004; Lieutier et al. 2004; Lee et al. 2006a). This method is time and labor intensive since a high density of holes are generated manually and then inoculated with fungi. Testing threshold densities, the density of inoculations necessary to result in tree death (Christiansen et al. 1987), typically requires a fungal inoculation level up to 800 holes/m^2 (Solheim et al. 1993; Bois and Lieutier

2000; Solheim et al. 2001; Lee et al. 2006a). Testing enough trees at high inoculation levels requires a large amount of time and manpower, and may not be feasible when financial or time resources are limited. However, it is important to deliver massive inoculations of fungi when testing pathogenic symptoms since increasing levels of inoculation tend to result in stronger pathogenic symptom development and likely simulate decreases in tree defense resources facing massive beetle attacks (Christiansen 1985a,b; Solheim et al. 1993; Krokene and Solheim 1998). An alternative to the cork borer method is the bark flap inoculation method, where flaps of bark are peeled back from trees and inoculated with fungi. Two studies have used this technique to demonstrate the virulence of *G. clavigera* (Yamaoka et al. 1995) and *O. montium* (Strobel and Sugawara 1986) to lodgepole pine. Although the cork borer and the bark flap inoculation have been used for assessing fungal virulence, the efficiency of the two methods has not been compared.

The aim of this study was to compare the pathogenic effects of fungal inoculation when performed at a density of 200 or 800 cork borer holes/m^2 and using bark flap inoculations. Measures of virulence included reductions in sapwood moisture content, percentage occluded sapwood and needle discoloration. Results are discussed with regard to the time needed to perform each technique.

Materials and methods

Growth of inoculum

The fungus *G. clavigera,* strain SL-KW 1407 (=DAOM 234193) was grown on 2% Oxoid malt extract agar (OMEA, 33 g Oxoid malt extract agar, 10 g technical Agar #3, 1 L distilled H_2O, Oxoid Ltd., Hants, England) for one week before being inoculated into trees (Lee et al. 2006a). This strain was isolated in Kamloops, British Columbia in 2001 from lodgepole pine sapwood that had been attacked by the mountain pine beetle.

Fungal inoculation of lodgepole pine

In both years, all trees used for testing fungal virulence were healthy prior to inoculations. The field site was located near Kamloops, British Columbia. Between September 29th and October 1st, 2003, 12 lodgepole pines were inoculated with *G. clavigera* and 6 with sterile agar. Half of the trees were inoculated at a density of 200 cork borer holes/m^2, and the other half were inoculated at a density of 800 cork borer holes/m^2. The field site was inaccessible earlier in the year due to forest fires in the region. Trees from 2003 had a mean diameter at breast height (DBH) of 18.8 (14.5-26) cm and an average age of 116 (98-130) years. Starting at DBH, a 5 mm metal cork borer was used to remove sections of wood from the outer bark down to the cambium in a 60 cm wide band encircling the tree. Bark pieces were replaced over holes after inoculum was inserted and the entire area was covered with duct tape. The average number of holes per tree was 71 and 283 respectively for trees inoculated at 200 and 800

cork borer holes/m^2. The distance between evenly spaced inoculation points was 3.1 cm and 7.1 cm at high and low density respectively. On July 7th, 2004, 280 days after inoculation, trees were felled and bolts of approximately 1.4 m were transported back to the lab for further analysis.

On July 5th, 2004, 5 lodgepole pines were inoculated with *G. clavigera* and 4 with sterile agar using the bark flap inoculation method. To insert fungal inoculum, a three-sided flap (2cm x 10cm) was cut into the bark (Figure 2.1) and peeled back using a chisel. Flaps were evenly spaced in a ring around the tree, with 4-5 cm between flaps. A second ring was cut 20 cm below the first ring. Rings were spaced to avoid vertical lesion overlap between upper and lower rings. Under each flap, a square of MEA (2cm x 2cm) with fungal inoculum was smeared from the top to the bottom of the inner bark. The flap was flattened back over the inoculated area. The area inoculated with fungi, comprising both rings of bark flap inoculations and the intervening space, was covered with duct tape to prevent infection or invasion by secondary beetles. Each tree received, on average, 6-7 bark flap inoculations per ring for a total of 12-14 total bark flap inoculations per tree, resulting in approximately 260 cm^2 of area inoculated with fungi in a 40 cm band completely circling the tree at DBH. This is equivalent to 48 flaps/m^2 or 960 cm^2 of fungal inoculum/m^2 of tree surface. Control trees received the same peeling as treated trees but were inoculated with fungus-free media. Trees inoculated in 2004 had an average DBH of 18 (13 – 22) cm and an average age of 145 (121 – 161). All trees were felled on July 3rd, 2005, 363 days

after inoculation. The weather conditions during these incubation periods are shown in Table 2.1.

Measuring pathogenic symptoms

Occlusion area and moisture content were determined using the same procedures as Lee et al. (2006a). In brief, tree bolts of 1.4m were cut and transported back to the lab. The occluded sapwood area on stem disks cut near inoculation points or flaps was determined after tracing the area on Mars Vellum paper (Staedtler Inc., Mississauga, ON, Canada), cutting the drawings and weighing them. To determine the living sapwood area, stem disks were immersed overnight in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, Oakville, ON, Canada) solution in the dark. Regions showing red staining were considered viable, as TTC stains living plant cells red while non-viable cells remain whitish in appearance (Towill and Mazur 1975).

Moisture content was determined by removing pieces of sapwood near fungal inoculation areas and weighing them immediately. Pieces of wood were then dried overnight at 95º C and reweighed the following day. Percent moisture content was determined as a percentage by subtracting the dry weight from the wet weight and then dividing by the dry weight. Normal sapwood moisture content in lodgepole pines ranges from 85-150% (Reid 1961).

In November 2003 and April 2004, the foliage color was assessed for trees inoculated using the cork borer. Trees were left standing to allow symptoms to further develop. The foliage color was assessed in October 2004, April 2005

and May 20th, 2005 for trees inoculated using the bark flap method. Trees with green crowns were considered still viable, while trees with yellow or red needles were considered to be dead. Yellow needles are a precursor to red needles, which is usually an indicator of successful beetle attack and tree mortality (Amman et al. 1990).

Fungal re-isolation

Re-isolation points near fungal inoculum were selected from bolts and transported back to the lab. Three points were selected from each tree. The wood was placed on 2% malt extract agar and incubated at 22º C for one week before being re-examined for the presence of fungi.

Statistical analysis

The normality of data sets was verified using the Shapiro-Wilk p-value and visually inspecting normality plots of residuals for outliers. Comparisons between all treatment and control means were performed using an ANOVA (GLM procedures) using S.A.S. software followed by a Bonferonni correction for multiple pair-wise comparisons.

Treatments were categorized by inoculum type (control or *G. clavigera*), and method and density (200 holes/m^2, 800 holes/m^2 or bark flap inoculation).

A significance threshold of 0.05 was used. Pairwise comparisons were limited to 1) treatment versus control for the same inoculation level (3 comparisons), 2) within treatment (3 comparisons) and 3) within controls (3

comparisons), for a total of 9 comparisons. As such, a Bonferonni significance level of 0.0055 (number of total comparisons divided by 0.05 acceptance level of error) was used when comparing pair-wise significance levels.

Results

The average occlusion area for trees inoculated at 200 cork borer holes/m^2 was much lower compared to trees inoculated at 800 cork borer holes/m^2 or using flap inoculations (Figure 2.2). Occlusion area between trees inoculated at 800 holes/m^2 and using bark flap inoculations were not significantly different (p-value = 0.595), whereas the occlusion area of trees inoculated at 200 holes/m^2 were significantly different (p-value < 0.0001 in both cases) from the other two treatments. Control trees inoculated at 200 cork borer holes/m^2 had an average occlusion of 1.2%, an average occlusion area of 1.5% for trees inoculated at 800 holes/m^2 and an average occlusion area of 1.6% for trees inoculated using flap inoculations. The occlusion area for controls of all levels was not significantly different from each other (p-values range from 0.847 to 0.947). These values are grouped together in Figure 2.2. The occlusion area in treated trees was always significantly higher than it was in control trees (p-value < 0.0001 in all cases).

The average moisture content of trees receiving 200 cork borer holes/m^2 of fungal inoculum was higher compared to trees receiving 800 cork borer holes/m^2 of fungal inoculum or for trees receiving bark flap inoculations (Figure 2.3). The sapwood moisture content of disks in trees that received bark flap inoculations was not different from trees that received cork borer inoculations at

800 holes/m^2 (p = 0.922). Using a significance level of 0.0055 based on

Bonferonni corrections, comparisons between sapwood moisture content for

inoculation at 200 cork borer holes/m^2 and 800 cork borer holes/m^2 (p-value =

0.006) or bark flap inoculations (p-value = 0.007) were borderline significant. No

differences were found between levels of moisture content in control trees

receiving either cork borer density or flap inoculations (p-values range from 0.799

to 0.984). The moisture content in control trees was always significantly higher

than in treatment trees (200 cork borer holes/m^2 p-value = 0.0003; 800 cork borer

holes/m^2 p-value < 0.0001; bark flap inoculation p-value = 0.0002). The sapwood

moisture content of control trees was within the 85-150% moisture content range

that is considered normal for lodgepole pine trees (Reid 1961), while trees

inoculated with *G. clavigera* at either density or with either technique had

sapwood moisture contents well below 85%. *G. clavigera* was successfully

recovered from all trees in at least one of the three inoculation points sampled

from all three treatments but was not re-isolated from agar control trees.

In November 2003 and April 2004, the foliage color remained unchanged

for trees inoculated at 200 and 800 cork borer holes/m2. Trees were left standing

to allow symptoms to further develop. By July 7th, 2004, 280 days after

inoculation, for trees inoculated with G. clavigera at 800 holes/m2, four of six

trees had yellow needles, one tree had yellow and green needles, and one tree

had only green needles. All trees inoculated at 200 cork borer holes/m2 and all

control trees had green needles.

In October 2004 and April 2005, the foliage color remained unchanged for trees inoculated using the bark flap method. By May 20th, 2005, foliage discoloration had started to occur. By July 3rd, 2005, 363 days after inoculation, four of five trees inoculated using the bark flap method had yellow needles and one tree had green needles. All control trees had green needles.

It took six people 15 hours (i.e. 90 person hours) to inoculate nine trees (6 with *G. clavigera* and 3 controls) at a density of 800 holes/m^2. The same six people took 6 hours (i.e. 36 person hours) to inoculate nine trees at a density of 200 holes/m^2. It took five people a total of 5 hours (i.e. 25 person hours) to inoculate nine trees using flap inoculations.

Discussion

Inoculating the fungus *G. clavigera* at a density of 800 cork borer holes/m^2 produced pathogenic symptoms (sapwood occlusion, moisture content decrease and foliage discoloration) that were comparable to inoculations using the bark flap method. Pathogenic symptoms were less intense in trees inoculated at 200 cork borer holes/m^2. However, results between inoculation methods must be interpreted with caution because of differences in the length of time for fungal colonization and in yearly weather conditions. Fungi inoculated using the bark flap technique had almost three additional months of summer weather to colonize trees, while fungi inoculated using the cork borer method were inoculated in October, when the average mean monthly temperature was only 11ºC. *G. clavigera* has an optimal growth rate between 22.0 – 25.0 ºC and showed

reduced growth at lower temperatures (Solheim and Krokene 1998). This could result in virulence symptoms being stronger in the second season when the bark flap method was used. The amount of precipitation, especially during the spring and summer months, was much higher during the second field season (July 2004 to July 2005) than it was for first field season (October 2003 to June 2004). More precipitation is likely to reduce the stress on trees, which could lead to trees responding more vigorously to the presence of fungi in the second field season when the bark flap method was used. Horntvedt (1988) has shown that the resistance of *Picea abies* to *Ips typographus* varied significantly during different months of the summer and Krokene and Solheim (1998) noticed considerable variation between two consecutive years of treatments. Our results might have been affected by similar variations.

Declines in tree health and foliage discoloration were observed in the bark flap technique used by Yamaoka et al. (1995). They found that one of two trees receiving *G. clavigera* inoculations had turned brown after 84 days, while the crown of the second tree turned brown within one year. Control trees and trees inoculated with *O. montium* had green crowns after one year. The dimensions of their bark flaps differed from the ones used in this study. They were shorter and wider. Despite the small sample size used, the authors indicated that the inoculation method used seemed reliable to assess the ability of a fungus to kill mature lodgepole pine trees within one year. Our results also suggested that the majority of trees receiving bark flap inoculations would have died because of the small percent of viable sapwood and foliage discoloration, although our foliage

discoloration (yellow) was not as severe as the brown discoloration observed by Yamaoka et al. (1995). Strobel and Sugawara (1986) also used a bark flap technique to inoculate 20 year old lodgepole pines with *O. montium*. Their flaps were shorter and wider than the ones used in this study. Trees appeared healthy after one growing season but after two growing seasons, three of eight trees were dead and another four trees showed signs of decline. While this work is not directly comparable to our work because of the age of the trees and the fungal species used, the results showed that the bark flap inoculation can be used to determine fungal virulence and tree mortality.

Although both inoculation techniques are capable of inducing tree mortality, each technique has its limitations. A low inoculation density of *G. clavigera* (200 cork borer holes/m^2) was insufficient to kill mature lodgepole pines after one year while a higher density (800 holes/m^2), *G. clavigera* was lethal to trees (Lee et al. 2006a). However, Waring and Pitman (1983) found that successful tree attacks were characterized by beetle densities of 40 to 160 per m^2, depending on the health of the tree, which they estimated by wood production/unit leaf area. Thus, inoculating fungi into small holes may not give a good indication of the beetle density needed to overcome host defense mechanisms. Krokene and Solheim (1998) noted that one beetle gallery of *Ips typographus* was probably a more severe challenge to the tree's defenses compared to one cork borer inoculation. It is likely that the effects of a mountain pine beetle boring a vertical gallery are more detrimental to the tree than an individual point inoculation. Neither inoculation technique mimics the full area that beetles naturally attack. Much

larger sections of a host tree compared to the 0.6 m or 0.4 m bands of inoculations used in this study are normally attacked but regardless of pattern, it is likely the total number of inoculations is more important than the inoculation pattern (Christiansen 1985b; Krokene and Solheim 1998). The cork borer method also does not simulate how beetles disseminate fungal spores while constructing galleries. The bark flap method more accurately reflects this, but does not simulate how larvae spread fungi in constructing galleries perpendicular to the main gallery.

Bark flap inoculations required 30.6% less time than using a density of 200 cork borer holes/m^2 and 72.2% less time than using a density of 800 cork borer holes/m^2. Given the efficiency with which this method delivers fungal inoculum to a tree, it makes it practical for a small team to characterize fungal virulence on a relatively large number of hosts, which may be important for understanding genetic variability in fungal virulence or host resistance. In contrast, cork borer inoculations produce small wounds, seem less damaging to a tree and may be more appropriate at low densities for two applications: monitoring individual symptom development in hosts (Krokene and Solheim 2001; Rice et al. 2007a,b) and inducing tree resistance to subsequent beetle or pathogen invasion (Christiansen et al. 1999; Krokene et al. 1999; Krokene et al. 2000).

To further assess the new inoculation approach in the context of characterizing large scale genetic variability, follow-up studies should examine the repeatability of these results between field seasons, and should address whether the approach could be used with other beetle-fungal-tree complexes.

Acknowledgements

References

Amman GD, McGregor MD, and Dolph Jr RE (1990). Mountain pine beetle forest insect and disease leaflet 2. US dept. of agriculture. Last updated July 2002. Available online at http://www.barkbeetles.org/mountain/fidl2.htm.

Basham HG (1970). Wilt of loblolly pine inoculated with blue-stain fungi of the genus *Ceratocystis*. Phytopathology. **60**: 750–54.

Bois E, Lieutier F (2000). Resistance level in Scots pine clones and artificial introductions of *Tomicus piniperda* (Coleoptera., Scolytidae) and *Leptographium wingfieldii* (Deuteromycetes). *Journal of Applied* Entomolgy. **124**: 163-167.

Christiansen E (1985a). Ips/Ceratocystis infection of Norway spruce: what is a deadly dosage? Journal of applied entomology. **99**: 6-11.

Christiansen E (1985b). *Ceratocystis polonica* inoculated in Norway spruce: blue-staining in relation to inoculum density, resinosis and tree growth. European journal of forest pathology. **15**: 160-167.

Christiansen E, Waring RH, Berryman AA (1987). Resistance of conifers to bark beetle attack: searching for general relationships. Forest Ecology and Management. **22**: 89-106.

Christiansen E, Krokene P, Berryman AA, Franceschi VR, Krekling T, Lieutier F, Lonneborg A, Solheim H (1999). Mechanical injury and fungal infection induce acquired resistance in Norway spruce. Tree physiology. 19(6): 399-403.

Fernandez MMF, Garcia AE, Lieutier F (2004). Effects of various densities of *Ophiostoma ips* inoculations on *Pinus sylvestris* in north-western Spain. Forest Pathology. **34**: 213-223.

Horntvedt R (1988). Resistance of Picea abies to Ips typographus: Tree response to monthly inoculations with Ophiostoma polonicum, a beetle transmitted blue stain fungus. Scandinavian Journal of Forest Research. **3**: 107-114.

Krokene P, Solheim H (1998). Pathogenicity of four blue stain fungi associated with Aggressive and Nonaggressive bark beetles. Phytopathology. **88**(1): 39-44.

Krokene P, Christiansen E, Solheim H, Franceschi VR, Berryman AA (1999). Induced resistance to pathogenic fungi in Norway spruce. Plant Physiology. **121**: 565-569.

Krokene P, Solheim H, Langstrom B (2000). Fungal infection and mechanical wounding induce disease resistance in Scots pine. European Journal of Plant Pathology. **106**: 537-541.

Krokene P, and Solheim, H (2001). Loss of pathogenicity of the blue-stain fungus *Ceratocystis polonica*. Plant Pathology. **50**(4): 497-502.

Langstrom B, Solheim H, Hellqvist C, Gref R (1993). Effects of pruning young Scots pines on host vigour and susceptibility to *Leptographium wingfieldii* and *Ophiostoma minus*, two blue-stain fungi associated with *Tomicus piniperda*. European Journal of Forest Pathology. **23**: 400-415.

Lieutier F, Yart A, Ye H, Sauvard D, Gallois V (2004). Variations in growth and virulence of *Leptographium wingfieldii* Morelet, a fungus associated with the bark beetle *Tomicus piniperda*. L. Annals of Forest Science. **61**: 45-53.

Lee S, Kim JJ, Breuil C (2006a). Pathogenicity of *Leptographium longiclavatum* associated with *Dendroctonus ponderosae* to pinus contorta. Canadian Journal of Forest Research. **36**: 2864-2872.

Lee S, Kim JJ, Breuil C (2006b). Fungal diversity associated with the mountain pine beetle, *Dendroctonus poderosae* and infested lodgepole pines in British Columbia. Fungal Diversity. **22**: 91-105.

Mathre DE (1964). Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa* pine. Contributions of the Boyce Thomson Institute. **22**: 363-388.

Ministry of Forests and Range. 2007. 9.2 million hectares affected by the mountain pine beetle. Bulletin number 2007FOR0011-000152. Released February 19th.

