CHANGES IN FUNGAL ASSOCIATE ABUNDANCE OVER MOUNTAIN PINE BEETLE LIFECYCLE USING TARGET-SPECIFIC PRIMERS AND QUANTITATIVE PCR

by

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Abstract

The mountain pine beetle (MPB) is a native bark beetle of western North America that attacks pine tree species, in particular, lodgepole pine. It is closely associated several ophiostomatoid fungi, with which it has a mutually beneficial relationship.

This thesis is comprised of two sections. The first objective was to develop target-specific PCR primers that could identify the major fungal species associated with MPB: the pathogenic Grosmannia clavigera and Leptographium longiclavatum, the less pathogenic Ophiostoma montium, and an un-described Ceratocystiopsis species (Cop. sp.1). Growing, isolating and extracting DNA from fungi vectored by MPB can be time and labour intensive, and these associates can be difficult to differentiate morphologically. I designed three rDNA primer sets that specifically amplify short rDNA amplicons from O. montium, Cop. sp.1. and the pine Leptographium clade (i.e. G. clavigera and L. longiclavatum). I also designed two primer sets, from another gene, that can differentiate G. clavigera and L. longiclavatum. The primers reliably identify their targets from DNA obtained from pure fungal cultures, pulverized beetles, beetle galleries, and tree phloem inoculated with G. clavigera.

The second objective was to use these target-specific primers in conjunction with qPCR to compare the relative abundance of MPB fungal associates during the beetle life cycle. To determine the changes in relative abundance of the fungal species, MPB galleries were sampled at four phases in the beetle life cycle: eggs, larvae, pupae and teneral adults. Multivariate analysis of covariance indicated that changes in the relative abundance of the fungi over the lifecycle of the MPB were statistically significant. Univariate analysis of covariance showed a statistically significant difference in the abundance of Cop. sp.1 through the lifecycle, and pairwise analysis showed that the difference occurs after the larval phase. The staining fungi O. montium and the Leptographium species did not change significantly through the MPB lifecycle.

The work described in this thesis contributes to our understanding of the interactions between the MPB and its fungal associates and provides a tool for further studies that require rapid detection, identification and quantification of MPB fungal associates.
Preface

A version of Chapter 2 has been published:


The authors contributed in the following ways:

Lily Khadempour designed the methods and carried them out, analyzed the data and compiled it and wrote and compiled the manuscript.

Sepideh Massoumi Alamouti provided DNA sequences for designing target-specific primers, helped select isolates to test and helped with revising the manuscript.

Richard Hamelin helped with initial project conception and helped revise the manuscript.

Jörg Bohlmann helped with initial project conception and helped revise the manuscript.

Colette Breuil helped with initial project conception, helped with designing the methodology, and contributed to the manuscript both in writing and in revising.

A version of Chapter 3 is being submitted for publication. The authors contributed in the following ways:

Lily Khadempour conceived of the idea, developed the methodology, carried it out, analyzed the data and wrote the manuscript.

Valerie LeMay helped analyze the data and reviewed the manuscript.

David Jack helped design the experiment, was responsible for fieldwork, helped with lab work and he reviewed the manuscript.

Jörg Bohlmann obtained funding for the project and revised the manuscript.

Colette Breuil obtained funding for the project, advised, helped to design the experiment and helped with writing and reviewing the manuscript.
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Chapter 1: Introduction

The mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) is a native bark beetle that infests pine species in western North America. In outbreaks or epidemics, the beetle and its associated microflora are major forest pests that attack and kill healthy living trees, particularly lodgepole pine (*Pinus contorta* Doug. var. *latifolia* Engelm.). The current MPB epidemic in British Columbia (BC), Canada has affected an estimated 17.5 million hectares of pine forest (BC Ministry of Forests, 2011a). Like many other bark beetles, the MPB is associated with diverse organisms that include nematodes, mites, bacteria, yeasts and filamentous fungi. Some of the associated micro-organisms have a symbiotic relationship with the beetle, while others are generalists that can be found in diverse ecosystems. The fungi that are specific and symbiotic are usually ophiostomatoids. Ophiostomatoid fungi have been described since the early 1900’s, and a number of methods have been used to characterize them as species associated with insects or host plants or trees (Wingfield et al. 1993). In the introduction of my thesis, I will provide some background on this ecosystem and will report previous work on MPB-associated fungi. I will also describe the methods that have been used to study the ophiostomatoid fungi. However, many of these methods are not necessarily appropriate to carry out large-scale ecological work. The objectives of the thesis are: 1) to develop a method to efficiently characterize MPB fungal associates (Chapter 2) and 2) to use the developed method in an ecological study to identify and quantify the range of associated fungi through the MPB lifecycle (Chapter 3). The final chapter summarizes the major findings and recommends further research into the relative abundance of the MPB fungal associates over different time and space scales.