Owen DR, Lindahl Jr KQ, Wood DL, Parmeter JR (1987). Pathogenicity of fungi isolated from *Dendroctonus valens*, *D. brevicomis* and *D. ponderosae* to ponderosa pine seedlings. Phytopathology. **77**: 631-636.

Paine TD, Raffa KF, Harrington TC (1997). Interactions among scolytid bark beetles, their associated fungi and live host conifers. Annual Review of Entomology. **42**: 179-206.

Raffa KF, Berryman AA (1982). Physiological differences between lodgepole pines resistant susceptible to the mountain pine beetle and associated microorganisms. Environ. Entomol. **11**: 486–492.

Reid RW (1961). Moisture changes in lodgepole pine before and after attack by the mountain pine beetle. Forest chronicle. **37**: 368-375.

Reid RW, Whitney HS, Watson JA (1967). Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Canadian Journal of Botany. **45**: 1115-1126.

Rice A, Thormann MN, Langor DW (2007a). Mountain pine beetle associated blue-stain fungi cause lesions on jack pine, lodgepole pine, and lodgepole pine-jack pine hybrids in Alberta. Canadian Journal of Botany. **85**: 304-315.

Rice A, Thormann MN, Langor DW (2007b). Virulence of, and interactions among, mountain pine beetle associated blue-stain fungi on two pine species and their hybrids in Alberta. Canadian Journal of Botany. **85**: 316-323.

Solheim H, Langstrom B, Hellqvist C (1993). Pathogenicity of the blue-stain fungus *Leptographium wingfieldii* and *Ophiostoma minus* to Scots pine: effect of tree pruning and inoculum density. Canadian journal of forest research. **23**: 1438- 1443.

Solheim H (1995). Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. Canadian Journal of Botany. **73**: 70-74.

Solheim S, Krokene P (1998). Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. Canadian Journal of Botany. **76**: 561-566.

Solheim H, Krokene P, Langstrom B (2001). Effects of growth and virulence of associated blue-stain fungi on host colonization behaviour of the pine shoot beetles *Tomicus minor* and *T. piniperda*. Plant Pathology. **50**: 111-116.

Strobel GA, Sugawara F (1986). The pathogenicity of *Ceratocystis montia* to lodgepole pine. Canadian Journal of Botany. **64**: 113-116.

Towill LE, Mazur P (1975). Studies on the reduction of TTC as an availability assay for plant tissue cultures. Canadian Journal of Botany. **53**: 1097-1102.

Waring RH, Pitman GB (1983). Physiological stress in lodgepole pine as a precursor for mountain pine beetle attack. Journal of applied entomology. **96**: 265-270.

Whitney HS, Bandoni RJ, Oberwinkler F (1987). *Entomocorticium dendroctoni* gen. et. sp. nov. (Basidiomycotina), a possible nutritional symbiote of the

mountain pine beetle in lodgepole pine in British Columbia. Canadian Journal of Botany **65**:95–102

Yamaoka Y, Swanson RH, Hiratsuka Y (1990). Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine beetle, monitored by a heat pulse velocity (HPV) instrument. Canadian Journal of Forest Research. **20**: 31-36.

Yamaoka Y, Hiratsuka Y, Maruyama PJ (1995). The ability of *Ophiostoma clavigerum* to kill mature lodgepole pine trees. European Journal of Forest Pathology. **25**: 401-404.

Yamaoka Y, Wingfield J, Ohsawa M, Kuroda Y (1998). Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenecity to Japanese larch. Mycoscience. **39**: 367-378.

	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	April	May	June
Avg. monthly temperature (ºC)												
season 1				11.1	-1.6	-0.7	-4.3	0.1	7.2	12.1	14.8	20.8
season 2	23.4	22.2	14.4	8.7	3.3	0.1	-5.6	1.1	6.8	10.9	16.5	17.2
total monthly precipitation (mm)												
season 1				15.1	26	3.9	32.4	13.4	11.2	11.5	52.1	23.4
season 2	39.6	55.5	38.9	30.8	39.1	31	24.1	17	16.6	0.2	19.7	86.2

Table 2.1. Average monthly temperature and total monthly precipitation during

the two field seasons. Season 1 started October 2003 and ended June 2004.

Season 2 started July 2004 and ended July 2005. Temperatures were similar

between the two field seasons, but season 2 was wetter than season 1.
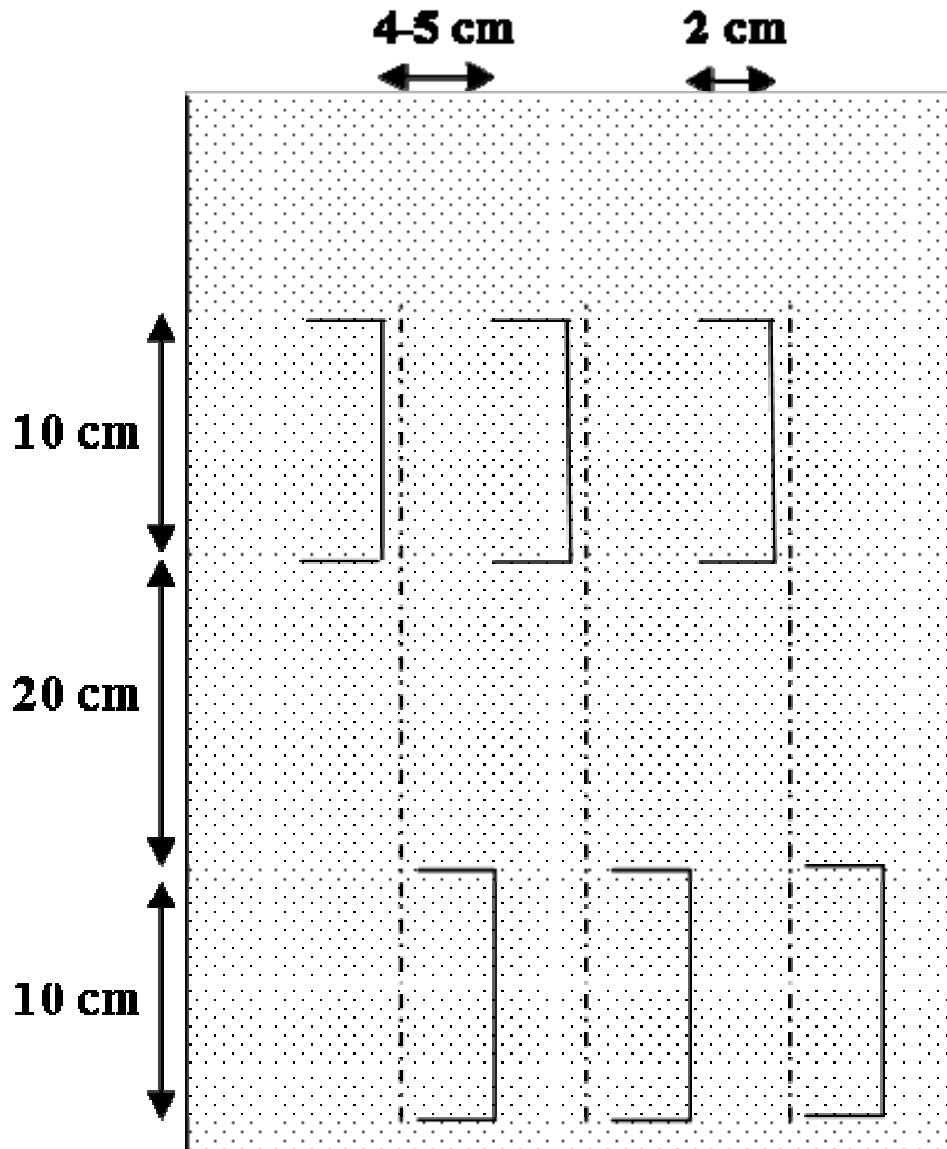
Figure 2.1. Bark flap inoculation pattern used for inoculating *G. clavigera* into *Pinus contorta* trees. Each bark flap measured 2 cm by 10 cm with 4-5 cm between flaps. Two rings of flaps separated by 20 cm were made around the tree. Since the majority of lesion development occurs in a vertical manner, the upper and lower flaps were spaced vertically to avoid overlapping fungal growth.
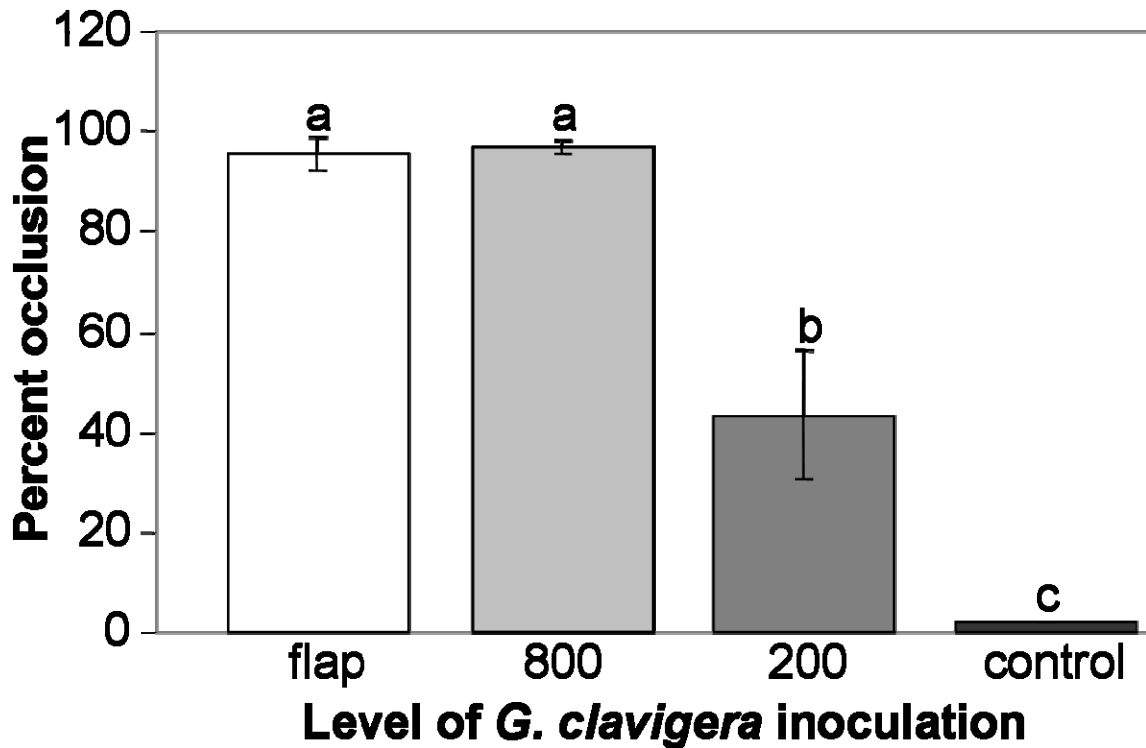
Figure 2.2. Percent occlusion in stem disks of trees inoculated with *G. clavigera* at three inoculation levels: 200 and 800 cork borer inoculations/m^2 and bark flap inoculation. Error bars represent means +/- standard deviations. Bars with the same letter above them are not significantly different at the 0.05 level. Flap = bark flap inoculation. 800 = 800 cork borer inoculations/m^2. 200 = 200 cork borer inoculations/m^2. Control = sterile agar control.

Figure 2.3. Percent sapwood moisture content in pieces of sapwood taken from trees after inoculation treatment. Error bars represent means +/- standard deviations. Bars with the same letter above them are not significantly different at the 0.05 level. Flap = bark flap inoculation. 800 = 800 cork borer inoculations/m^2. 200 = 200 cork borer inoculations/m^2. Control = sterile agar control.

Chapter 3

Intraspecific pathogenic variation in *Grosmannia clavigera*

Introduction

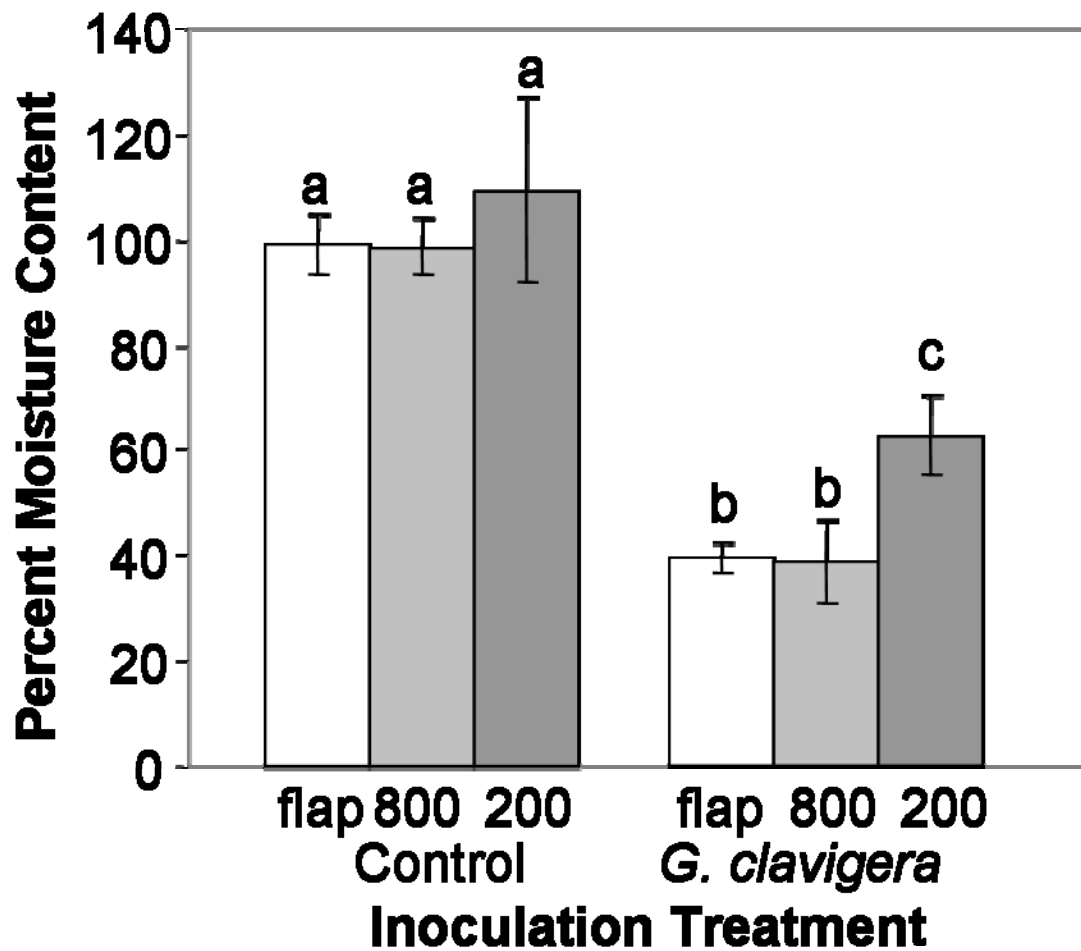[3]The sap-stain fungus *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel de Beer and Wingfield (previously known as *Ophiostoma clavigerum* (Robinson-Jeffrey and Davidson) Harrington) is the most pathogenic fungus associated with the mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) (Reid 1967; Lee et al. 2006). The MPB and its pathogenic fungal associates are able to kill many pine species (Mathre 1964; Strobel and Sugawara 1986; Owen et al. 1987; Yamaoka et al. 1990; Yamaoka et al. 1995; Lee et al. 2006; Rice et al. 2007a, b). In British Columbia (BC) alone, the MPB and its fungal associates have infected over 9.2 million hectares of mature pure and mixed lodgepole pine (*Pinus contorta*) stands (Ministry of Forests and Range 2007). As the fungal associates grow in and stain the sapwood of infected trees, they impede water and mineral transport in these tissues (Amman 1978). The reduction of viable sapwood decreases the tree's ability to synthesize and mobilize secondary compounds, reducing the speed at which a tree can deliver defense compounds to the infection site, which benefits the MPB (Christiansen et al. 1987; Huber et al. 2004). Variation in a tree's susceptibility to fungal colonization and variation in fungal pathogenicity are anticipated to be important determining factors for epidemic dynamics.

.

---

[3] A version of this chapter has been submitted for publication

Infected trees respond to fungi and beetles through defenses that consist of preformed (primary) and induced (secondary) resins. Host trees attempt to flush out MPB and its fungal associates through the flow of preformed resins, while induced resins reduce the suitability of host tissues for growth and development of MPB fungi and beetle brood (Shrimpton and Whitney 1967; Raffa and Berryman 1982; Huber et al. 2004). The induced resin response involves the synthesis of *de novo* secondary compounds, which requires energy and nutrients (Christiansen et al. 1987). Lodgepole pine trees that resist MPB attack produce a strong induced response, while susceptible trees show a weaker response (Reid et al. 1967, Shrimpton 1978). Younger trees tend to produce stronger resin responses than older trees (Shrimpton 1973; Raffa and Berryman 1982, 1983; Christiansen et al. 1987). During a MPB attack, beetles land on trees and release aggregation pheromones to attract conspecifics. As attacking beetles accumulate, a lodgepole pine's ability to respond with preformed or induced resins decreases until the tree can no longer secrete additional resin (Raffa and Berryman 1982, 1983). The threshold density for a successful MPB attack is determined by the total defensive capabilities of a tree (Christiansen et al. 1987) but is likely influenced by the spread of pathogenic fungi as the MPB builds its gallery.

Fungal pathogens can overcome tree defenses, cause disease and eventually kill a host tree. Characterizing pathogenic variation over large geographic areas in a forest epidemic is important for understanding epidemic dynamics and for identifying resistant trees. This task is challenging since pathogenicity testing involves inoculating many trees at various densities with

fungi. Usually fungal inoculations are performed manually with metal cork borers (Yamaoka et al. 1995; Lieutier et al. 2004). Pathogenicity tests on ophiostomatoid fungi have been carried out for short (2-3 months) and long (one year) incubation times. Researchers using short incubation times tend to measure lesion length and/or occlusion area as these symptoms develop relatively quickly (Reid et al. 1967; Solheim 1995; Solheim and Krokene 1998; Yamaoka et al. 1998; Krokene et al. 1999; Bois and Lieutier 2000; Lieutier et al. 2004; Rice 2007a,b). For longer incubation times, researchers tend also to measure moisture content and tree death as these symptoms take longer to develop (Yamaoka et al. 1995; Kim et al. 2005; Lee et al. 2006a). Shorter incubation periods are comparable to the length of time that colonizing beetles and fungi spend in trees before temperatures drop in the fall, reducing activity and growth. Longer incubation periods allow more time for symptoms to develop and permit assessing the over-wintering survivability of the fungi being studied.

Variation in pathogenicity is poorly characterized for many fungal species. Among MPB-associated fungi, only one study examined intraspecific variability (Rice et al. 2007a). However, that study focused on the ability of *G. clavigera* and *O. montium* to induce lesions after a short incubation time in mature jack pine, jack-lodgepole pine hybrids, and lodgepole pine. Jack pine is a major component of boreal forests east of BC and is now threatened by MPB. Rice et al. (2007a) reported that differences in lesion length among three isolates were not significant on lodgepole pine or jack pine, while on hybrid pine one of the three

isolates induced shorter lesions. Thus intraspecific variability was not observed on mature lodgepole pine.

In contrast to previous epidemics, in which most of the MPB-infested trees were old, in the current BC epidemic young tree plantations have been heavily attacked (MacLaughlan and Brooks 2007). In the work reported here we addressed this difference by characterizing the pathogenicity of five isolates of *G. clavigera* that we inoculated into young lodgepole pine trees. We measured three pathogenic indicators and collected data for both short and long fungal growth periods (7 and 48 weeks). We compared pathogenic symptom development trends in young vs. mature lodgepole pines by assessing lesion length development in older trees over a short time period (7 weeks) for two of the isolates. Finally, we characterized the growth rates of the five isolates in a low oxygen environment and over a range of temperatures using artificial media in order to determine intraspecific variation in a more controlled environment than living trees.

Materials and methods

Fungal isolates: history, maintenance and growth tests on artificial medium

Five isolates of *G. clavigera* were used for pathogenicity testing (Table 3.1). ATCC 18086 is the type specimen of *G. clavigera* and was isolated from ponderosa pine during a MPB outbreak in 1965 (Robinson-Jeffrey and Davidson 1968). The other four isolates were obtained from lodgepole pine trees infested with MPB during the early 2000's. KW 1407 has been used to measure

pathogenicity indicators in older lodgepole pine trees (Lee et al. 2006a), while B5, B20 and H55 have been used in a population genetic study using amplified fragment length polymorphism (Lee et al. 2006b). This study found that the majority of isolates (over 100) belong to genetic group 1, while only nine isolates belonged to genetic group 2. H55 and B20 are part of genetic group 1, while B5 is part of group 2. B5 and B20 were isolated from trees harvested near Banff AB, while H55 was isolated from a tree near Houston, BC. Thus, a small amount of genetic and geographic variation was incorporated in pathogenicity testing.

Isolates were stored at -80 ℃ in 20% glycerol. For field inoculation, plugs were taken from isolates which had been grown on 2% Oxoid malt extract agar (OMEA, 33 g Oxoid malt extract agar, 10 g technical Agar #3, 1 L distilled H_2O, Oxoid Ltd., Hants, England) for 5-7 days at 20 ℃. For growth at different temperatures and in low oxygen environments, plugs were taken from cultures that had been stored at 4 ℃ for 1-3 months.

The growth rates of each fungal isolate was tested at 4.0 ℃, 10.0 ℃, 15.0 ℃, 20.0 ℃, 22.5 ℃, 25.0 ℃, 27.5 ℃, 30.0 ℃ and 37.0 ℃. Fungal plugs were placed in the center of 2% OMEA plates. Two radial measurements per plate were taken daily for five days. Each isolate was plated in triplicate, and the experiment was replicated once. For statistical analysis, only growth rates at day four were used because this was usually the last day of growth before some fungal isolates reached the edges of plates. To measure growth in low oxygen

environments, two small pieces of Tygon tubing were placed on the edges of Petri dishes to allow for air circulation. Dishes were placed in 2 L glass bells that were then sealed with silicone grease (Dow Corning Corp. Michigan, USA). Air in the glass bells was replaced by pure nitrogen (99.99% N2 – Praxair, Ontario, Canada) that was circulated for at least one minute prior to sealing. *G. clavigera* encounters low oxygen conditions as it is introduced into the water-saturated environment of host pines. The glass bells were sealed for four days at 20 °C, after which 2 growth measurements per plate were taken. In control Petri dishes, no nitrogen was circulated into the glass bells. Each isolate was plated once per experiment and the experiment was done in triplicate.

Study sites and inoculation of lodgepole pine trees

Young lodgepole pine trees were selected from the 1986 Dardanelles Lake Plantation, located NE of Merritt off the 5A highway near the Stump Lake Ranch turnoff. The site slopes gently downward from the northwest. Trees were spaced 1.0 m from each other in straight lines. In 2005, trees were selected at random from four tree families, two of which had much higher 10 year DBH (diameter at breast height) growth than the other two tree families. Trees were inoculated between 4-7 July 2005 and felled on 9 June 2006 after approximately 48 weeks. This long incubation permits pathogenic symptoms to develop more fully while allowing fungi to overwinter in trees. In 2006, trees were all located within a 15 tree x 15 tree grid on the flatter, southeast side of the test site to minimize micro-site variation since no differences in tree family background were

detected the year before (results not shown). Trees from 2006 were inoculated on 6-7 July 2006 and felled on 23-24 August 2006. These 7 weeks of incubation after inoculation is similar to the length of time used in other studies (Solheim and Krokene 1998) and is approximately equal to the amount of time fungi in the colder regions of the MPB range grow in trees before the decrease of temperatures, when fungal growth slows down or does not occur.

Forty-three 20 year-old lodgepole pine trees were inoculated in the summer of 2005 and thirty-six 21 year-old lodgepole pine trees were inoculated in the summer of 2006. The DBH of trees inoculated in 2005 ranged from 4.5 cm to 14.6 cm, while the DBH of trees inoculated in 2006 ranged from 6.7 cm to 16.6 cm. None of the trees used in field tests or in the plantation showed signs of MPB attack. However, the area around the plantation, consisting predominantly of older lodgepole pines, was heavily infested with MPB.

Old lodgepole pine trees were selected from Ketchan Mountain, located about 35 km south of Merritt. Ten trees with diameter greater than 18 cm at DBH from a 200 m x 200 m stand were randomly selected and inoculated with either isolate B5 or KW 1407. In this stand trees with a DBH of at least 18 cm were on average 145 years old (Dr. John McLean, pers. comm.). Fungi were inoculated into the trees at DBH with spacing between inoculations similar to that of young trees; the DBH of trees ranged from 19.1 cm to 31.8 cm. Trees were inoculated on 24 June 2006 and lesions were measured on 8 August 2006, nearly 7 weeks after inoculation. Old trees were not harvested. No controls were used in this experiment since the aim was to compare relative lesion size between a) B5 and

KW 1407 and b) young and old trees. Control lesions on mature trees from this site used in other experiments have shown minimal (< 10 mm) lesion development (pers. obs.).

To inoculate all trees, a 0.5 cm cork borer was used to remove sections of bark from trees as described by Yamaoka et al. (1995). Plugs of 0.5 cm in diameter from 2% OMEA without (control) or with fungal hyphae were removed from Petri dishes and inoculums were inserted into the opening with the mycelium facing the cambium. The bark section was replaced to seal the opening. For young trees at Dardanelles Lake, depending on the circumference of the tree, trees had between 4-7 rings of inoculations and each ring contained between 4-10 inoculations. Rings were separated vertically by 10-18 cm, depending on the spacing of the whorls of branches on the lower bole of the tree. Inoculations were spaced, on average, 3 cm apart. This is approximately equal to an inoculation density of 200 inoculations/m^2, although the exact total area of inoculation differed slightly between trees. Allowances had to be made when the presence of tree branches or stumps interfered with fungal inoculation. For older trees at Ketchan Mountain, due to time constraints, each tree received only one ring of inoculations, although due to the much larger DBH, young trees (38.9 +/- 7.7) only received about 1.5x the number of inoculations compared to old trees (23.8 +/- 4.5). In the area to be inoculated, all non-living branches were trimmed using a branch cutter and the bark was smoothed using a rough brush. No noticeable resin secretion occurred from the removal of these branches. After all inoculations were made, each ring of inoculations was covered with duct tape.

Measurements of pathogenicity indicators

Phloem lesions were measured after peeling bark off trees in the field with a chisel and blade. Lesions were measured from the top of the inoculation point to the darkened visible leading edge of the lesion. Only the uppermost inoculation points were used to measure lesion length since many lesions overlapped with one another. This technique differs from studies where only a single ring of inoculations is performed on each tree. In these studies, the total lesion length above and below the inoculation point is often measured as opposed to measuring half of the lesion from the inoculation point to the top of the lesion. This may lead to lesion length inconsistency when data are reported in the literature.

Felled tree bolts of young lodgepole pine trees measuring approximately 1.3 m long were transported back to the lab and stored at -4 °C for 1-3 days before indicators of pathogenicity were analyzed. Ends of bolts were sealed with silicone. Three disks of approximately 3-6 cm in height were cut from the middle of each bolt. Two disks were sectioned at points where rings of fungal inoculations were present. One of these disks was used for measuring occlusion area, while the other was used for measuring moisture content. Disks were also used to re-isolate fungi. The third disk was used in cases where one of the first two disks was damaged.

Disks for assessing sapwood occlusion area were soaked overnight in the dark in 1% TTC (2,3,5-triphenyltetrazolium chloride) (Sigma-Aldrich, Oakville,

Ontario). Wood tissues stained red were considered alive (Towill and Mazur 1975), while tissues remaining white were occluded and indicated fungal growth in the area. The following day, all disks were digitally photographed. The percentage of sapwood occlusion area was calculated as:

$$[1] \quad \text{Percent occlusion area} = \left(\frac{TA - HWA - RA}{TA - HWA} \right) \cdot 100$$

where TA = total area, HWA = heartwood area and RA = red area. Areas were calculated using the imaging software in ImageJ (Rasband 2006, Maryland, USA).

Sapwood moisture content was measured by removing 2 pieces of wood that did not include bark or heartwood area from a disk. These pieces were weighed, placed for 24 h at 105 ºC and weighed again. Moisture content was calculated as:

$$[2] \quad \text{Percent moisture content} = \left(\frac{WW - DW}{DW} \right) \cdot 100$$

where WW = wet weight, and DW = dry weight.

For the trees from 2005 and 2006, three random wood samples were taken near inoculation points with or without fungi and were plated onto 2% OMEA for re-isolating the fungi.

Statistical analyses

Comparisons between means were performed using an ANOVA (GLM procedures) with S.A.S. software (S.A.S. institute, North Carolina, USA) followed by a Bonferonni correction for multiple pair-wise comparisons. Data from obvious outliers were removed before the analysis. These included cases where fungi failed to germinate on plugs inoculated into trees or where the presence of tree branches prevented the spread of fungal induced lesions. Residual variances almost always followed a normal distribution, but when variances did not satisfy tests of normality, data sets were power transformed according to Kuehl (1994) to improve the fit of the model. Arithmetic averages and standard deviations are still reported even if power transformed averages were used for tests of significance. All field measurements were nested within tree and tree family (first field season) or just tree (second field season). For temperature related growth rates, comparisons were made at the individual temperature level. Average growth rates at different temperature levels were not compared. Values for temperature related growth rates and oxygen deficient growth rates were nested within Petri Dish number and replicate number.

For Figures 3.1 and 3.2, values are standardized as follows:

$$[3] \quad \text{Standardized value} = \left(\frac{\text{Avg of Pathogen indicator}}{\text{Highest avg indicator value}} \right) \cdot 100$$

where the numerator is the average value of a pathogenic indicator and the denominator is the highest average indicator value from the five different fungal

isolates and one agar control average. For moisture content, since lower moisture content instead of a higher one indicates a more pathogenic isolate, the ratio was then subtracted from 100. Hence, on each graph, the most pathogenic isolate will have a lesion length and an occlusion area value of 100 and a moisture content value of 0. Standard deviations were standardized in the same manner to keep the linear relationship between average values and standard deviations.

Results

Pathogenicity indicators measured in young trees 48 weeks after inoculation with five isolates of *G. clavigera*

For young trees harvested 48 weeks after fungal inoculation, isolates ATCC 18086, B5 and H55 consistently induced longer lesion lengths, larger occlusion areas and lower moisture content than isolates KW 1407 and B20. Pathogenicity indicators were lower in agar controls for lesion length and occlusion area and higher in moisture content compared to any of the fungal isolates tested (Figure 3.1). Longer lesions, more occluded area and low moisture content are indicative of aggressive fungal pathogens. ATCC 18086, B5 and H55 induced stronger pathogenic symptoms. They had average values for pathogenicity indicators of 201.1 mm ± 103.0 mm, 90.0% ± 15.2% and 39.5% ± 13.0% for lesion length, occlusion area and moisture content respectively. Isolates KW 1407 and B20 induced less pathogenic symptoms. They had average values of 44.6 mm ± 26.6 mm, 4.0% ± 4.1% and 143.7% ± 21.3%. Agar

controls had lower average values for lesions lengths and occlusion area: 5.0 mm ± 1.4 mm, 1.0% ± 1.9%, and a higher moisture content of 181.7% ± 25.4%.

Pathogenicity indicators measured in young trees 7 weeks after inoculation with five isolates of *G. clavigera*

For young trees harvested 7 weeks after fungal inoculations, isolate ATCC 18086 induced significantly longer lesions and larger occluded areas than all other isolates (Figure 3.2). This isolate also induced a decrease in the sapwood moisture content more so than other isolates, but the differences were not statistically significant (Table 3.2). For all other isolates, lesion length, sapwood occlusion area and moisture content were not significantly different from each other. For lesion length and occlusion area, controls produced significantly lower measures compared to all isolates except for the lesion lengths of KW 1407. For moisture content, none of the isolates or controls was significantly different from one another. For ATCC 18086, the average values were: 57.3 mm ± 14.9 mm, 27.8% ± 7.2% and 88.1% for lesion length, occlusion area and moisture content, respectively. For the other four isolates, the values were: 15.2 mm ± 10.3 mm, 3.7% ± 1.4% and 127.7% ± 29.2%. For controls, the values were: 1.9 mm ± 0.6 mm, 0.2% ± 0.5% and 125.3% ± 27.2%.

All isolates after both 48 and 7 week incubation periods were successfully re-isolated near inoculation points at least twice from tree disks.

The pathogenicity indicator, lesion length, measured in old trees 7 weeks after inoculation with *G. clavigera* isolates B5 and KW 1407

In older trees (~ 145 years) after 7 weeks after fungal inoculation, isolate B5 induced significantly longer lesions than trees inoculated with isolate KW 1407(Figure 3.3). The average lesion length from all five trees inoculated with isolate B5 was 48.9 mm ± 29.1 mm, while the average lesion length for all five trees inoculated with isolate KW 1407 was 10.46 mm ± 10.8 mm. For isolate B5, average lesion lengths ranged from 30.2 mm ± 19.3 mm to 75.7 mm ± 26.3 mm per tree. For isolate KW 1407, average lesion lengths ranged from 6.9 mm ± 5.3 mm to 16.2 mm ± 16.9 mm. Significant variation for isolate B5 was observed between trees.

Growth rates of five isolates of *G. clavigera* at varying temperatures and in a low oxygen environment 4 days after inoculation

ATCC 18086 grew significantly faster than all other isolates from 10.0 ℃ to 22.5 ℃. However, at 15.0 ℃, there was no significantly difference between the growth rates of KW 1407 and ATCC 18086. At 25.0 ℃, there were no differences in growth rates between all fungal isolates. At 27.5 ℃, ATCC 18086 grew the slowest, although it was not significantly different from KW 1407 (Figure 3.4). The other isolates had similar growth rates at 10.0 ℃, 22.5 ℃, 25.0 ℃ and 27.5 ℃. At 15.0 ℃ and 20.0 ℃, there was some variation among the growth

rates of isolates. Isolates did not grow or grew poorly (< 4 mm growth after 4 days) at 4.0 ℃, 30.0 ℃ and 37.0 ℃; thus, these temperatures were not used for statistical comparisons. When fungi were incubated at 4.0 ℃ or 30.0 ℃ for four days and transferred to room temperature (~22.0 ℃), normal growth resumed. Fungi incubated at 37.0 ℃ did not grow at all even after being transferred to room temperature.

Under low oxygen conditions, all isolates except ATCC 18086 grew faster than under normal oxygen conditions (Figure 3.5). ATCC 18086 grew faster under low oxygen conditions than under normal conditions, but this was not statistically significant. In both the low oxygen and normal environments at 20.0 ℃, ATCC 18086 grew significantly faster than all other isolates. The growth rates of the other isolates did not differ significantly from each other.

Notes from the first field season

During the first field season, all trees used for field testing originated from four tree families, two of which had very high 10 year DBH (diameter at breast height) growth rates, and 2 of which had lower than normal 10 year DBH growth rates. Trees with a high 10 year DBH are more likely to resist MPB attack than trees with a low 10 year DBH. Tree family background was initially used as a covariate, but there was no significant difference in lesion length, moisture content or occlusion area between trees coming from high 10 year DBH growth

73

rates or low 10 year DBH growth rates that had been inoculated with the same fungal isolate (results not shown). Tree family background was ignored for field season two. Instead, trees were selected from a smaller area to minimize micro-site differences between trees. During the first season, to accommodate tree family background trees were selected up to 1 km apart and they were either above, on, or at the bottom of a slope. All trees from field season 2 were located at the bottom of the slope. DBH was also used as a covariate for both field season 1 and field season 2, but DBH was not found to be related to any of the three pathogenic symptoms measured (results not shown, all p-values > 0.70).

Discussion

This work addressed a concern related to large-scale ecological issues in the current MPB epidemic — heavy MPB attack on young lodgepole pine trees. The results described are the first to characterize intraspecific variability in the pathogenicity of a MPB associate towards young lodgepole pine. For *G. clavigera*, we found statistically significant variation for all pathogenic symptoms measured, except for moisture content after 7 weeks.

Only one other study has examined intraspecific variation on lodgepole pine (Rice et al. 2007a). Our results differed from those of Rice et al., who found no intraspecific variation on mature lodgepole pine and jack pine that had been inoculated with three isolates of *G. clavigera*. The differences in results may be due to differences in objectives and methods. Rice et al. (2007a) inoculated older trees (~50 years in age), which are currently the major class of trees threatened

by the MPB in Alberta. To characterize the ability of *G. clavigera* and *Ophiostoma montium* to colonize mature pines from three species, they inoculated eight samples (three *G. clavigera isolates*, three *O. montium* isolates, and a positive and negative control) per tree and measured only lesion development. As noted above, we worked with younger lodgepole pine trees. We reduced the risk of cross contamination and interactions between fungal isolates by inoculating a single fungal isolate per tree. We used a high inoculation density (200 holes/m^2) and measured three pathogenicity indicators. Some authors have suggested the beneficial link between corroborating results from lesion length to other pathogenic metrics (Solheim 1988; Langstrom et al. 1993). Rice et al. (2007a) reported that *G. clavigera* induced lesion length variation on hybrid pines. In her study, isolate B20 induced shorter lesions than B5 but not KW 1407 on hybrid pine. Finally, while in the discussion the authors suggested that 'isolates of *G. clavigera* varied significantly (p < 0.0001) in their abilities to cause lesions on all pine species', this statement is difficult to interpret because the intraspecific analysis of variance test included both fungal species and controls.

Using large numbers of isolates to examine intraspecific variability has resulted in pathogenicity metrics being distributed across a range of values (Lieutier et al. 2004; Salle et al. 2005). However, characterizing such variability requires an appropriate experimental design, such as using a large number of isolates and hosts, and this may not always be feasible for fieldwork. In the work reported here, pathogenicity metrics after 48 weeks were not distributed across a range but rather appeared either pathogenic or less pathogenic. Sapwood

occlusion area was either greater than 85% or less than 5%, phloem lesion length was either around 200 mm or 45 mm and sapwood moisture content was either around 40% or 145%. ATCC 18086, B5 and H55 caused greater pathogenic symptom development and were most likely to be capable of killing young trees after a long incubation period at the density tested. In contrast, KW 1407 and B20 caused weaker pathogenic symptom development. They were usually contained in the reaction zone or spread minimally in the phloem or sapwood. However, only 5 isolates of *G. clavigera* were tested. It is possible that increasing the number of isolates would result in some isolates producing intermediate values for pathogenicity indicators.