1.1 The MPB ecosystem

As noted, MPB occurs in lodgepole pine and other pine ecosystems, in both pure and mixed-species, primarily pine, stands (Pedersen 2004; Kim et al. 2005; McFarlane and Stumpf-Allen 2006). Climate (i.e., seasonal temperature and precipitation) and abundance of susceptible host trees are important factors in the distribution and size of the beetle population (Safranyik 1978). During endemic phases that can last for many decades, the number of beetles is low with fewer than 10 trees per ha attacked. Commonly, MPB affects mature pine trees (i.e. ~ 80 years or older), colonizing weakened or dying trees that have been attacked by other beetles (Parker and Clancy 2006); thus, this assemblage of beetles participates in removing non-sound trees from
healthy forests (Safranyik et al. 2010). Fungal associates are commonly found with MPB. During outbreaks or epidemic phases that can last 5 to 20 years, MPBs again target preferred mature lodgepole pine trees and, with fungal associates, can kill healthy individuals causing widespread tree death (Cole and Cahill 1976; Amman et al. 1990; Elkin and Reid 2004; Stahl and Moore 2006). The current epidemic in BC started in the early 1990’s in the west central region of the province and spread eastward, while small endemic populations in southern BC, simultaneously expanded, and these two populations coalesced (Aukema et al. 2006). While it is the largest MPB epidemic reported in western Canada, during the past century there have been at least four major MPB outbreaks in BC (Taylor and Carroll 2004).

1.1.1 Pine forests and MPB impacts in BC

As noted, lodgepole pine is the primary species attacked by MPB, although MPB has attacked 22 different pine species with varying levels of success (Safranyik et al. 2010). Lodgepole pine grows in a wide range of environments from mid-elevation to sub-alpine sites and across a wide climatic range from Alaska in the US through BC to northern California, with a small isolated population in southern US, extending to San Bernardino (Massoumi Alamouti et al. 2011). Lodgepole pine has adapted to regions with frequent stand-replacing fires. The species burns easily and has serotinous cones that require fire or very hot temperatures to open. Relative to other northern temperate forest species, lodgepole pine has a faster growth rate; it is also shade intolerant and is one of the first colonizers after a major stand-replacing disturbance, particularly fire.

All pine species have developed anatomical structures for producing, transporting, and storing defence chemicals that can be deployed against a broad range of insects and fungal pathogens. This defence system is composed of specialized polyphenolic parenchyma cells localized in the phloem that synthesize and store phenolic compounds (Franceschi et al. 2005), and possesses a complex resin duct and blister system, which stores preformed or induced oleoresin (Keeling and Bohlmann 2006). Oleoresin is mainly composed of monoterpenes, diterpenes and sesquiterpenes (Trapp and Croteau 2001). Preformed defence compounds protect host tissues from primary colonizers. These chemicals are the first obstacle encountered by insects including MPB. In
particular, oleoresins are released when the beetle bores an entrance hole and the flow of oleoresin can drown a beetle and stop an attack. Then, trees further respond by producing additional resins (induced oleoresins), and may survive unless attacked by a host of beetles.

Forest products accounted for approximately 40% of BC’s exports (Baxter and Ramlo 2002). Five pine species are present in interior BC: lodgepole and the related variety shore pine (*Pinus contorta* Dougl. ex Loud var. *contorta*), ponderosa pine (*Pinus ponderosa* Laws), western white pine (*Pinus monticola* Dougl. ex D. Don), whitebark (*Pinus albicaulis* Engelm.) and limber (*Pinus flexilis* James). However, lodgepole pine stands is the most abundant pine by area in BC. A combination of forest fire suppression and longer periods between harvests (i.e., 80 or more years) resulted in an increase in the area of mature lodgepole pine stands over the last 90 years. Further, in the past, large areas were left unharvested because of forecasted low economic return in harvesting lodgepole pine relative to other species (Wagner et al. 2007). In 2004, 65% of timber harvested in the Lakes, Quesnel and Prince George timber supply areas of BC was lodgepole pine (Bunnell et al. 2004). Pulp, lumber, poles, plywood, and railroad ties have all been produced from lodgepole pine and used in paper-making and as construction material and furniture. Even with harvest of lodgepole pine in these timber supply areas, the susceptibility of the species to MPB and the remaining large areas of mature trees resulting from past management provided an optimal environment for the current MPB outbreak. As well as having a large impact on the forest product industry, the current MPB epidemic has had a large impact on environmental services including water and carbon sequestration through loss of trees and on the tourism industry through changes in forest views. By 2013, 80% of the lodgepole pines in BC are expected to be killed by MPB resulting in severe economic losses.

1.1.2 MPB life cycle and characteristics

Bark beetles and their fungal associates have been recognized as the most economically and ecologically damaging pests of forests in the northern hemisphere; they have inhabited conifer hosts since at least the Mesozoic era (Seybold et al. 2000). Bark beetles are members of the family Curculionidae (subfamily Scolytinae), within the order Coleoptera (Farrell et al. 2001). Among these beetles, the native North American MPB lives under the bark of lodgepole pine trees, whereas the related Jeffrey pine beetles (JPB; *Dendroctonus jeffreyi* Hopk.) live under the
bark of Jeffrey pine (*Pinus jeffreyi* Hopk.) trees (Six and Paine 1997). MPB is found in the same range as lodgepole pine in Canada and US, but also extends to other pine species as far east as South Dakota, US, while JPB is restricted to the range of Jeffrey pine, mainly from southwest Oregon, US through California, US. During endemic phases, most bark beetles colonize weak or dying trees, but may kill healthy trees during outbreaks, as noted for MPB.

These small black MPB beetles of ~ 5-7 mm long attack the lower two thirds of the tree trunk, usually in the summer (July to September) when the trees are often under stress due to water deficiency. The pioneer female beetles bore through the bark to the cambium and attract males and other females by emitting pheromones. When into the tree, parental beetles move upwards and produce a long and narrow vertical tunnel called an egg gallery. Each female lays ~ 60-80 eggs, enabling populations to expend quickly. Both sexes of MPB are associated with a diversity of fungi that are introduced into the tree during their attack. The MPB is mycophagous (Harrington 2005), as well as phytophagous (Berryman 1989). Before emerging, newly adult beetles feed on fungi that line the pupal chamber. The adult beetles bore exit holes and fly out to find another suitable host. The number of beetles emerging from a single tree is large enough to attack 15 new trees and fungi associated with the beetle are dispersed. As beetles attack the trees, the cycle is repeated with each generation generally taking one year. The beetles can be potentially dispersed over great distances (30 to 100 kilometres or more) when transported by wind currents (Safranyik et al. 2010).

At the onset of a successful beetle attack, the tree foliage becomes discoloured to yellow and then red in the first and second year after attack, as leaves are deprived of nutrients. Foliage then dies two to three years post-attack, resulting in what is termed “grey-attack”. Pitch tubes, visible symptoms of MPB attack, are solidified resin around beetle-attacked spots on the bark. The infested trees will also show boring dust in bark crevices and around their base. Once a tree has been infested with MPB, it becomes vulnerable to infestation by secondary beetles such as *Ips* and ambrosia species.
1.1.3 Fungal associates of the mountain pine beetle

Bark beetles are commonly associated with different organisms, including nematodes, mites, yeasts, and ascomycete and basidiomycete fungi (Harrington 2005). Ascomycete ophiostomatoids are the beetles’ most frequent fungal associates (Wingfield et al. 1993). Some of the ophiostomatoid associates have specific relationships with their beetle vectors and are restricted to one beetle species (e.g. _Grosmannia clavigera_ an MPB associate, or _Leptographium fruticetum_ an _Ips perturatus_ associate) (Six and Paine 1999; Massoumi Alamouti et al. 2007). Other ophiostomatoid associates are generalists (e.g. _Ophiostoma bicolor_ or _Leptographium abietinum_ on _Ips_ spp. and _D. affaber_) and are found with a variety of beetle species (Massoumi Alamouti et al. 2007). Past research on MPB fungal associates has focused mainly on the ophiostomatoid fungi. When these fungi colonize the phloem and sapwood they produce the pigment melanin that gives a dark blue colour to wood. The pigment is found in the fungal hyphae and in the fruiting body where it protects the sexual spores. Although stained wood products do not have lower strength qualities than regular wood, their properties result in higher processing costs and the end product has lower value due to customer preference for unstained wood (Byrne et al. 2007). As well, the presence of stain fungi in wood can result in phytosanitary and quarantine consequences.

The MPB ophiostomatoid fungi include species from the genera _Grosmannia, Ophiostoma_ and _Ceratocystopsis_. These fungi reproduce both sexually and asexually and their asexual and sexual spores (conidia and ascospores) are produced in slimy masses that are easily dispersed by insects (Harrington 1993a). The three species isolated most frequently from beetle bodies (mycangia, gut and exoskeleton) and stained wood include two pathogenic species and a weak pathogen. The pathogens are: a) _Grosmannia clavigera_ (Zipfel et al. 2006) and b) _Leptographium longiclavatum_ (Lee et al. 2005). The weak pathogen is _Ophiostoma montium_ (Rumbold 1941; Whitney and Farris 1970; Six 2003a; Lee et al. 2006a). Another associate, _Ceratocystiopsis_ sp.1 (Cop. sp.1), is a slow-growing, white (i.e. non-staining) fungus that preferentially colonizes beetle galleries and rarely colonizes the surface of the sapwood. It resembles _Cop. minuta_ from MPB-infested lodgepole pine described in 1960 (Robinson 1962; Upadhyay 1981; Plattner et al. 2009). These four fungal species are specifically associated with MPB and have not been
isolated from other beetles, even when the diverse beetles inhabit the same tree. MPB fungal associates from the current epidemic have been extensively sampled in BC by the University of British Columbia (UBC) research group headed by Professor Colette Breuil (hereafter termed the UBC research group) between 2001-2003 (Kim et al. 2005; Lee et al. 2006a; Plattner 2008) and more recently in Alberta and the Rocky Mountain area by Roe et al. (2011).