Variation in intraspecific pathogenicity has been reported for other species of bark beetle-associated ophiostomatoid fungi. Of these, the most extensively studied are *Ceratocystis polonica* and *Leptographium wingfieldii* Morelet. The former is a fungal associate of *Ips typographus* L. that kills Norway spruce trees. The latter is a fungal associate of the pine shoot beetle *Tomicus piniperda* that affects Scots pine and other pines and has been found recently in North America (Krokene and Solheim 2001; Lieutier et al. 2004; Hausner et al. 2005). While *L. wingfieldii* kills healthy trees when artificially inoculated at a density of 400 inoculations per m^2, its ecological role in beetle-attacked trees is still unresolved. Previous studies have reported a range of variation when examining intraspecific variation. When Krokene and Solheim (2001) inoculated six isolates of *C. polonica* into Norway spruce, lesion length and occlusion area allowed isolates to be characterized as non-pathogenic, of intermediate pathogenicity or pathogenic.

Lieutier et al. (2004) worked with *L. wingfieldii* isolates over two years of tests. In the first year, using six isolates, the occlusion area covered approximately 0%, 25% or 50% of sapwood area, but the differences were not statistically significant because of the small sample size. In the second year, using 15 isolates, lesion length and occlusion area varied over a large range of values (Lieutier et al., 2004). Using more isolates resulted in pathogenic metrics having a broader distribution, as opposed to being pathogenic or less pathogenic.

Patterns in pathogenicity symptoms differed between short and long incubation periods of *G. clavigera* in younger trees. After seven weeks, ATCC 18086, but not H55 and B5, consistently appeared to be more pathogenic than the other isolates, inducing longer lesions and occluding more sapwood area. For sapwood moisture content, no isolates or controls differed significantly. ATCC 18086 had lower moisture content, but this was not statistically significant. Moisture content was the least sensitive metric tested. More time is likely needed for the sapwood colonizing fungi to affect water conducting cells and moisture content. In previous studies where moisture content was measured, data were recorded after approximately one year, allowing fungi more time to impede water transport and lower moisture content (Kim et al. 2005; Lee et al. 2006a). However, trees used in previous studies were older than trees used in the present study.

Using younger trees appeared to affect the rate of pathogenic symptom development. Younger trees tend to respond more vigorously to fungal invasion

than older trees (Shrimpton 1973; Raffa and Berryman 1982, 1983; Christiansen et al. 1987). Overcoming a stronger resin response likely requires more time. After seven weeks, lesion lengths were about three times longer in older trees than in younger trees, indicating that lesions developed faster in older trees. Consistent with the pattern observed in younger trees after 48 weeks, isolate B5 induced significantly longer lesions in older trees than isolate KW 1407. This trend was evident but not statistically significant in younger trees after seven weeks. The more rapid development of pathogenic indicators in older trees is consistent with results from the literature. Shrimptom (1973) reported that trees aged between 41-60 years were about three times more resistant to inoculations with blue-stain fungi than trees aged 111-140 years. In other studies, lesions were longer and sapwood area was nearly totally occluded after 6-8 weeks for 50-150 year old lodgepole pines (Reid et al. 1967, Solheim 1995, Rice et al. 2007a). Older trees are more likely to have less energy reserves that can be mobilized in defense responses (Christiansen et al. 1987). For future field experiments, researchers should be aware of these differences and allow for a longer time for symptoms to develop in younger trees. It also suggests that younger trees are better able to defend themselves against MPB attack and that a larger number of MPB are needed in order to colonize young trees. Unfortunately, the number of MPB present in BC is now sufficiently high to allow for the successful attack of young lodgepole pine trees. However, the ability of MPB to overwinter in younger trees with thinner bark is unknown. It is possible that younger trees are a sink for MPB rather than a source.

Intraspecific variability in ophiostomatoid fungi has also been examined *in vitro*, where conditions are more controlled. Salle et al. (2005) observed intraspecific variation for two ophiostomatoid species in their ability to tolerate resveratrol. For MPB-related fungi, Solheim and Krokene (1998) found some visual variation among isolates in their ability to grow in oxygen deficient conditions or at different temperatures, but only 2 isolates of *G. clavigera* and *O. montium* were used and differences between isolates were not compared statistically. A low oxygen environment is typical of the phloem and sapwood of healthy trees before bark beetle attack. Our results on *G. clavigera* are consistent with previous work. This primary sapwood invader grows well in a low oxygen environment, giving it an advantage when colonizing host trees (Solheim and Krokene 1998). Intraspecific growth rate variability of *G. clavigera* was observed in this environment. This is possibly influenced by the more rapid growth rate of ATCC 18086 at 20 °C rather than this isolate tolerating a low oxygen environment better than other isolates. The intraspecific variation in growth rates over a range of temperatures suggests that at least some of the isolates associated with the beetle are well suited to accompany the MPB as it expand its geographic range.

Isolates from the current epidemic are more likely to be representative of the range of pathogenicity in current populations of *G. clavigera*. Our results suggest that among isolates from the current epidemic, H55 and B5 were more pathogenic than KW 1407 or B20. Differences were likely not observed in young trees after a short incubation time because not enough time was given for

symptoms to develop. In older lodgepole pine, B5 induced longer lesions than KW 1407. In the study by Rice et al. (2007a) on older hybrid pine, B5 induced longer lesions than B20.

Overall, ATCC 18086 was the most pathogenic isolate, while isolate KW 1407 was the least pathogenic. However, when inoculated at a density of 800 inoculations/m^2, KW 1407 was capable of killing mature lodgepole pine trees (Lee et al. 2006a), demonstrating its pathogenicity. This indicates that some isolates of *G. clavigera* may be more pathogenic than previously reported. If MPB carry more virulent isolates, then fewer beetles may be needed to successfully colonize trees. However, ATCC 18086 was isolated during a previous MPB epidemic and from a different host tree. No isolates tested from the current epidemic so far are as virulent as ATCC 18086.

Isolates of lower pathogenicity could be useful when managing small urban or plantation areas, where individual trees are of importance. Raffa and Berryman (1983) showed that low to medium density inoculations of *G. clavigera* prior to beetle attack improve a tree's ability to respond to mass attack. A similar enhanced defense reaction was reported for Norway spruce inoculated with *Ceratocystis polonica* (Krokene et al. 1999) and for Scots pine trees inoculated with either *Leptographium wingfieldii* or *Ophiostoma canum* (Krokene et al. 2000). Using isolates of lower pathogenicity may stimulate and enhance the tree's defensive capabilities against future MPB invasion without largely affecting overall tree productivity and health. Intraspecific variation may also be beneficial for research aiming to link genetic factors with pathogenicity. For example,

differences in expression profiles between highly pathogenic and less pathogenic isolates may help to locate fungal genes associated with pathogenicity.

The current study has demonstrated that five isolates of *G. clavigera* inoculated into lodgepole pine varied in their ability to induce pathogenic responses. Intraspecific variation was detected for all metrics tested, both in the field and in the lab. Isolate KW 1407 was less pathogenic than other *G. clavigera* isolates and may be a good candidate for future tests aiming to enhance a tree's defensive capabilities. Isolate ATCC 18086 was highly pathogenic and would be a good candidate for future studies on gene expression involved in pathogenicity. The development of pathogenicity indicators appears to take longer in younger trees than in older trees.

Acknowledgements

References

Amman GD (1978). Biology, ecology and causes of outbreaks of the mountain pine beetle in lodgepole pine forests. In theory and practice of mountain pine beetle management in lodgepole pine forests. University of Idaho, Pullman, Washington. p. 39-53.

Bois E, Lieutier F (2000). Resistance level in Scots pine clones and artificial introductions of *Tomicus piniderpa* and *Leptographium wingfieldii*. Journal of Applied Entomology **124**: 163-167.

Christiansen E, Waring RH, Berryman AA (1987). Resistance of conifers to bark beetle attack: searching for general relationships. Forest Ecology and Management **22**: 89-106.

Hausner G, Iranpour M, Kim JJ, Breuil C, Davis CN, Gibb EA, Reid J, Loewen PC, Hopkin AA (2005). Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada and comments on the taxonomy of *Leptographium lundbergii*, *L. terebrantis*, *L. truncatum and L. wingfieldii.* Canadian Journal of Botany **83**: 1222-1237.

Huber DPW, Ralph S, Bohlmann J (2004). Genomic hardwiring and phenotypic plasticity of terpenoid-based defenses in conifers. Journal of Chemical Ecology **30**: 2399-2418.

Kim JJ, Allen EA, Humble LM, Breuil C (2005). Ophiostomatoid and basidiomycetous fungi associated with green, red, and grey lodgepole pines after mountain pine beetle infestation. Canadian Journal of Forest Research **35**: 274-284.

Krokene P, Christiansesn E, Solheim H, Franceschi VR, Berryman AA (1999). Induced resistance to pathogenic fungi in Norway Spruce. Plant Physiology **121**: 565-569.

Krokene P, Solheim H, Langstrom B (2000). Fungal infection and mechanical wounding induce disease resistance in Scots pine. European Journal of Plant Pathology. **106**: 537-541.

Krokene P, Solheim H (2001). Loss of pathogenicity in the blue-stain fungus *Ceratocystis polonica*. Plant Pathology **50**: 497-502.

Kuehl RO (1994). Statistical principles of research design and analysis, 2nd edition. Belmont, CA. Duxbury Press.

Langstrom B, Solheim H, Hellqvist C, Gref R (1993). Effects of pruning young Scots pine on host vigour and susceptibility to *Leptgraphium wingfieldii*

and *Ophiostoma minus*, two blue-stain fungi associated with *Tomicus piniperda*. European Journal of Forest Pathology **23**: 400-415.

Lieutier F, Yart A, Ye H, Sauvard D, Gallois V (2004). Variations in growth and virulence of *Leptographium wingfieldii* Morelet, a fungus associated with the bark beetle *Tomicus piniperda* L. Annals of Forest Science **61**: 45-53.

Lee S, Kim JJ, Breuil C (2006a). Pathogenicity of *Leptographium longiclavatum* associated with *Dendroctonus ponderosae* to *pinus contorta*. Canadian Journal of Forest Research **36**: 2864-2872.

Lee S, HamelinR, Six D, Breuil C **(**2006b). Genetic diversity and the presence of two distinct groups in *Ophiostoma clavigerum* associated with *Dendroctonus ponderosae* in British Columbia and the Northern Rocky Mountains. Phytopathology 97: 1117-1185.

MacLaughlan LE, Brooks J (2007). Determining susceptibility of young pine stands to the mountain pine beetle, *Dendroctonus ponderosae*, and manipulating future stands to mitigate losses. FSP project # Y072003. Final technical report (unpublished).

Mathre DE (1964). Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa* pine. Contributions of the Boyce Thomson Institute **22**: 363-388.

Ministry of Forests and Range. 2007. 9.2 million hectares affected   by the mountain pine beetle. Bulletin number 2007FOR0011-000152. Released February 19th.

Owen DR, Lindahl KQ Jr, Wood DL, Parmeter JR Jr (1987). Pathogenicity of fungi isolated from *Dendroctonus valens*, *D. brevicomis* and *D. ponderosae* to ponderosa pine seedlings. Phytopathology **77**: 631.636.

Raffa KF, Berryman AA (1982). Physiological differences between lodgepole pines resistant susceptible to the mountain pine beetle and associated microorganisms. Environmental Entomology **11**: 486–492.

Raffa KF, Berryman AA (1983). Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, *Dendroctonus ponderosae*. Canadian Entomologist **115**: 723-734.

Rasband WS (2006). ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006.

Reid RW, Whitney HS, Watson JA (1967). Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Canadian Journal of Botany **45**: 1115-1126.

Rice AV, Thormann MN, Langor DW (2007a). Mountain pine beetle-associated blue-stain fungi cause lesions on jack pine, lodgepole pine, and lodgepole-jack pine hybrids in Alberta. Canadian Journal of Botany **85**: 307-315.

Rice AV, Thormann MN, Langor DW (2007b). Virulence of, and interactions among, mountain pine beetle-associated blue-stain fungi on two pine species and their hybrids in Alberta. Canadian Journal of Botany **85**: 316-323.

Robinson-Jeffrey RC, Davidson RW (1968). Three new Europhium species with Verticicladiella imperfect states on bluestained pine. Canadian Journal of Botany **46**: 1523–1527.

Salle A, Monclus R, Yart A, Lieutier F (2005). Effect of phenolic compounds on the invitro growth of two fungi associated with *Ips typographus*. Forest Pathology **35**: 298-304.

Shrimpton DM, Whitney HS (1967). Inhibition of growth of blue stain fungi by wood extractives. Canadian Journal of Botany **46**: 757-761.

Shrimpton DM (1973). Age- and size-related response of lodgepole pine to inoculation with *Europhium clavigerum*, a blue-stain fungus. Canadian Journal of Botany **51**: 1155-1160.

Shrimpton DM (1978). Resistance of lodgepole pine to mountain pine beetle infestation.In theory and practice of mountain pine beetle management in lodgepole pine forests. University of Idaho, Pullman, Washington. p. 64-76.

Solheim H (1988). Pathogenicity of some *Ips typographus*-associated blue-stain fungi to Norway spruce. Medd. Nor. Inst. Skogforsk **40**: 11pp.

Solheim H (1995). Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. Canadian Journal of Botany **73**: 70-74.

Solheim H, Krokene P (1998). Growth and virulence of mountain pine beetle associated blue-stain fungi, *Grosmannia clavigera* and *Ophiostoma montium*. Canadian Journal of Botany **76**: 561-566.

Strobel GA, Sugawara F (1986). The pathogenicity of *Ceratocystis montia* to lodgepole pine. Canadian Journal of Botany **64**: 113-116.

Towill LE, Mazur P (1975). Studies on the reaction of TTC as an availability assay for plant tissue cultures. Canadian Journal of Botany **53**: 1097-1102.

Yamaoka Y, Swanson RH, Hiratsuka Y (1990). Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine beetle, monitored by a heat pulse velocity (HPV) instrument. Canadian Journal of Forest Research **20**: 31-36.

Yamaoka Y, Hiratsuka Y, Maruyama PJ (1995). The ability of *Ophiostoma clavigerum* to kill mature lodgepole pine trees. European Journal of Forest Pathology **25**: 401- 404.

Yamaoka Y, Wingfield M, Ohsawa M, Kuroda Y (1998). Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese Larch. Mycoscience **39**: 367-378.

Isolate	Location	Date	Host
ATCC 18086	Cache Creek, BC	1965	*Pinus ponderosae* infested by *Dendroctonus ponderosae*
KW 1407	Kamloops, BC	2001	*Pinus contorta* infested by *D. ponderosae*
B5	Banff, Alberta	2003	*P. contorta* infested by *D. ponderosae*
B20	Banff, Alberta	2003	*P. contorta* infested by *D. ponderosae*
H55	Houston, BC	2003	*P. contorta* infested by *D. ponderosae*

Table 3.1. *Grosmannia clavigera* isolates used in this study.

G. clavigera isolate:	ATCC 18086	B5	H55	B20	KW 1407	Control
48 week incubation						
Statistical grouping	A	A	A	B	B	C
Phloem lesion length (mm)	193.6	200	207.8	45.4	43.2	6
Std. deviation	85.44	103.4	116.2	30.9	18.3	3.5
No.Trees	7	7	8	8	5	6
No. Measurements	35	35	45	49	37	37
Statistical grouping	A	A	A	B	B	C
Sapwood moisture content (%)	38.8	40.1	39.7	143.3	144.5	181.7
Std. deviation	13.5	14.4	12.3	22.3	20.7	25.4
No.Trees	8	6	8	8	5	6
No. Measurements	16	12	16	16	9	12
Statistical grouping	A	A	A	B	B	B
Sapwood occlusion area (%)	89.1	94	87.5	4.3	3.6	1
Std. deviation	15.4	8.5	19.3	3.2	5.3	1.9
No.Trees	8	7	8	8	6	6
No. Measurements	16	14	16	16	12	12
7 week incubation						
Statistical grouping	A	B	B	B	BC	C
Phloem lesion length (mm)	57.3	15	21.6	19	5.3	1.9
Std. deviation	14.9	7.1	11.6	10.5	2.4	0.6
No.Trees	6	6	6	6	6	6
No. Measurements	39	36	39	34	38	34
Statistical grouping	A	A	A	A	A	A
Sapwood moisture content (%)	88.1	106.2	135.4	139.9	129.7	125.3
Std. deviation	41.3	19.8	25.4	26	33.2	27.2
No.Trees	6	6	6	6	6	6
No. Measurements	12	12	12	12	12	12
Statistical grouping	A	B	B	B	B	C
Sapwood Occlusion area (%)	27.8	5.1	3.8	3.8	3.4	0.2
Std. deviation	7.2	1.2	0.8	1.4	2.1	0.5
No.Trees	6	6	6	6	6	6
No. Measurements	12	12	12	12	12	12