1.1.3.1 *Grosmannia clavigera*

*Grosmannia clavigera* is the most thoroughly studied MPB fungal associate. This fungus was described as a wood-staining and symbiotic associate of MPB that inhabits *Pinus ponderosa*. Initially it was named *Europhium clavigerum* by Robinson-Jeffrey and Davidson (1968), then was renamed *Ophiostoma clavigerum* (Rob.-Jeffr. & R.W. Davidson) T.C. Harr. in 1987, and recently it was moved to the genus *Grosmannia* under the name *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. (Zipfel et al. 2006). Since the early 1960’s, substantial information has been generated on the morphology, physiology and ecology of this fungal species. Studies have shown that *G. clavigera* can overcome pine tree defences (i.e. terpenoids and phenolic compounds) of mature and young trees (e.g. 60-80 and 20 years old, respectively) and can kill these trees when inoculated at high enough inoculation densities (Yamaoka et al. 1995; Plattner et al. 2008). The relationship between MPB and *G. clavigera* is mutualistic and highly specific. The beetle not only vectors the fungus between different trees, but also maintains and protects the fungal spores in specialized structures, the mycangia (Six 2003b). *G. clavigera* has been isolated only from MPB and the related species JPB, and has not been shown to be associated with any other bark beetles (Six and Paine 1997). *G. clavigera* produces both asexual and sexual fruiting bodies; however, it seems to grow in the beetle gallery and in the wood as a haploid anamorph. The sexual phase has rarely been found (Robinson-Jeffrey and Davidson 1968; Lee et al. 2003) and cannot be obtained on artificial media in laboratory. Even when observed in nature, the fruiting bodies are only found after the MPB has left the tree. In the absence of sexual fruiting bodies (perithecia) the morphological identification is difficult, since the identification is based on variable anamorph structures and a broad range of conidia sizes and shapes. This fungal species grows well on solid media in a low oxygen environment at 20-22°C. Recently, the genome of *G. clavigera* has been sequenced (DiGuistini
et al. 2009) and the information generated is being used to understand how the fungus bypasses the tree defences, detoxifies the terpenoids and uses some of them as carbon sources (DiGuistini 2010). Using AFLP, population studies on *G. clavigera* have indicated that its genetic variability is low (Lee et al., 2007). However, using genomic resources, Massoumi Alamouti et al. (2011) showed that this species consists of two different lineages or species. One of the species has been isolated from JPB and also from MPB that colonized localized populations of ponderosa pine in the US; this species is genetically similar to the holotype described by Robinson-Jeffrey and Davidson (1968), and should retain the name *G. clavigera*. The other, which was isolated from MPB in the epidemic areas in BC and Alberta, Canada, and in the US, should be described as a new species. In this thesis, I mainly report work from this unnamed species, but I continue to refer to it as *G. clavigera*, since a version of Chapter 2 of this thesis was published before the paper by Massoumi Alamouti et al. (2011).

1.1.3.2 *Leptographium longiclavatum*

*Leptographium longiclavatum* is a close relative of *G. clavigera*. Because of these morphological, physiological and genetics similarities, it was only recently described (Lee et al. 2005). Like *G. clavigera*, *L. longiclavatum* is a blue staining and pathogenic fungus. While *G. clavigera* has both oval and clavate spores, *L. longiclavatum* only has long, clavate spores. Despite this difference, differentiating the species morphologically is unreliable. It is also difficult to differentiate these species through DNA sequencing. They differ by only two base pairs in the ITS2 LSU region of rDNA (Tsui et al. 2010). Therefore, in order to obtain reliable identification of these species, it has traditionally been necessary to sequence several genes.

1.1.3.3 *Ophiostoma montium*

*Ophiostoma montium* is frequently isolated from the MPB and associated attacked lodgepole pine trees (Kim et al. 2005; Lee et al. 2006a). It is usually isolated from galleries and beetle bodies more frequently than *G. clavigera* but less from the beetle mycangia (Six 2003b). While it is a staining fungus, it grows more slowly than *G. clavigera* or *L. longiclavatum*, and its pathogenicity to trees has not been clearly shown. It has been suggested that *O. montium* prefers higher growth temperatures than *G. clavigera* (Six and Bentz 2007; Rice and Thormann 2008). *O.*
montium has long been confused with a close relative, *O. ips* (Kim et al. 2003), which is a generalist fungus associated with several species of bark beetles (Six and Bentz 2007).

1.1.3.4 *Ceratocystiopsis* sp.1

As with the other fungi described here, *Cop.* sp.1 belongs to the ophiostomatoid fungi, but is mainly found on beetle bodies and in the beetle galleries and does not grow in or stain sapwood. The fungus is white, fluffy and grows very slowly in artificial media. It lines a beetle gallery and can be seen mostly in proximity to larvae (Kim et al. 2005). It is suspected that this fungus serves as a nutritional source for the developing larvae (Plattner et al. 2009). Because of its slow growth rate and because it does not penetrate and stain the sapwood of infested trees, *Cop.* sp.1 was only recently discovered and differentiated from other similar fungi (Plattner et al. 2009). The species has not been formally described, but it does occur consistently and exclusively in association with the MPB. Phylogenetically, it is a sister species of *Cop.* brevicomi, *Cop.* ranaculosa and *Cop.* collifera (Plattner et al. 2009).

1.1.3.5 Role of the fungi

The different fungal associates likely play different roles in the MPB-lodgepole pine ecosystem. During beetle attack, the fungi may help beetles overcome tree defences by modifying defence chemicals. Because they grow into the parenchyma cells and in the tracheids of the sapwood, they block the transport of water to the crown of the tree. By reducing the moisture content of phloem and sapwood they help the beetles to reproduce successfully. As noted, some fungal species may also serve as a food source for the beetle as the beetles have often been seen consuming fungal spores and mycelium (Craighead 1928; Caird 1935; Paine 1984; Paine et al. 1997; Hsiau and Harrington 2003; Adams and Six 2007). The fungi may supplement the nutrients of the beetle brood by providing ergosterol (Bentz and Six 2006) or increasing accessibility of nitrogen (Bleiker and Six 2007) in the beetle galleries.

*G. clavigera* and *L. longiclavatum* are both pathogenic fungi in the *Leptographium* clade; when they are inoculated into lodgepole pine at high enough concentrations, they can kill the trees without participation from the beetle (Yamaoka et al. 1995; Lee, Kim, and Breuil 2006b). The two species are closely related and have similar morphological characteristics. Both grow quickly
into the phloem and the sapwood of living trees. They produce a dark pigment, melanin, which stains the wood and reduces its commercial value. These pathogenic fungi may facilitate the colonization of the tree by the beetle and even by other fungi as they may breakdown tree defences and change the growth environment. Six and Wingfield (2011) challenged the original paradigm regarding the role that virulent/pathogenic fungi play in helping bark beetles colonizing healthy trees. They suggested that generally, pathogenic fungi do not always play a role in helping bark beetles to colonize trees. For MPB, the consistent association of the beetle with some fungal species that are highly pathogenic is strong evidence in support of their role as symbionts. It is possible that rather than helping the beetle in killing the tree, these fungi play a role in modifying the immediate environment of the beetle gallery by reducing the toxic defence chemicals and volumes of sap that the tree produces (Klepzig et al. 2009). Whether on a large scale (whole tree) or small scale (beetle gallery), it is becoming evident that these fungi participate in detoxifying tree defence chemicals (Paine et al. 1997; DiGuistini et al. 2011).

1.2 Molecular methods for identifying and detecting ophiostomatoid fungi

1.2.1 Identification problems associated with the ophiostomatoid fungi

The ophiostomatoid fungi are generally difficult to differentiate based on morphology. There are several examples pertaining to the MPB associates to show this difficulty. *L. longiclavatum* was only recently discovered (Lee et al. 2005) due to its morphological and genetic similarities to *G. clavigera*. It is possible that some researchers are still not differentiating the two fungi and simply report *L. longiclavatum* as *G. clavigera*, since the latter is more well-known. *O. montium* and a closely-related generalist fungus *O. ips* were also frequently misidentified (Kim et al. 2003). The MPB-associated *Cop. sp.1* has not been formally identified but until recently had been grouped incorrectly with other related fungi (Plattner et al. 2009). Due to these difficulties, various molecular methods have been used to study these fungi.

1.2.2 Sequencing

The initial molecular systematics of the ophiostomatoid fungi has mainly used the ITS2 and partial LSU regions of the ribosomal DNA (rDNA) (Hausner et al. 2000). Early sequencing relied heavily on rDNA because it is a multicopy region of DNA, which is easy to amplify. As
well, it contains regions that are relatively conserved and variable, which are used for identifying
distantly related and closely related fungi, respectively. Using this approach, a large number of
sequences were generated for the genera *Ophiostoma*, *Grosmannia*, *Ceratocystiopsis* and
*Ceratocystis* representing approximately 140 ophiostomatoid species. However, it has been
shown that rDNA was not appropriate to differentiate a number of closely related species that
infest pine (e.g. *O. robustum*, *O. aureum*, *L. terebrantis* and *L. pyrinum*) (Lim et al. 2004).

To improve rDNA phylogenies, which do not always provide enough resolution for the diagnosis
of ophiostomatoid fungi, protein-coding genes, like β-tubulin, actin and elongation factor-1α are
used alone or in combination. Similar to rDNA, protein-coding genes contain non-functional
introns and functional exons that vary in their rate of evolution. These genes have improved the
taxonomic resolution. Therefore, β-tubulin is more effective than rDNA for identifying closely
related species and for determining their evolutionary relationships (Lim et al. 2004). If time and
resources permit, DNA sequencing provides a reliable method for identifying ophiostomatoid
fungi (Zipfel et al. 2006). However, the process can be difficult and is not a method that can be
used to quickly identify an unknown species or detect it in its environment.