Caption on next page

Table 3.2. Lesion length, sapwood moisture content and sapwood occlusion area induced by *G. clavigera* in 20-21 year old lodgepole pine after either 48 or 7 weeks of incubation. Statistical groupings with the same letter are not significantly different from one another at the 0.05 level.
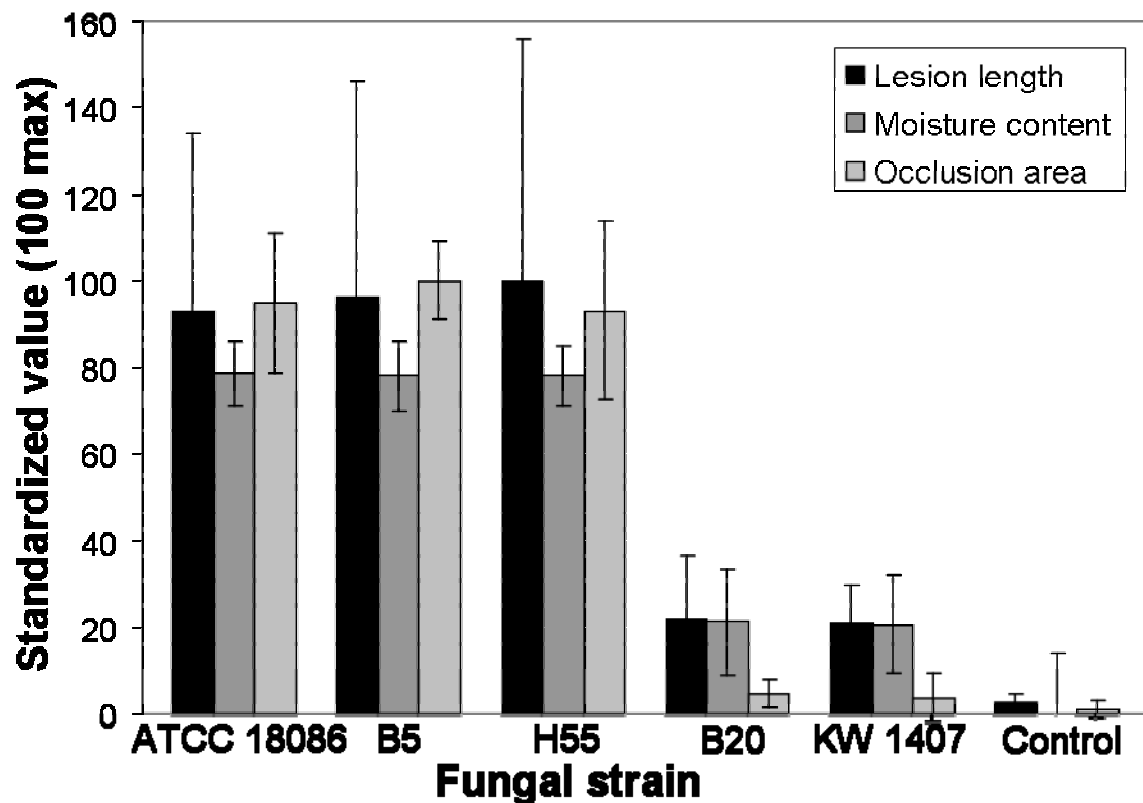
Figure 3.1. Standardized values after 48 weeks incubation with *G. clavigera* inoculated at 200 holes/m^2 in young trees for phloem lesion length, sapwood moisture content and sapwood occlusion area for five fungal isolates and agar controls.The maximum value was defined as the largest average value for each pathogenic indicator among the five isolates according to equation 3. Error bars are standardized standard deviations of pathogenic indicator means. Statistical groupings are located in Table 3.2.
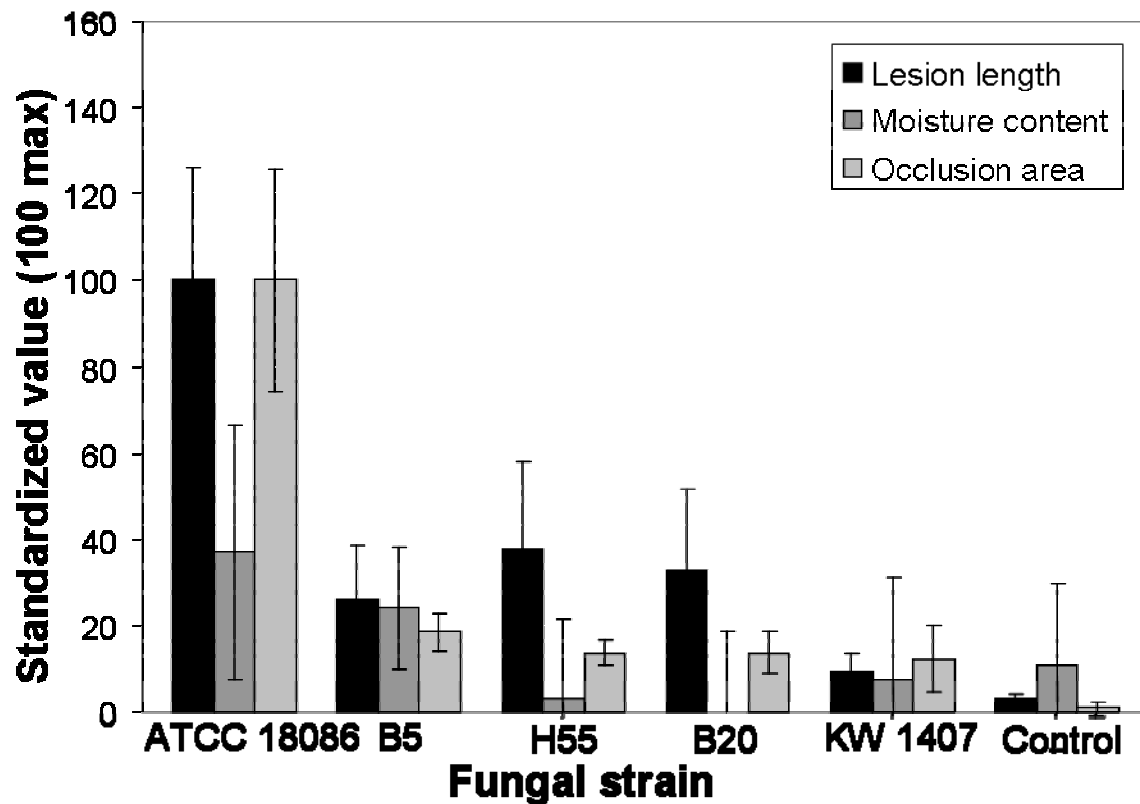
Figure 3.2. Standardized values after 7 weeks incubation with *G. clavigera*

inoculated at 200 holes/m^2 in young trees for phloem lesion length, sapwood

moisture content and sapwood occlusion area for five fungal isolates and agar

controls. The maximum value was defined as the largest average value for each

pathogenic indicator among the five isolates according to equation 3. Error bars

are standardized standard deviations of pathogenic indicator means. Statistical
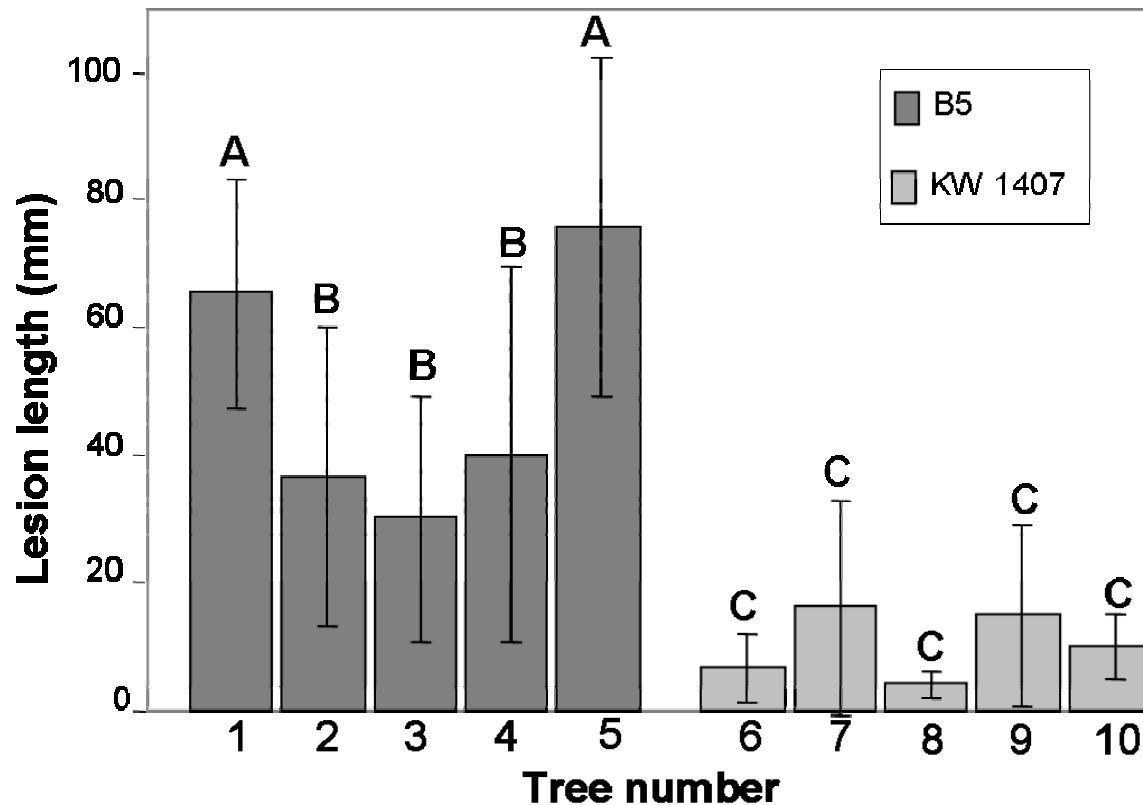
groupings are located in Table 3.2.

Figure 3.3. Average phloem lesion length in ~145 year old lodgepole pines inoculated with *G. clavigera* at 200 holes/m^2 after 7 weeks for two fungal isolates. Results from individual trees are shown due to the large amount of variation in lesion length between individual trees. Tree age, health, amount of sunlight received and DBH were more variable compared to young trees from the plantation and likely account for a large amount of the variation seen between individual trees. Lesion lengths induced by B5 were always significantly longer than lesions induced KW 1407. Bars with the same letter above are not significantly different at the 0.05 level. Error bars are standard deviations of mean phloem lesion length. Trees received between 18-30 holes/tree, depending on DBH.
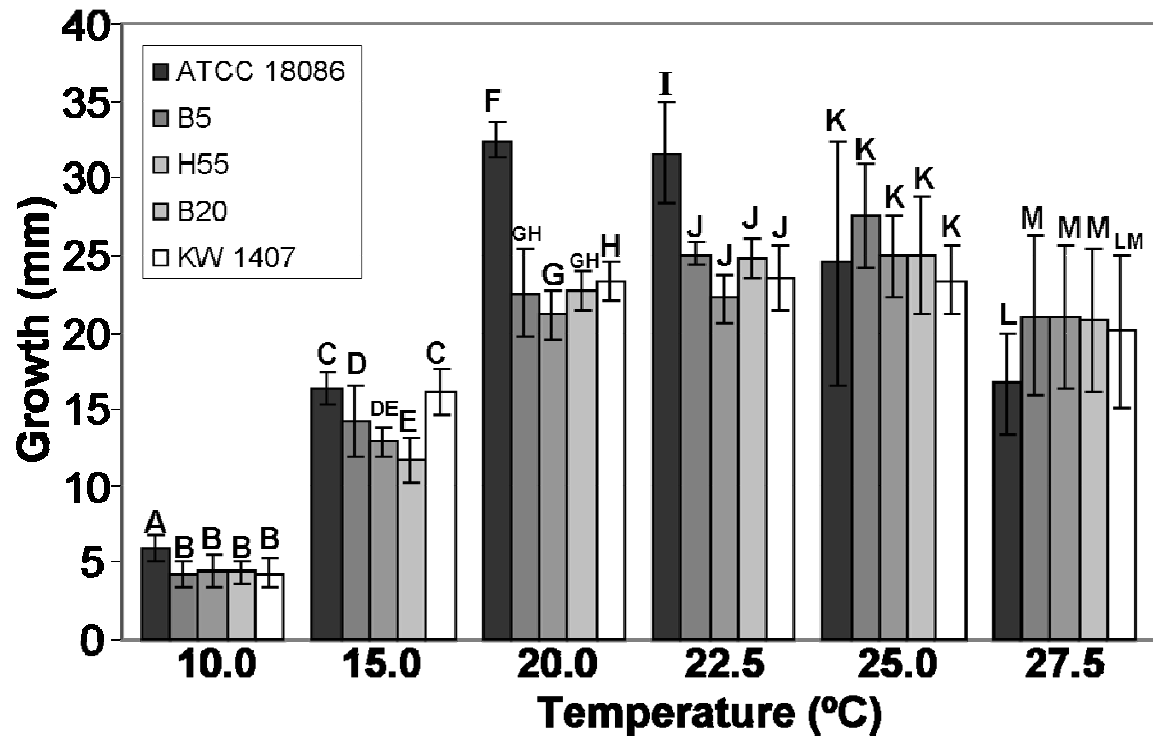
Figure 3.4. Total day 4 growth rates for five isolates of *G. clavigera* grown from

10 ℃ to 27.5 ℃ on 2% OMEA. Within each temperature, average growths with

the same letter are not statistically different at the 0.05 level. Error bars are

standard deviations of mean growth. No comparisons were made between fungal

isolates at different temperatures. Little to no growth was observed at 4 ℃, 30 ℃

and 37 ℃. For the experiment, triplicates of each fungal isolate were used at

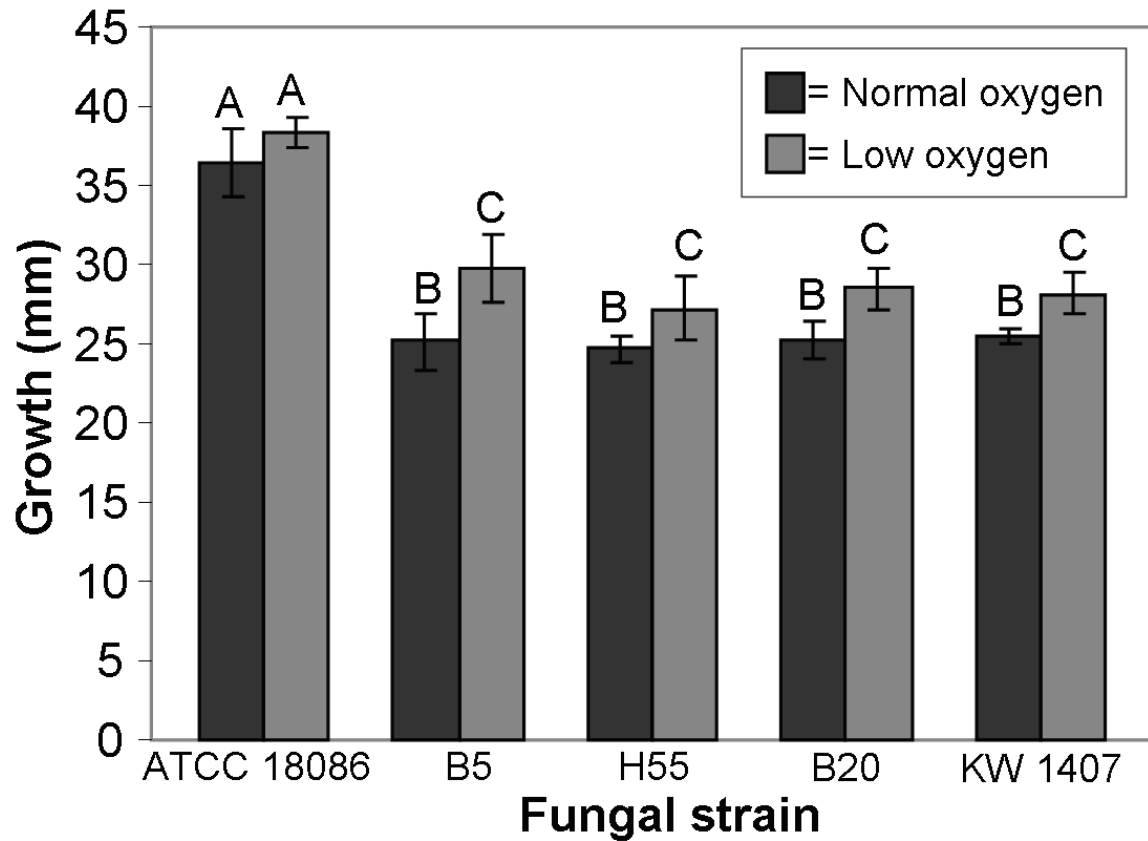each temperature, and the entire experiment was repeated once.

Figure 3.5. Total day four growth rates for five fungal isolates of *G. clavigera* grown at 20 ℃ on 2% OMEA in ambient and low oxygen environments. Bars with the same letter above are not significantly different at the 0.05 level. Error bars are standard deviations of mean day 4 total growth. Due to space constraints in glass bells, each fungal isolate was only plated once per experiment. The entire experiment was done in triplicate.

Chapter 4

Taxonomic history of *Ceratocystiopsis minuta*

Introduction

[4]The fungus *Ceratocystiopsis minuta* (Siezmasko) Zipfel de Beer and Jacobs is the type species of the genus *Ceratocystiopsis*, which was erected in 1975 (Upadhyay and Kendrick 1975). Members from this genus are characterized by falcate or elongate ascospores (Figure 4.1B) usually with a hyaline sheath, dark ascocarps with short necks (Figure 4.2), and sensitivity to cycloheximide (Upadhyay and Kendrick 1975; Zipfel et al. 2006). They grow slowly in comparison to species in the closely related genera Gro*smannia* and *Ophiostoma*, which can be pathogenic and cause sap stain. Morphological and genetic inconsistencies within the genus lead to synonymy of *Ceratocystiopsis* with the genus *Ophiostoma* (Wingfield 1993; Hausner et al. 1993). However, this amalgamation was reversed in 2006 (Zipfel et al. 2006) after extensive genetic analysis.

Ecologically, *C. minuta* is usually found in old bark beetle galleries (Mathiesen-Kaarik 1960), typically in spruce or pine. It grows in the range of 3°-37° C and optimally at 28° C (Solheim 1986). It was reported as nonpathogenic because it induced the smallest lesions out of six fungi tested, sometimes failing to induce any lesion formation (Yamaoka et al. 1998). *Ceratocystiopsis minuta* has been found on three continents and has been associated historically with at least five bark beetles and nine tree species (Table 4.1). In North America, it is a

---

[4] A more comprehensive version of this manuscript will be submitted for publication at a later date.

common associate of the mountain pine beetle (MPB), *Dendroctonus ponderosae*, and in is often found in MPB-infected pine trees such as lodgepole pine, *Pinus contorta* (Figures 4.1C and 4.3). It was found at high frequencies in brood galleries and pupal chambers, but at much lower frequencies in adult galleries and sapwood (pers. observation), leading to speculation that it may provide nutritional benefits to developing broods. However, its exact ecological role is not well understood. In Europe and Japan, it is often associated with Ips spp. and pine trees. It was first isolated and described by Siemaszko (1939) from *Picea abies* infested with *Ips typographus* in Bialowieza, Poland, but, as Hunt (1956) explained, 'no type material has been seen, nor is it known if a type collection has been designated' (Hunt 1956). This has resulted in confusion in the literature as to what constitutes '*C. minuta*.' Davidson (1942) noted that his perithecial measurements were smaller than those of Siemaszko (Table 4.2) but he still considered that his isolates belonged to the same species, even though they were isolated from different hosts on a different continent (Figure 4.2). In Sweden, Mathiesen-Kaarik (1951, 1960) like Davidson, noted that her perithecial measurements were smaller than Siemaszko's measurements and that the solid tendrils of asci exuded from the ostiole were different than the normal mucilaginous ooze that normally accumulates at the tip of the ostiole. In spite of this, she too considered her isolates to be *C. minuta*. Hsiau and Harrington (1997) found that the two different isolates of *C. minuta* used as outgroups in their study had significantly different isozyme profiles, leading them to believe that at least one of their specimens was misidentified. Hausner et al. (2003)

noticed that his isolates of *C. minuta* differed from other descriptions, but this could not be completely verified due to lack of fruiting in cultures or in nature. They noted other incongruencies in *C. minuta* described by previous authors, and suggested conducting comparative molecular analysis to resolve the taxonomic confusion surrounding the species.

The Minuta complex was genetically delineated via the large subunit (LSU) rDNA region (Hausner and Reid 2003). This region is highly conserved and has been recommended for determining differences between families or genera (Lim et al. 2004). However, the sequences used by Hausner and Reid (2003) for their analysis were short (~250 base pairs) and the neighbour-joining tree derived from these data had many polytomies. Ophiostomatoid phylogenies have also been constructed using partial regions of the LSU and internal transcribed spacer (ITS) regions (Hausner et al. 1993; Hausner and Reid 2003; Lim et al. 2004). The ITS2 region is often suggested for delineating groups at the species level, because it has both high interspecific variability and low intraspecific variability, while also containing highly conserved regions (Gardes and Bruns 1993). In addition, the beta-tubulin (βT) gene has been informative in analyses of closely related Ophiostomatoid fungi (Lee et al. 2003; Kim et al. 2003; Lim et al. 2004) and ascomycetous fungi (O'Donnell and Cigelnik 1997). Given this, in the current study we used the ITS and LSU regions from the rDNA operon, and one protein coding gene, the βT gene to phylogenetically re-evaluate *C. minuta*'s taxonomic incongruencies. From the results we recommend an epitype candidate for future studies of the species in the genus.