1.2.3 **Target-specific primer sets**

When species of interest are known and sufficient DNA sequence information is available,
target-specific primers can be designed to identify species isolated on specific media or directly
from environmental samples. This approach has been used widely to detect pathogenic fungi
from soil, wood, insects and nematodes, for example (Luchi et al. 2005; Guzman-Franco et al.
2008; Kelly et al. 2008; Sampietro et al. 2010). The major difficulty with this method is
obtaining DNA that is free from contaminants that can affect PCR or degrade the DNA template.
Further, some fungi (e.g. ophiostomatoid) have cell walls that are difficult to break down and the
DNA extraction protocol must be optimized with both mechanical and enzymatic steps.

Designing target-specific primer sets involves selecting genes, then aligning the gene sequences
from the target species with closely related species and others that may co-occur in particular
ecosystems. Regions with unique sequences for the targeted species are selected from the
sequence alignments, and primers are designed for these sequences. The primers are then
thoroughly tested to determine their effectiveness for specifically detecting only the targeted fungal species, and no other organisms that are potentially present in the ecosystem examined. Generally, target-specific primers are designed from rDNA or a few other genes whose gene sequences are available from a large number of species. However, genes need to show enough variation in order to be useful for differentiating closely related species.

Schweigkofler (2005) used this method for detecting *L. waganeri*, a fungus also associated with bark beetles. They designed an rDNA primer set that was specific to the *L. wageneri* species complex. While they intended to study several isolate variants in the complex, the probe designed from rDNA was not sufficiently specific. Consequently, they designed primers for the whole complex rather than for each variant. Detecting fungi from DNA extracted directly from environmental samples using target-specific primers is more efficient than isolating, purifying and identifying fungi using morphology and DNA sequencing. As such, many samples can be tested with little cost and expertise.

1.2.4 Quantitative PCR

Quantitative or real-time PCR (qPCR) methods use fluorescent dyes to measure the concentration of an amplified segment of DNA as a reaction proceeds (Heid et al. 1996). Several technologies are available for using fluorescent probes that bind to specific regions of DNA. These probes can increase the specificity of a reaction but they cost more and are relatively difficult to design. Other dyes, such as SYBR green and EVA green, bind to any double stranded DNA. The fluorescence is measured at the end of each PCR cycle, allowing tracking the amount of double stranded DNA produced. When compared to a standard curve of known DNA concentration, the concentration of the starting material in a sample can be calculated. This method can be used with target-specific primers in order to measure the amount of a particular species in an environmental sample. For efficient qPCR reactions, the region amplified should be short, i.e. less than 300 base pairs, to optimize fluorescence. Even if the end goal is only detection not quantification, qPCR is useful since it does not require that the PCR product be detected through an electrophoresis gel. In contrast to PCR, qPCR detects lower concentrations of DNA, identifies whether samples contain more than one species, and provides data more rapidly.
Currently, little work has been done to detect and quantify ophiostomatoid fungi. Schweigkofler et al., (2005) used SYBR Green for detecting and quantifying *L. wageneri* associated with bark beetles. They were able to quantify the spore load from this complex on beetle bodies. This was accomplished by correlating their qPCR results with those obtained from a known serial dilution of spores on beetle bodies. Using this method, they concluded that one bark beetle species was the preferred vector for this fungal tree pathogen. Wu et al. (2009) used rDNA target-specific primers and qPCR to detect *Ceratocystis fagacearum* from inoculated soil and wood samples. They have not yet designed a method to successfully detect the fungus naturally occurring in environmental samples.

### 1.2.5 Metagenomics

All of the methods described require some amount of culturing. Some micro-organisms cannot be cultured, however, and this limits our ability to fully capture the dynamics of complex ecosystems with a high diversity of micro-organisms. New sequencing technologies allow the development of high throughput sequencing projects using DNA extracted directly from ecosystems. These methods fall under the umbrella of metagenomics, or the genetic analysis of an assemblage of organisms.

One metagenomic method involves cloning and sequencing particular segments of DNA from the total genomic DNA in a sample. Typically, the 16S rDNA is sequenced, as it is a good marker for differentiating bacteria, which often do not grow in artificial media. The UBC research group assessed this approach using 454 pyrosequencing (GS FLX) for characterizing the diversity of micro-organisms associated with MPB (Khadempour et al. 2010a). The UBC research group found all the fungal associates reported in the literature, as well as additional fungi that had not been reported; in addition, the group detected some as nematodes and mites. Most surprising was the quantity and diversity of the associated yeast species. This high diversity would have been difficult to discover by any other method since yeasts are morphologically similar, but genetically and functionally yeast species associated with bark beetles are very different (Kurtzman and Robnett 1998). This diversity suggests that yeasts may play important roles in the interactive micro-organism-beetle-tree system. Because this method works with total extracted DNA, it has less bias than methods that sequence only micro-organisms that can grow.
on artificial media (Eisen 2007). However, this method still has some bias because sequencing of a single gene does not accurately represent the genetic diversity of the microflora.

A more holistic approach involves sequencing total DNA in a sample, rather than targeting a single gene or DNA segment. With this approach, random shotgun sequencing is used. Several groups have performed whole metagenome sequencing on a variety of substrates, including human feces (Breitbart et al. 2003), seawater (Venter et al. 2004) and biofilm (Tyson et al. 2004). In each case, the data showed many previously undescribed micro-organisms in the samples. With this method, much of what is sequenced is identified as hypothetical or unknown, but is still useful as a resource as environmental genomic databases grow (Vieites et al. 2009).

There are drawbacks to metagenomic sequencing. One is the high cost of processing a sample. Another is the amount of data that is produced and must be processed, for which expertise in bioinformatics is required (Eisen 2007). Metagenomic sequencing is already making an impact in several fields, and, as the cost of sequencing decreases and metagenomic analysis becomes more efficient, it will be used to further investigate the microbial community associated with the MPB.
Chapter 2: Target-specific PCR primers can detect and differentiate ophiostomatoid fungi from microbial communities associated with the mountain pine beetle *Dendroctonus ponderosae*

2.1 Introduction

Bark beetles, including the mountain pine beetle (*Dendroctonus ponderosae*, MPB), are associated with a diversity of micro-organisms that the beetles vector when they colonize host trees. Some of these micro-organisms are associated with specific beetle species, while others are associated with multiple vectors (Six 2003a; Kirisits 2004). The majority of filamentous fungi carried by bark beetles belong to a group of wood-colonizing fungi generally called ophiostomatoid fungi. For a few bark beetle ecosystems, these fungi have been isolated on artificial media and identified using morphological and molecular approaches (Harrington 1987; 1993b; Six and Paine 1999). However, these methods are impractical for population and quarantine work when large numbers of sample need to be analyzed.

Most ophiostomatoid associates of MPB are specific to this beetle vector and are rarely isolated from other ecosystems (Lim et al. 2005). Ophiostomatoid fungi represent a diverse group of species that are present in various ecological niches (Wingfield et al. 1993). Among the fungi carried by MPB, those in the *Leptographium* pine clade (Lim et al. 2004), *Grosmannia clavigera* and *Leptographium longiclavatum* are pine pathogens (Yamaoka et al. 1995; Lee et al. 2006b; Plattner et al. 2008) while *Ophiostoma montium* has been shown to be a weak pathogen of pine (Solheim and Krokene 1998). *Ceratocystiopsis* sp.1 (*Cop. sp.1*) from MPB has been reported as *Cop. manitobensis*-like or *O. minuta*. This species is mainly found on beetle bodies and in beetle galleries but does not colonise or stain pine sapwood (Kim et al. 2005; Lee et al. 2006a; Plattner et al. 2009). While these fungal species have been isolated from the exoskeleton and gut of MPB, *G. clavigera* and *L. longiclavatum* have also been found in beetle mycangia (Six 2003a; Lee et al. 2006a). When MPBs attack healthy trees, the fungi may benefit the beetle by modifying toxic tree defence chemicals, reducing sapwood and phloem moisture, making the environment favourable to beetle reproduction and development and providing a source of nutrients to beetle progeny (Whitney 1982; Harrington 1993b; Six 2003a). However, the specific roles of these
fungal species in the MPB-lodgepole pine ecosystem and in the beetle life cycle remain uncertain. To clarify the relative abundance and the roles of the different MPB associates throughout the beetle life cycle in endemic and epidemic landscapes requires identification methods that are specific, rapid and cost effective.

The diversity of fungi associated with MPB has been well established by culturing them on artificial media. Cultures are obtained by spreading washes of the beetle exoskeleton, gut and mycangia and by directly inoculating the medium by streaking the beetle across the media surface (Six 2003b; Lee et al. 2006a). The growing colonies need to be further purified and transferred to new media before morphological characteristics or DNA analyses can be conducted to identify each fungal species. All of these techniques are labour intensive, time consuming, and have other limitations (Harrington 1987; Jacobs et al. 2003; Lee et al. 2003; Lim et al. 2005). For example, isolation on artificial media can underestimate the presence or frequency of a fungal species. Yeasts are often present in high numbers and can prevent or mask the growth of filamentous fungi by competing for space and nutrients or by producing inhibitory metabolites (Luchi et al. 2005; Lee et al. 2006a; unpublished results).