Materials and methods

Taxon sampling

Information on individual isolates is listed in Table 4.1. All strains are maintained in the Breuil culture collection at the University of British Columbia. Strains were originally ordered from different culture collections from the Netherlands (CBS – Centraalbureau voor Schimmelcultures), Japan (YCC – Yamaoka's Culture Collection) and Canada (UM – University of Manitoba; Reid's culture collection; UAMH – University of Alberta Microfungus collection and Herbarium; and MPB – isolates from the University of British Columbia; Breuil's culture collection). Strains were selected based on their potential inclusion within the *Minuta* group in previous articles (Hausner et al. 1993; Yamaoka et al. 1997; Hsiau and Harrington 1997; Hausner and Reid 2003, Zipfel et al. 2006). Forty isolates representing fourteen species were assembled for analyzing sequences from the three genetic regions (Table 4.2).

Growth, DNA extraction, PCR, and sequencing

Isolates were grown on top of autoclaved cellophane at 22° C on 2% oxoid malt extract agar (OMEA). Mycelium was scraped from the surface of cellophane and DNA was extracted following the method described by Kim et al. (1999). Extracted DNA was stored at -20 C until it was analyzed. PCR amplification was performed as described by Lee et al. (2003). A list of primers used is shown in Table 4.3. Amplifying the ITS region was sometimes difficult, so additional primers were used if regions failed to amplify using the ITS1F primer. PCR

products were purified using a QiaQuick PCR Purification Kit (Qiagen, InC.) and sequenced with an ABI 3700 automated sequencer (Perkin-Elmer, InC. USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). The three regions were successfully amplified for all isolates used.

Phylogenetic analysis

Sequences were viewed using Chromas version 1.43 to ensure proper base pair assignment from chromatograms (http://www.technelysium.com.au/chromas.html for latest version, McCarthy 2004). Files were edited in PHYDIT version 3.2 (http://plaza.snu.aC.kr/~jchun/phydit/download.php, Chun 2001). Alignments were performed by CLUSTALX v1.83 (Thompson et al. 1997) using default settings. Alignments were manually improved by eye in Se-Al v. 2.0 al carbon (Rambaut 2002). Finished alignments were exported into PAUP v. 4.0.B10 (Swofford 2003). Phylogenetic analysis of the three genes combined was conducted using maximum parsimony (MP), maximum likelihood (ML), neighbour-joining (NJ) and Bayesian analysis (BA). MP, ML and NJ were performed in PAUP 4.0.B10, while BA was performed using Mr. Bayes 3.12 (Ronquist and Huelsenbeck 2003). In all cases, gaps were treated as missing data. Maximum parsimony was conducted using a heuristic search with TBR-branch swapping. The stability of clades was evaluated using 1000 bootstrap replicates. Maximum likelihood, using a Rogers-Swofford approximation and TBR branch swapping, was conducted using a GTR model with rates of base pair

substitutions and the proportion of invariable sites estimated by PAUP. The stability of clades was evaluated using 100 bootstrap replicates and no molecular clock was enforced. NJ analysis was conducted using default settings and 1000 bootstrap replicates were used to evaluate clade support. Bayesian analysis was conducted using a GTR + Γ + γ model based on ModelTest v. 3.7 (Posada and Crandall 1998) results. Posterior probability was conducted by using 1,000,000 cycles using 2 runs of 4 chains (1 hot 3 cold) and discarding the first 800,000 trees as burnin. This Bayesian process was used for both the combined data set and individual gene analysis. Using the phylogeny from Hausner and Reid (2003), *O. ips* was selected as the outgroup because it is near the genus *Ceratocystiopsis* within the Ophiostomatoid group of fungi.

Results

DNA sequence comparisons

DNA sequence alignments resulted in 609 characters from the LSU region, 700 characters from the ITS region and 928 from the βT region. There were 46 sites excluded from the ITS region and 24 sites from the βT region due to alignment ambiguity; no characters were excluded from the LSU region.

Phylogenetic analysis of the combined data set

Maximum parsimony

The combined data set contained 2137 sites, of which 1209 were constant, 254 were parsimony uninformative and 674 were parsimony informative. Each of

64 equally parsimonious trees had a tree length of 2665. The basal and terminal clades from one example of the 64 equally parsimonious trees was the same as shown in Figure 4.4, although certain isolates within clades were placed slightly differently (results not shown). Terminal clades from a 50% majority rules consensus tree after 1000 bootstrap replicates were the same as shown in Figure 4.4, but there was no basal resolution between clades (data not shown).

Maximum likelihood

The phylogram from this analysis is shown in Figure 4.4. Bootstrap replication supported terminal clades and the separation of western and eastern isolates seen at group H, but the basal resolution among North American isolates was unresolved (data not shown).

Neighbour joining

After 1000 replicates, the neighbour-joining analysis using default assumptions mostly supported groups A, D, E, K and I as seen in Figure 4.4, although *C. minuta* UM 1453, CBS 116963 and CBS 117566 have unresolved positions and were not placed within groups. *Ceratocystiopsis* sp. 1 grouped more closely to *C. manitobensis* than to *C. brevicomi* or *C. ranaculosa*. There was no basal resolution apart from separating *O. longisporum* and *O. fasciatum* from the rest of the isolates.

Bayesian analysis

The stationary phase of the markov chain was observed after 1,000 generations (100,000 runs) but discarding the first 8,000 generations as opposed to the first 1,000 improved posterior probability values slightly; only the last 2,001

generations (last 200,000 runs) were kept to produce a 50% majority rules consensus tree. This analysis was the only one to provide some support at the node between groups C and F in Figure 4.4. In addition to providing strong support for terminal group resolution, it also provided the most support for basal resolution and helped to resolve many of the polytomies from other analyses.

Phylogeny of Ceratocystiopsis species

Multi-gene analyses, especially using BA and ML, helped resolving basal positioning of groups. The phylogram showed that many isolates formed well resolved groups within the genus *Ceratocystiopsis*. Group H was well supported by Bayesian posterior probability (99%) and included many closely related European and Japanese isolates. This bipartition was unresolved or did not have strong support from the ML, MP and NJ analyses. Within this group, five isolates of *Ceratocystiopsis minuta* from Poland clustered with one Japanese isolate with high support (group I), while other European and Japanese isolates formed a separate and well supported group (group K). The sister group to group J contained two isolates of *C. minuta* from Poland and 2 isolates of *C. rollhanseniana* from Norway (group G). Basal to group H were several well supported groups that were isolated in North America. Four isolates of *O. minimum* from Canada and one isolate of *C. minuta* from the USA formed a well supported group (group E). Two isolates of *C. manitobensis* and two isolates of *Ophiostoma* sp. 3 that resembled *C. manitobensis* formed a well supported group (group D). *Ceratocystiopsis* sp. 1, *C. ranaculosa*, *C. collifera* and *C. brevicomi*,

grouped separately with less support (93% Bayesian support, < 50% for all other support values) but within this group (group B), *C.* sp. 1 was distinct from *C. ranaculosa*, *C. collifera* and *C. brevicomi*. The last well supported group contained three isolates of *C. minuta-bicolor* and one isolate of *C. minuta* (group A). *C. pallidobrunneum* was placed basally within the genus *Ceratocystiopsis*. *O. longisporum*, and *O. fasciatum* were located outside the *Ceratocystiopsis* genus and did not cluster within groups.

Out of the 21 isolates of *C. minuta* used in the analysis and that have been called *C. minuta* or that resembled *C. minuta* (Ophiostoma sp. 1 and sp. 3), 14 isolates formed a monophyletic group supported strongly by Bayesian analysis. All of these isolates were isolated in Europe or Japan and were found in group H. North American isolates of *C. minuta* grouped with other *Ceratocystiopsis* species but did not group closely to European or Japanese isolates of *C. minuta*.

Phylogenetic analysis of single gene regions

Only results from single gene Bayesian analysis are shown (Figures 4.5, 4.6 and 4.7) although MP and ML were also used (results not shown). The phylogeny resulting from the LSU analysis (Figure 4.5) showed the bipartition seen at group H in the combined analysis as having 82% support, although *O. longisporum* and *C. pallidobrunnea* grouped with isolates of *C. minuta*. These two species did not group in the same region for other single gene analyses or in the combined data set analysis. Group G in the combined analysis grouped more distantly from isolates of *C. minuta* and more closely to other species within the

genus *Ceratocystiopsis*. *C. minuta* isolate CBS 145.59 grouped with isolates of *C. minuta* from Europe and not as closely to isolates of *C. minima*, as it did in the combined analysis. Overall, there were more polytomies and only several groupings showed greater than 95% support.

The βT analysis (Figure 4.6) showed group H in with 81% support. There was little support and resolution for groups of *C. minuta* from Europe and Japan. There were many polytomies and no basal resolution. *C. minuta* isolate CBS 463.77 grouped closely to isolates of *C. minutabicolor*, while *C. minuta* isolate CBS 145.59 grouped closely to isolates of *C. minima*. For *Ceratocystiopsis* species aside from *C. minuta,* the terminal clade and intermediary clade resolution was much stronger.

The ITS analysis (Figure 4.7) showed group H with 88% support. There were very few polytomies in the analysis, although support values were often much stronger for terminal clades than for basal clades. *C. minuta* isolate CBS 145.59 grouped closely to isolates of *C. minima* while *C. minuta* isolate CBS 463.77 grouped closely to isolates of *C.minutabicolor*.

Discussion

Morphological identification of *C. minuta* is difficult because there is no holotype and no description of the anamorph. This study identifies the most appropriate isolate of *C. minuta* to use in future research.

CBS 116796 was the best candidate to represent *C. minuta* in future research for three reasons. First, it was isolated from Bialowieza National Park,

Poland, where the original *C. minuta* was found. Second, it groups closely to other isolates of *C. minuta* from Poland. Third, it is available from CBS, an internationally recognized culture collection. No other isolates of *C. minuta* had the three criteria. However, a major problem with this isolate is that it did not produce perithecia in culture. This is a common problem with many *C. minuta* isolates. The few isolates that did produce perithecia in culture took several months to do so. These isolates were usually not from Poland, and in many cases, perithecia were sparse. At this time, for morphological work we recommended using values from the literature until measurements from a new epitype from Poland are available.

Other isolates of *C. minuta* are not as suitable to represent *C. minuta* in future research. Two isolates, CBS 116796 and CBS 116795 were isolated from *Picea abies* in Bialowieza National Park and are available from CBS. However, CBS 116795 does not group as closely to other isolates of *C. minuta* as CBS 116796, making it less appropriate phylogenetically. CBS 116795 did produce a few perithecia (< 15) in culture on autoclaved lodgepole pine sapwood after several months of incubation. However, the number of perithecia was insufficient to produce a thorough description of the species. Other isolates of *C. minuta* from Poland (UM 1532-1535) did not fruit in culture, were not from Bialowieza National Park and were not available from international culture collections. CBS 116963 was isolated from Bialowieza National Park and is available from CBS, but did not fruit in culture and was not as closely related to other *C. minuta* isolates phylogenetically. The remaining isolates of *C. minuta* from other parts of Europe

and from Japan were monophyletic (group H) but were not appropriate to represent *C. minuta* in future research.

Five isolates of *C. minuta* or *C. minuta*-like species (CBS 145.59, CBS 463.77 and *C.* sp. 1i, *C.* sp. 1ii, *C.* sp. 1iii) found in North America are not in group H. Isolates CBS 145.59 and CBS 463.77 were isolated by Davidson in the 1940s. Drawings of these isolates show larger ostioles than in other descriptions and photographs of *C. minuta*. According to Davidson, perithecia from these isolates began to form slowly after 2-3 weeks, but in the present study perithecial formation of other isolates, notably CBS 116795 and YCC 251, took between 2-4 months to develop. Many other isolates failed to produce perithecia even after four months. CBS 145.59 groups closely with four isolates of *O. minima* from Canada, a species first described in 1974 (Olchowecki and Reid 1974; Upadhyay 1981). CBS 463.77 groups closely with three isolates of *O. minuta-bicolor* from Canada and was first described in 1966 (Griffin 1966; Upadhyay and Kendrick 1975; Upadhyay 1981). Both species resemble *C. minuta* morphologically; they have overlapping perithecial and ascospore sizes and similar *Hyalorhinocladiella* anamorphs. It is possible that these isolates were misidentified, although morphological work is needed to confirm this. These isolates were used by Hausner et al. (1993) to represent *C. minuta* in their phylogenetic work. The remaining three isolates of *Ceratocystiopsis* sp. 1 isolated from mountain pine beetles in BC, Canada, closely resembled *C. minuta* morphologically (Figure 1C, 3). When grown on 2% OMEA, they formed white colonies, grew slowly and had a *Hyalorhinocladiella* anamorph. However, genetically, they grouped closely to,

but were distinct from *C. ranaculosa* and *C. brevicomi*. They were even more distantly related to European and Japanese isolates of *C. minuta*. Robinson (1962) and Upadhyay (1981) have described *C. minuta* being isolated from mountain pine beetles. It is possible that the isolates were misidentified and that the authors were observing *Ceratocystiopsis* sp. 1. In our work, we observed a few perithecia on bark of white pine that was infested by the mountain pine beetle (Figure 2 E,F). Although these perithecia resembled those of *C. minuta*, they were smaller than those from other *C. minuta* isolates and they yielded no ascospores. We observed perithecia only once on MPB-infested lodgepole pine bark. Because we were not aware at the time of the rarity of perithecia, we took only one picture of perithecia and ascospores (Figure 3). Attempts to produce perithecia in culture have been unsuccesful. Conidia from anamorphs of *Ceratocystiopsis* sp. 1 also resembled conidia from *C. minuta* (Figure 1 C,D). It is likely that this species belongs to the genus *Ceratocystiopsis*; however, a formal description cannot be made until perithecia are found and measured. Isolates resembling *C. minuta* but from mountain pine beetle infested wood should be interpreted cautiously, as it is possible that they represent a separate species.

Two isolates of *C. minuta*, C112 from Louisiana, USA (used as an outgroup by Hsiau and Harrington 1997), and IMI 212115 from Sweden (isolated and described by Mathiesen-Kaarik in the 1950's), were not used in our phylogenetic analysis. While the LSU sequences of these isolates were conserved and could be readily aligned for all isolates, the βT and ITS sequences were variable and could not be aligned; as a result, we excluded

these isolates from the analysis. Hsiau and Harrington (1997) noted that C112 had a very different isozyme profile than the other isolate of *C. minuta* that they used as an outgroup; however, the other isolate was no longer viable and could not be included in the current analysis. Alignment results suggested that isolate IMI 212115 was incorrectly identified as *C. minuta*. Photographs of the anamorph from this isolate (Mathiesen-Kaarik 1951, p. 207) did not closely resemble other anamorph descriptions/photographs of *C. minuta*. It is likely that these isolates were misidentified and genetic information used from them could be misleading.

The separation of North American versus European and Japanese isolates at group H was well supported by Bayesian analysis but not by ML, MP, or NJ analyses. Within the North American grouping, the basal position of groups was usually not well supported, except in some cases by Bayesian analysis. This was evident in the many polytomies that were more common in MP and NJ analyses, and to a lesser extent, ML analysis. Caution should be used when inferring information from the basal areas of Figure 4.

Similar to results from Zipfel et al. (2006), *C. minuta, C. ranaculosus, C. minuta-bicolor, C. minima, C. rollhanseniana* and *C. manitobensis* grouped within the *Ceratocystiopsis* genus. Isolates of *C.* sp. 1 from mountain pine beetles grouped closely to *C. brevicomi*, *C. ranaculosum* and *C. colliferum* species (group B), all of which grouped within the genus. *Ceratocystiopsis pallidobrunnea* is part of the minuta group, as mentioned by Hausner and Reid (2003). Our analysis placed it basally within the group, but we consider this placement to be tentative because we had only one isolate for the analysis.

Consistent with previous literature, *Ophiostoma longisporum* and *O. fasciatum* did not group within the *Ceratocystiopsis* genus (Hausner et al. 1993; Hausner and Reid 2003; Zipfel et al. 2006). *Ophiostoma retusum* and *O. carpenteri* were also initially included in the analysis but failed to align with other taxa and did not group within the minuta complex during early phylogram construction (results not shown, but in agreement with Zipfel et al. 2006); we excluded these taxa from the final analysis. Zipfel et al. (2006) placed *C. parva* and *C. concentrica* in this genus. In our work, at least one genetic region failed to amplify in these two species, resulting in their exclusion from the analysis.

The taxonomic history of *C. minuta* is confusing. Afterthe first description of *C. minuta* by Siemaszko in 1939, researchers used different isolates and reported different perithecial measurements. It has been suggested that the original culture from Poland, dated 1939, which was not discussed by Siemaszko, may have been lost during World War II. Siemaszko also neglected to describe the anamorph and conidia of the species (Figure 1 C, D). The inconsistencies from early records are problematic because figures and descriptions of the anamorph from early authors do not closely resemble one another, suggesting that these isolates may represent different species (see descriptions in Davidson 1942; Mathiesen-Kaarik 1951; Hunt 1956). No subsequent descriptions of *C. minuta* reported perithecial base measurements as large as those originally described by Siemaszko (Table 4.2), making it difficult to define *C. minuta*. Should one consider all subsequent measurements of perithecia as too large, or might there have been a problem with the original description? Could authors be

comparing different but closely related species? Without either a dried or living

sample of the original culture, it is impossible to answer such questions. From

1939 to 2007, researchers have also used many different isolates to represent *C.

minuta*. For example, Hsiau and Harrington (1997), in their phylogenetic analysis,

used two isolates of *C. minuta* collected in Louisiana, USA by Bridges and Perry

(1987) as outgroups, while Hausner et al. (2000) used CBS 134.51 from Scotland,

UK, an isolate is now re-identified as *Ophiostoma capilliferum*. In Japan,

Yamaoka et al. (1997) included isolates YCC 139 and YCC 251 from Japan

when identifying new *Ophiostoma* species. Hausner et al. (1993) used isolates

CBS 145.59 and CBS 463.77, collected by Davidson during the 1940s in the

USA, when examining the phylogeny of Ophiostomatoid species. Zipfel et al.

(2006) used isolate CMW 4586 from Scotland, UK. Unfortunately, none of these

isolates originate in Poland, the site of the original collection. It is therefore

difficult to determine if these isolates properly represent *C. minuta*.

Conclusion

Our analysis identifies the most suitable isolate of *C. minuta* to be used for

future research and helps resolve the phylogenetic relationships of species within

the genus *Ceratocystiopsis*. For future genetic work within the genus

Ceratocystiopsis, we recommend using isolate CBS 116796 until a Polish isolate

from Bialowieza is available that is genetically closely related to CBS 116796 and

produces perithecia in culture. The complicated history and broad distribution of

C. minuta make it unlikely that all isolates labeled as *C. minuta* are the same

species; our genetic analysis identified several isolates that may have been misidentified. *C. minuta* isolates from Europe and Japan were genetically similar, while isolates from North America appeared to be either misidentified or separate species.

References

Bridges JR, Perry TJ (1987). *Ceratocystiopsis ranaculosus* sp. nov. associated with the southern pine beetle. Mycologia **79**(4): 630-633.

Davidson RW (1942). Some additional species of ceratostomella in the United States. Mycologia **34**: 650-662.

Gardes M, Bruns TD (1993). ITS primers enhance specificity of basidiomycetes: an application to the identification of mycorrhizae and rusts. Molecular Ecology **2**: 113-118.

Griffin HD (1966). The genus *Ceratocystis* in Ontario. Can. J. Bot **46**: 689-718.

Hausner G, Reid J, Klassen GR (1993). *Ceratocystiopsis*: a reappraisal based on molecular criteria. Mycol. Res. **97**(5): 625-633.

Hausner G, Reid J, Klassen GR (2000). On the phylogeny of members of *Ceratocystis s.s.* and *Ophiostoma* that possess different anamorphic states, with emphasis on the anamorph genus *Leptographium*, based on partial ribosomal DNA sequences. Can. J. Bot. **78**: 903-916.

Hausner G, Eyjolfsdottir GG, Reid J (2003). Three new species of Ophiostoma and notes on Cornuvesica falcata. Can. J. Bot. **81**:40-48.

Hausner G, Reid J (2003). Notes on *Ceratocystis brunnea* and some other Ophiostoma species based on partial ribosomal DNA sequence analysis. Can. J. Bot. **81**: 856-876.

Hsiau PT-W, Harrington TC (1997). *Ceratocystiopsis brevicomi* sp. nov., a mycangial fungus from *Dendroctonus brevicomis*. Mycologia **89**(4): 661-669.

Hunt J (1956). Taxonomy of the genus *Ceratocystis*. Lloydia **19**: 1-59.

Lee S, Kim JJ, Fung S, Breuil C (2003). A PCR-RFLP marker distinguishing *Ophistoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Can. J. Bot. **81**: 1104-1112.

Lim YW, Alamouti SM, Kim JJ, Lee S, Breuil C (2004). Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. FEMS Microbiology Letters. **237**: 89-96.

Kim JJ, Kim SH, Lee S, Breuil C (2003). Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated fungi. FEMS Microbiology Letters **222**: 187-192.

Kim SH, Uzunovic A, Breuil C (1999). Rapid detection of *Ophiosotma piceae* and *O. quercus* in stained wood by PCR. Applied and Environmental Microbiology: 287- 290.

Mathiesen (-Kaarik) A (1951). Einige neue *Ophiostoma*-Arten in Schweden. Svenske Botanisk Tidskrift. BD **45**(1): 204-231.

Mathiesen-Kaarik A (1960). Studies on the ecology, taxonomy and physiology of Swedish Insect-associated blue stain Fungi, especially the genus *Ceratocystis*. Oikos **11**(1): 13-25.

Olchowecki A, Reid J (1974). Taxonomy of the genus *Ceratocystis* in Manitoba. Can. J. Bot **52**: 1675-1711.

O'Donnell K, Cigelnik E (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous. Mol. Phylogenet. Evol. **7**: 103-116.

Robinson RC (1962). Blue stain fungi in lodgepole pine (*Pinus contarta Dougl. Var. latifolia*) infested by the mountain pine beetle (*Dendroctonus moticolae Hopk.*). Can. J. Bot **40**: 609-614.

Siemaszko W (1939). Fungiassociated with bark-beetles in Poland. Planta Polonica **7**(3): 1-59.

Solheim H (1986). Species of Ophiostomataceae isolated from Picea abies infested by the bark beetle Ips typographus. Nord. J. Bot. **6**(2): 199-207.

Upadhyay HP, Kendrick WB (1975). Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. Mycologia **67**: 798-805.

Upadhyay HP (1981). A monograph on *Ceratocystis* and *Ceratocystiopsis*. The University of Georgia Press. Athens, Georgia.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J. Bacteriol. **172**: 4238-4246.

White TJ, Bruns TD, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols, Methods and Amplifications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds.), pp. 315-322. Press, San Diego, CA.

Wingfield MJ (1993). Problems in delineating the genus *Ceratocystiopsis*. From Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity. Chapter 3: Pages 21-25. APS Press, St. Paul. Minnesota.

Yamaoka Y, Wingfield MJ, Takahashi I, Solheim H (1997). Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus f. japonicus* in Japan. Mycol. Res. **101**(10): 1215-1227.

Yamaoka Y, Wingfield MJ, Ohsawa M, Kuroda Y (1998). Ophiostomatoid fungi associated with Ips cembrae in Japan and their pathogenicity to Japanese Larch. Mycoscience **39**: 367-378.

Zipfel RD, de Beer ZW, Jacobs K, Wingfield B, Wingfield MJ (2006). Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* as distinct from *Ophiostoma*. Studies in Mycology **55**: 75-97.
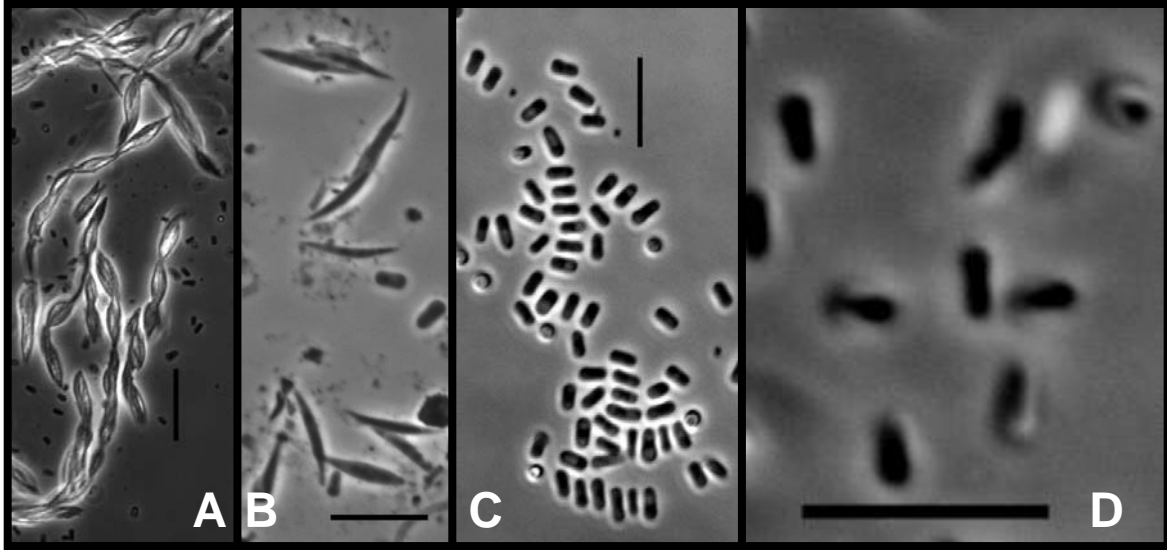
Figure 4.1. A. Asci from CBS 117562 from Austria. scalebar = 20 µm. **B.** Ascospores from CBS 117562. scalebar = 10 µm. **C.** Conidia from WY 13x-1-3 (*C.* sp. 1i) from Canada. scalebar = 10 µm. **D.** Conidia from CBS 117562. scalebar = 10 µm.
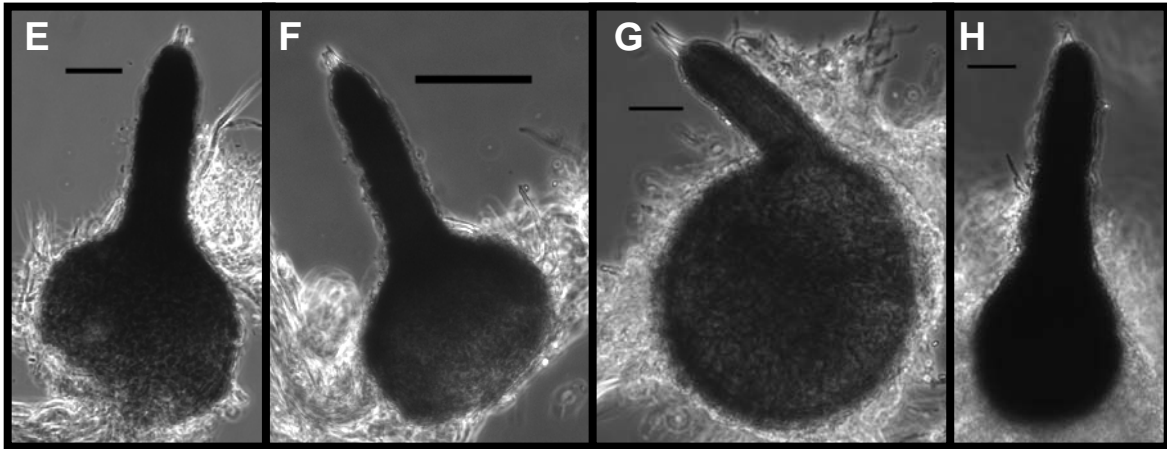
Figure 4.2. E and F. Perithecia from unknown sample isolated from Whitebark

pine infested with *Dendroctonus ponderosae* in Canada. scalebar E = 10 µm.

Scalebar F = 20 µm. **G.** Perithecia from CBS 116795 from Poland. scalebar = 20

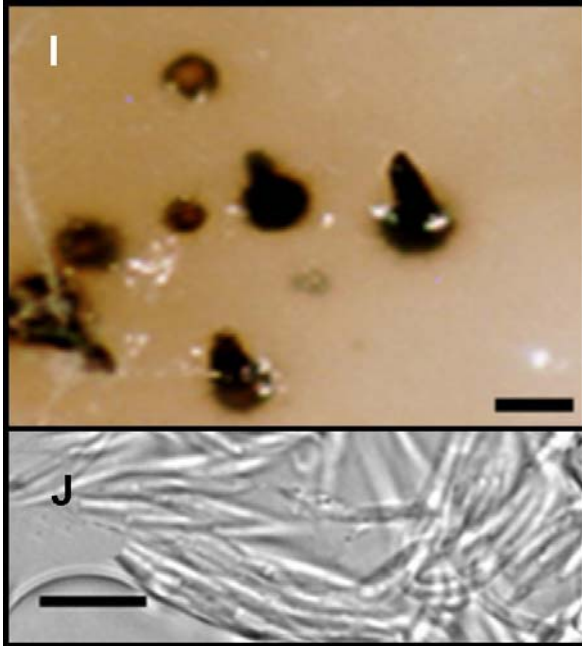µm. **H.** Perithecia from CBS 117566 from Scotland. scalebar = 20 µm.

Figure 4.3. I. Perithecia from *C.* sp. 1 isolated from Lodgepole pine infested with *Dendroctonus ponderosae* in Canada. scalebar I = 70 μm. **J.** Ascospores from the same isolate. scalebar = 5 μm.

Species name	Isolate No.	Host	Isolation Source	Geo. region	Collector
*Ceratocystiopsis brevicomi (*Hsiau and Harrington) Zipfel et al. 2006	UM1452 (CBS 333.97)	Unknown	*Dendroctonus brevicomis*	CA, USA	T. Harrington
*Ceratocystiopsis manitobensis (*Reid &Hausner) Zipfel et al. 2006	UM237	*Pinus resinosa*	Galleries of bark beetle	MB, Canada	J. Reid
*Ceratocystiopsis manitobensis (*Reid &Hausner) Zipfel et al. 2006	UM214	*Pinus resinosa*	Unknown	MB, Canada	J. Reid
Ophiostoma fasciatum (Olchowecki. & Reid) Hausner, et al. 2006	UM 56	***Pseudotsuga menziesii***	Unknown	BC, Canada	A. Olchowecki
Ophiostoma longisporum (Olchowecki. & Reid) Hausner et al. 2006	UM 48	***Pinus banksiana***	Unknown	MB, Canada	A. Olchowecki
Ceratocystiopsis minima (Olchowecki. & Reid) Zipfel et al. 2006	UM85	*Pinus resinosa*	Unknown	MB, Canada	J. Reid
Ceratocystiopsis minima (Olchowecki. & Reid) Zipfel et al. 2006	UM235	*Pinus banksiana*	Unknown	MB, Canada	J. Reid
Ceratocystiopsis minima (Olchowecki. & Reid) Zipfel et al. 2006	UM1501	Pinus banksiana	Unknown	MB, Canada	J. Reid
Ceratocystiopsis minima (Olchowecki. & Reid) Zipfel et al. 2006	UM1462 (CBS182.86)	*Pinus banksiana*	Uknown	WI, U.S.A.	M.J. Wingfield
Ceratocystiopsis minuta-bicolor (R.W. Davidson) Zipfel et al. 2006	CBS635.66 (UM844)	*Pinus contorta*	Galleries of *Ips* sp.		R.W. Davidson
Ceratocystiopsis minuta-bicolor (R.W. Davidson) Zipfel et al. 2006	UM480	*Pinus contorta*	Unknown	Alta. Canada	J. Reid
Ceratocystiopsis minuta-bicolor (R.W. Davidson) Zipfel et al. 2006	UAMH9551 (UM479)	*Pinus contorta*	Unknown	Alta. Canada	J. Reid
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	CBS145.59	Unknown	Unknown	USA	R.W. Davidson
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	CBS463.77 (UM846)	*Picea engelmannii*	Unknown	NM, USA	R.W. Davidson
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	CBS116795	*Picea abies*	Perithecia in galleries of *Ips typographus*	Poland	T. Kirisits
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	CBS117562	*Larix decidua*	Perithecia in galleries of *Ips cembrae*	Austria	T. Kirisits
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	CBS117566	*Larix decidua*	Perithecia in galleries of *Ips cembrae*	Scotland, UK	T. Kirisits et al.
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	UM1453 (CBS 441.94)	*Picea abies*	*Ips typographus*	Austria	T. Kirisits
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	YCC139	*Picea jezoensis*	Adult beetle of *Ips typographus japonicus*	Japan	Y. Yamaoka
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	YCC251	*Picea jezoensis*	Egg gallery of *Ips typographus japonicus*	Japan	Y. Yamaoka
*Ceratocystiopsis minuta (*Siemaszko) Zipfel et al. 2006	YCC294	*Larix kaempferi*	Gallery of Ips cembrae	Japan	Y. yamaoka

Table 4.1. List of species used in phylogenetic analysis and their isolation source, geographic location and collector

Species name	Isolate No.	Host	Isolation Source	Geo. region	Collector
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	CBS 116796	*Picea abies*	Sapwood of Ips typographus	Bialowieza, Poland	T. Kirisits
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	CBS 116963	*Picea abies*	Perithecia in Ips typographus galleries	Bialowieza, Poland	T. Kirisits
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	UM 1532	Unknown	Ips typographus	Biebrzanski National Park, Poland	R. Jankowiak
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	UM 1533	Unknown	Ips typographus	Krynki Forest District, Poland	R. Jankowiak
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	UM 1534	*Picea abies*	Ips typographus	Biebrzanski National Park, Poland	R. Jankowiak
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	UM 1535	*Picea abies*	Ips typographus	Limnowa Forest District, Poland	R. Jankowiak
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	IMI 212115	*Pinus sylvestris*	Unknown	Sweden	A. Kaarik
Ceratocystiopsis minuta (Siemaszko) Zipfel et al. 2006	C112	Unknown	Unknown	Louisiana	Bridges and Perry
Ceratocystiopsis ranaculosa (Bridges & Perry) Zipfel et al. 2006	CBS216.88	*Pinus taeda*	Tree infested with *Dendroctonus frontalis*	LA, USA	J.R. Bridges
Ceratocystiopsis ranaculosa (Bridges & Perry) Zipfel et al. 2006	UM113	*Pinus sylvestris*	Beetle galleries of standing tree	Akershus, Norway	J. Reid
Ceratocystiopsis ranaculosa (Bridges & Perry) Zipfel et al. 2006	UM110	*Pinus sylvestris*	Beetle galleries of standing tree	Hedmark, Norway	J. Reid
Ceratocystiopsis sp. 1 (C. *minuta*-like)	C. sp. 1i	*Pinus contorta*	Log infested with *Dendroctonus ponderosae*	BC, Canada	J.-J. Kim
Ceratocystiopsis sp. 1 (C. *minuta*-like)	C. sp. 1ii	*Pinus contorta*	Log infested with *Dendroctonus ponderosae*	BC, Canada	J.-J. Kim
Ceratocystiopsis sp. 1 (C. *minuta*-like)	C. sp. 1iii	*Pinus contorta*	Log infested with *Dendroctonus ponderosae*	BC, Canada	J.-J. Kim
Ceratocystiopsis sp. 2 (C. *minuta*-like)	YCC329	*Larix kaempferi*	Adult beetle of *Ips cembrae*	Japan	Y. Yamaoka
Ceratocystiopsis sp. 2 (C. *minuta*-like)	YCC330	*Larix kaempferi*	Adult beetle of *Ips cembrae*	Japan	Y. Yamaoka
Ceratocystiopsis sp. 2 (C. *minuta*-like)	YCC513	*Larix kaempferi*	Adult beetle of *Ips cembrae*	Japan	Y. Yamaoka
Ceratocystiopsis sp. 3 (*O. manitobense*-like)	C. sp3i (SWT1)	*Picea glauca*	Body of *Ips perturbatus*	Canada	S. M.-Alamouti
Ceratocystiopsis sp. 3 (*O. manitobense*-like)	C. sp3ii (SWT3)	*Picea glauca*	Body of *Ips perturbatus*	Canada	S. M.-Alamouti
Ceratocystiopsis pallidobrunnea (Olchow. & Reid) Zipfel et al. 2006	UM51	*Populus tremuloides*	Unknown	MB, Canada	J. Reid
Ophiostoma Ips	CBS 137.36 type	Unknown	*Ips* beetle	USA	Rumbold
Ceratocystiopsis collifera (Marmolejo & Butin) Zipfel et al. 2006	UM537	*Pinus teocote*	*Dendroctonus valens*	Santiago, Mexico	J. Marmolejo

Table 4.1. continued

Author	Year	Location	Tree Host	Insect Vector	Perithecia (µm)	Ostiole (µm)	Ascospore (µm)
Siemaszko	1939	Poland	*Picea abies*	*Ips typographus*	84-140 x 74-126	98-140	8-10(12.5) x 1-1.5
Davidson	1942	USA	dead pine trunk'	*Monochamus titillator*	60-80	45-90	12 x 1.1.5
Mat-Kaarik[1]	1951/60	Sweden	Pine/Spruce	"Bark beetles"	58-106	60-100	13.3 x 1.7
Hunt	1956	USA?	Not mentioned	Not mentioned	50-75	50-150	8-13 x 0.5-1.5
Upadhyay	1981	N. America	*Pinus* and *Picea* spp.	*Dendroctonus, Ips* spp.	50-87.5	45-150	6.5-13 x 1.2-2.0
Yamaoka et al.	1997	Japan	*Picea jezoensis*	*I. typographus japonicus*	48-87	67-151	9.6-13.6 x 1.4-2.4
Yamaoka[2]	2006	Japan	*P. jezoensis*	*I. typographus jap.*	48-75	69.9-112.1	9.6-12.8 x 1.4-1.6
Yamaoka[3]	2006	Japan	*P. jezoensis*	*I. typographus jap.*	56-75	83-147	10.4-12.1 x 1.6-2.4
Plattner[4]	2007	Austria	*Larix decidua*	*Ips cembrae*	62.5-75	100-142.5	6.8-9.4 x 0.9-1.2

Table 4.2. Historical measurements of *Ceratocystiopsis minuta* and associated tree/bark beetle hosts

Notes

[1] Mathiesen-Kaarik
[2] Previously unpublished measurements from 15 samples from YCC139
[3] Previously unpublished measurements from 10 samples from YCC 251
[4] Previously unpublished measurements from 50 samples from CBS 117562

Region	Name	Direction	Primer	Reference
LSU	LR0R	forward	5'-ACCCGCTGAACTTAAGC-3'	Vilgalys and Hester (1990)
LSU	LR3	reverse	5'-CCGTGTTTCAAGACGGG-3'	Vilgalys and Hester (1990)
BT	T10	forward	5'-ACGATAGGTTCACCTCCAGAC-3'	O'Donnell and Cigelnik (1997)
BT	BT12	reverse	5'-GTTGTCAATGCAGAAGGTCTCG-3'	Kim et al. (2003)
ITS	ITS1F	forward	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Gardes and Bruns (1993)
ITS	ITS1	forward	5'-TCCGTAGGTGAACCTGCGG-3'	White et al. (1990)
ITS	ITS3	forward	5'-GCATCGATGAAGAACGCAGC-3'	White et al. (1990)
ITS	ITS4	reverse	5'-TCCTCCGCTTATTGATATGC-3'	White et al. (1990)

Table 4.3. List of Primers used for phylogenetic sequencing

Figure 4.4. Phylogram of *Ceratocystiopsis minuta* and associated species. Support values from Bayesian analysis,

maximum likelihood, maximum parsimony and neighbourr-joining respectively are overlaid on a maximum likelihood tree.
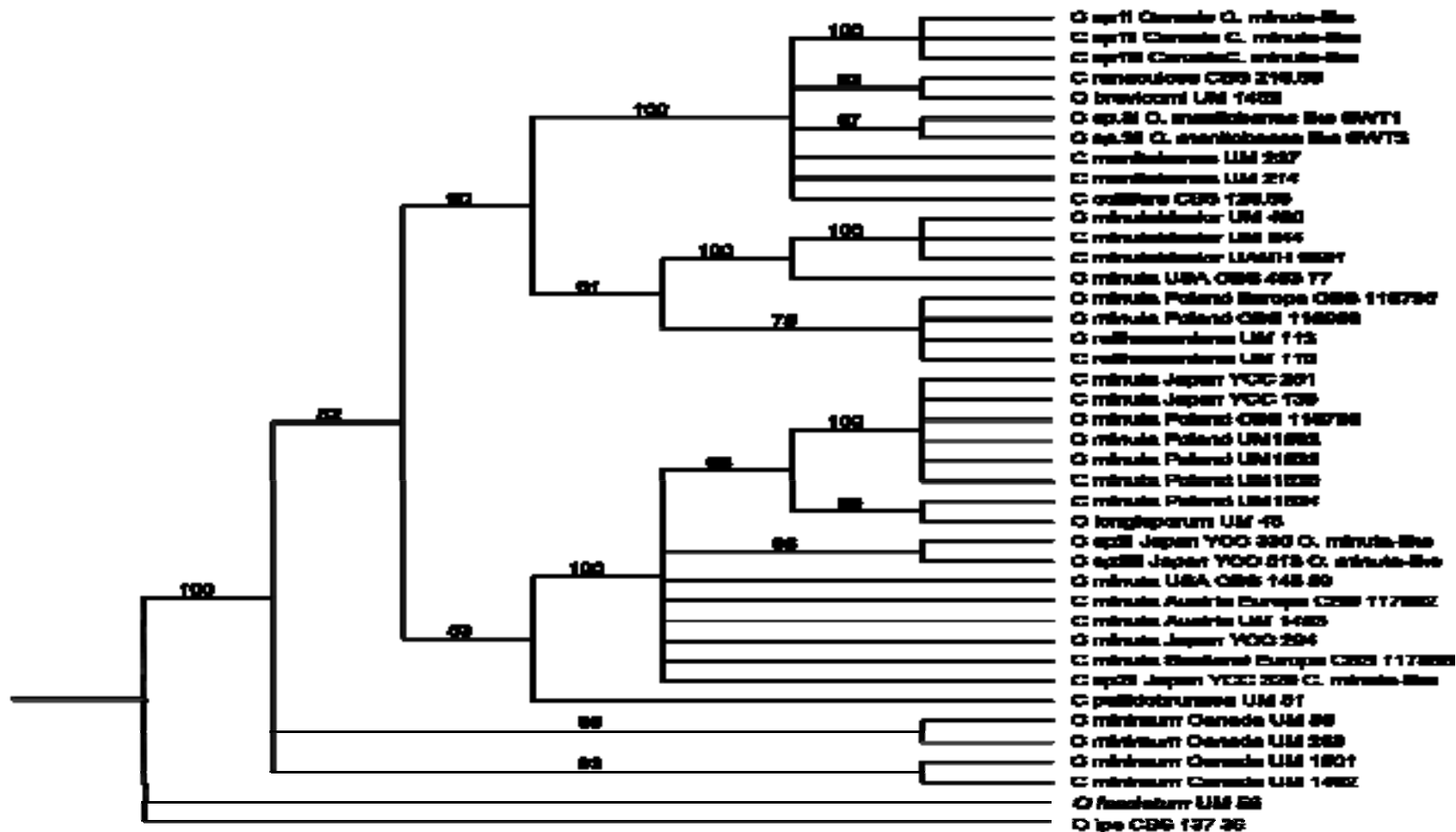
Figure 4.5. Phylogram of *Ceratocystiopsis minuta* and associated species using only sequences from the LSU region. Support values are from Bayesian analysis.
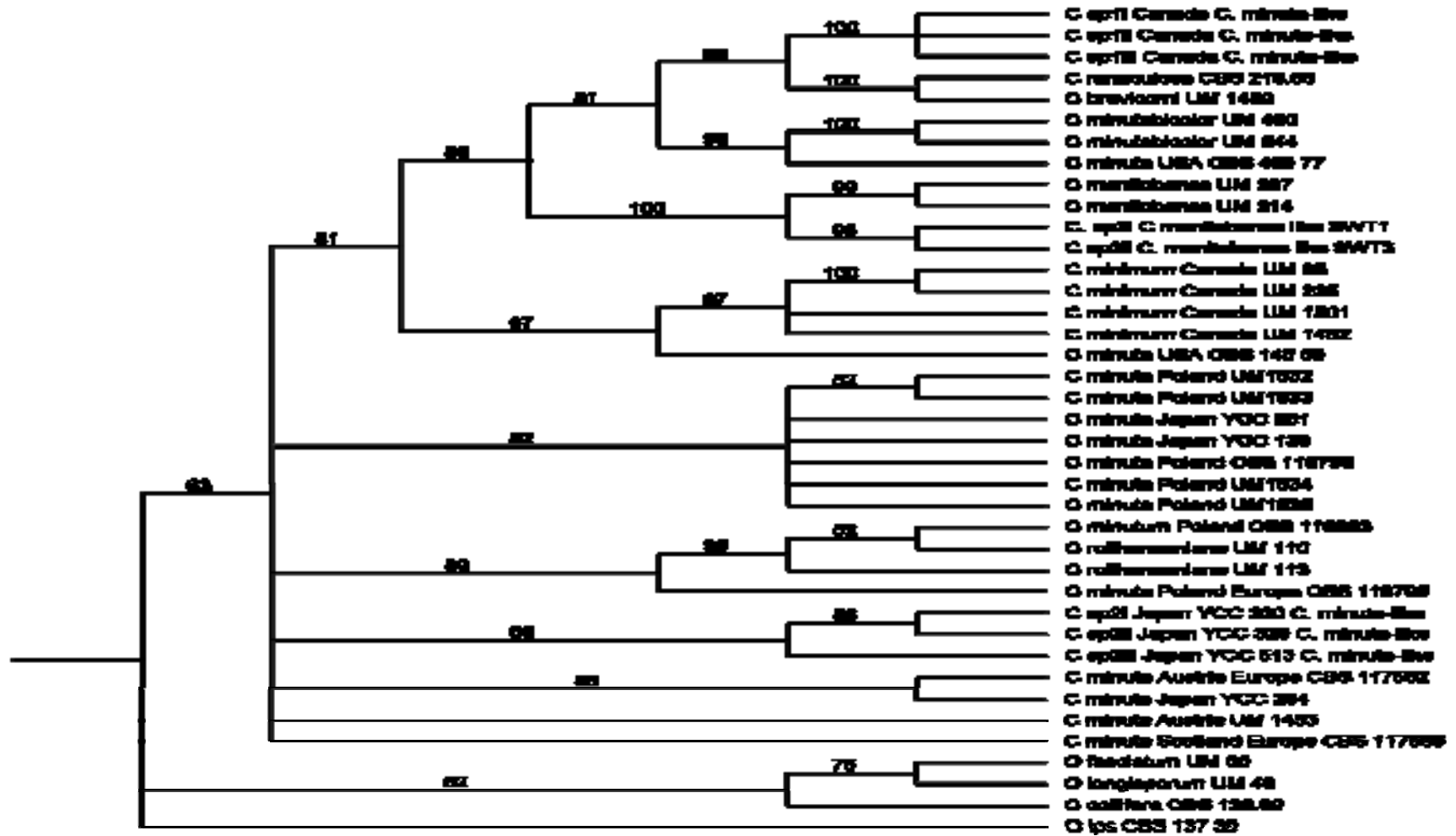
Figure 4.6. Phylogram of *Ceratocystiopsis minuta* and associated species using only sequences from the βT region. Support values are from Bayesian analysis.
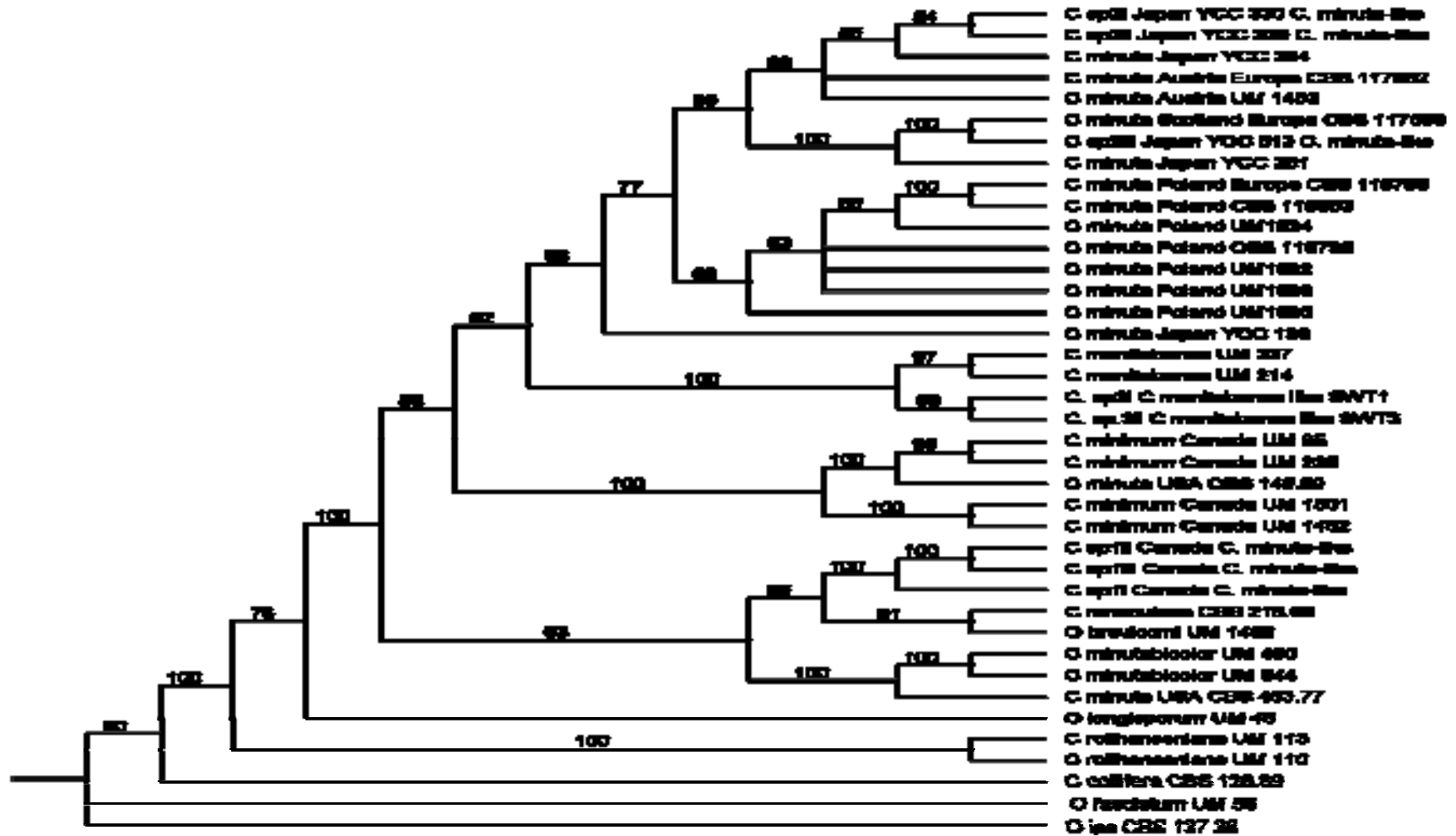
Figure 4.7. Phylogram of *Ceratocystiopsis minuta* and associated species using only sequences from the ITS region. Support values are from Bayesian analysis.

Chapter 5

Concluding chapter

The mountain pine beetle is associated with a diverse number of fungal associates (Kim et al. 2005). The exact roles of most of these fungi are either unknown or not fully understood. The work presented by this thesis improves the knowledge base of two fungal associates of the mountain pine beetle, *Grosmannia clavigera* and *Ceratocystiopsis* sp. 1, referred to in previous literature as *Ceratocystiopsis minuta*.

Aspects relating to the pathogenicity of *Grosmannia clavigera* were examined and described in chapters 2 and 3. The ability to evaluate pathogenicity in an efficient, time-saving manner is important when assessing a large number of specimens on different hosts. The alternating flap technique (AFT) presented in chapter 2 may be such a methodology that could be used when new beetle outbreaks occur. The strength of this technique is that it allows for the rapid inoculation of trees to quickly answer questions regarding the pathogenic effects of fungi. This is beneficial when fungi encounter new potential hosts, new environmental conditions or when new fungi are discovered. However, the applicability of this test to other fungal systems has not yet been demonstrated. It is uncertain if the AFT will produce similar results if other pathogenic fungi are used. In my work, the specific testing of the AFT to the cork borer method also suffered from lack of consistency between years. Inoculations using the cork borer method and the AFT occurred during alternate years. This is a weakness because it is uncertain how comparable the results are, given that

environmental factors were not consistent between years. Future work involving the AFT should aim to compare results during the same year between the AFT and the cork borer method. Subsequent work should examine the applicability of the test outside the MPB-*Grosmannia clavigera*-lodgepole pine system to see if similar results are obtainable in other bark beetle-fungal systems.

Because the AFT has not been rigorously tested within the field, the cork borer method was used to evaluate the variation in pathogenic symptom development in young lodgepole pine trees inoculated with *Grosmannia clavigera* (Chapter 3). Because of the consistency in results within years and the extensive statistical tests performed, the data are robust and provides compelling evidence of intraspecific variation existing among isolates of *G. clavigera*. This has not been demonstrated previously and has only been shown in a couple of other bark beetle-fungal systems. The results could be improved by using more isolates and the same time period of incubation. For future studies in pathogenicity, researchers should be aware that the period of incubation and number of isolates tested may affect their results. Results from my tests are based on younger trees; it is uncertain how results can be applied to economically valuable mature trees. However, symptom development has typically occurred at a faster rate in older trees. Although not conclusively proven, we may speculate that this would be the case in the given *G. clavigera*-lodgepole system. Both B5 and KW 1407 developed much longer lesions in mature trees compared to young trees in the same time frame.

The results drawn from the conclusions in chapter 3 lead to interesting and speculative questions. In other bark beetle systems, low level inoculations of fungi have induced tree defense responses, leading to a larger survival percentage in trees subsequently attacked by beetles. If KW 1407 is less pathogenic than other isolates, would not KW 1407 make a useful candidate for testing this question in the MPB-lodgepole pine system? Using more pathogenic isolates may inadvertently kill the tree, but KW 1407 seems incapable of killing mature trees as well as young trees when inoculated at 200 cork borer inoculations/m^2. It is also interesting to search for genetic reasons underlying phenotypic variation. The complete genome of KW 1407 is currently being sequenced. While this may be useful for searching for genes related to pathogenicity, it would be even more useful if the genome of ATCC 18086 was sequenced afterwards for comparative purposes. Differences in putative pathogenicity genes may be better answered when comparing more pathogenic to less pathogenic isolates. This may help us to link ecological observations with genetic fundamentals.

While it is known that *Grosmannia clavigera* is pathogenic, we are ecologically unsure of the role of *Ceratocystiopsis* sp. 1. However, before more specific questions are addressed, it is fundamentally important first to define the species being tested. Initially, the work presented in chapter 4 was a means of placing *C.* sp. 1 phylogenetically within the *Ceratocystiopsis* genus. However, the inconsistencies within the genus lead to the search for a suitable epitype of *C. minuta*. Future work within the genus *Ceratocystiopsis* will greatly benefit from

having an epitype and problematic isolates identified. Chapter 4 focuses on the phylogenetics and historical inconsistencies in the genus *Ceratocystiopsis*. Since the work was originally conducted to differentiate *C.* sp. 1 from *C. minuta*, morphological work on the anamorphs of selects isolates of *C. minuta* and of *C.* sp. 1 will be conducted to help formally describe *C.* sp. 1. The sensitivity of these isolates to cycloheximide will also be examined. The completed manuscript should contain all the historical and phylogenetic information presented in chapter 4 as well as formally present *C.* sp. 1 as a new species. Subsequent to this, ecological work can begin. As mentioned in chapter 1, there is speculation that *C.* sp. 1 may be nutritionally beneficial to the MPB. Understanding its ecological role may help us understand why it is so often found in association with the MPB.

Future research will benefit from the inferences drawn from the chapters in this thesis. During subsequent beetle epidemics, researchers will have at their disposal a technique to potentially rapidly evaluate the pathogenic status of fungi. It should now be clear that variation likely exists and tests of pathogenicity should seek to examine as many isolates of the same species as possible. *C.* sp. 1 is likely not pathogenic since it grows very slowly. This species has also proven very useful for aiding to point out the taxonomic problems associated with the genus *Ceratocystiopsis*. In the future, work within this genus will benefit greatly from using a consistent isolate of the type species and from avoiding potentially erroneously labeled isolates of *C.minuta*.

References

Kim JJ, Allen EA, Humble LM, Breuil C (2005). Ophiostomatoid and
basidiomycetous fungi associated with green, red, and grey lodgepole
pines after mountain pine beetle infestation. Canadian Journal of Forest
Research **35**: 274-284.