To resolve these limitations and to address high throughput identification that is necessary for ecological and other biological studies, as well as for phytosanitary issues, our objectives were to establish rapid protocols for extracting DNA from fungal cultures and microbial communities from MPB bodies and tree phloem, develop primer sets for identifying the ophiostomatoid fungi associated with MPB and its galleries and to compare the efficacy of the DNA detection method with the traditional technique of isolation and identification. We anticipated that direct detection methods could be used to resolve the diversity of MPB fungal associates in various ecological niches and to rapidly confirm the presence or absence of the fungi in forest products destined for export.
2.2 Materials and methods

2.2.1 Study design and sampling

We compared the traditional isolation and culture technique to direct DNA extraction and PCR amplification for detecting the presence or absence of specific fungi on MPB bodies, beetle galleries and inoculated pine phloem. To assess the robustness of our methodology, the samples were obtained from different locations and were harvested at different times during the year. We also tested the methodology on a more controlled system: lodgepole pine phloem inoculated with a single pathogen, *G. clavigera*.

Twenty MPBs were collected from mature lodgepole pine trees from Bear Service Road near Merritt, British Columbia (BC) that had been baited with Mountain Pine Beetle Tree Bait (Contech Enterprises, Delta BC) and attacked by MPB in the summer of 2009. The trees were harvested in September 2009 and logs were incubated at room temperature in plastic bags for three months until adult beetles emerged. New adults were removed from the incubated logs and used for fungal isolation and direct DNA extraction.

Trees from the same site (Bear Service Road near Merritt, BC) attacked by MPB in the summer of 2009 were harvested during November 2009 and kept at 4°C for two weeks before MPB galleries were sampled. Two adjacent disks of phloem containing MPB galleries were removed with a 7.0 mm diameter cork borer; one disk was used to isolate the fungi on 1% Oxoid malt extract agar (OMEA) while the other was used to extract the DNA directly. In total, there were 10 sample locations. At each location, one disk was for isolation and the other was for direct DNA extraction.

Additional MPB galleries were collected during October 2009 from trees attacked by an endemic population of MPB, in Prosser Creek, California, US. Samples were stored at 4°C for four weeks prior to fungal isolation and DNA extraction. Sampling and isolation were carried out as earlier described. Thirteen sample locations were tested.
Finally, lodgepole pine trees were inoculated at the end of August 2009 with *G. clavigera*, following the method of Lee et al. (2006b). For this test, the inoculation was performed at a density of eight inoculation points per tree, which does not cause tree death. Two months after inoculation with *G. clavigera*, bark pieces including phloem that contained fungal lesions of ~10 cm and the surrounding tissues were collected. To confirm the presence or absence of *G. clavigera*, isolations were carried out with ~1-2mm phloem samples located either inside the lesion or at 1-2 cm outside the lesion edge. From the same bark/phloem samples, additional phloem disks were removed with a 7.0 mm diameter cork borer from both inside and outside the lesions to extract and amplify DNA with *G. clavigera*-specific primers. Ten sample locations from different lodgepole pines inoculated with *G. clavigera*, were tested.

### 2.2.2 Fungal isolation

For MPB gallery isolation, one of the two gallery disks was cut into four pieces, placed on 1% OMEA plates and incubated. Growth of yeasts and filamentous fungi was monitored. Fungi were re-isolated and identified through morphology and DNA characterization, using the specific primers developed in this study. All the fungal isolations and cultures were incubated at ~22°C in the dark.

For microbial isolation from MPBs, two beetles were placed in a 1.5ml microfuge tube with 500µl of 0.01% Tween 20. The beetles were crushed with a pipette tip and vortexted for 10 minutes at medium speed. Then 50µl of the supernatant were serially diluted 10, 100 and 1000 times. 50 µl of each dilution were spread onto 1% OMEA. Colonies of yeast/bacteria and fungi were monitored. Fungal colonies were transferred into new media as soon as hyphal growth occurred. The initial inoculated media plates continued to be monitored for one or two more weeks to allow the growth of the slow growing species; these species were also transferred to new media. The fungi were identified as earlier described.

### 2.2.3 Primer design, testing and PCR conditions

Target-specific primers were designed from rDNA sequences of target species and close relatives that were downloaded from GenBank and aligned using the MUSCLE algorithm in CLC Main Workbench 5 (Edgar 2004; CLC bio, Cambridge MA). Potential primer sites were selected from
the alignment and were tested to minimize self-complementarities using Primer3 v. 0.4.0 (Rozen and Skaletsky 2000).

To further differentiate *G. clavigera* from *L. longiclavatum* which are close relatives, a more specific set of primers was designed within a gene of unknown function (GenBank Accession number EE729832) that we selected from EST libraries (DiGuistini et al. 2007). This gene is currently being used in work to characterize *G. clavigera* populations (Massoumi Alamouti et al. 2011). We found that *G. clavigera* has a deletion of 21 bp in this gene whereas *L. longiclavatum* does not. Primer 3 was used to design specific primers for *L. longiclavatum*; however, while Primer 3 did not provide satisfactory primers for *G. clavigera*, Visual OMP (DNA Software, Ann Arbor MI; Hausner et al. 2003; Six et al. 2003; Kim et al. 2005; Lee et al. 2006a; Zipfel et al. 2006; Massoumi Alamouti et al. 2009) generated an appropriate and specific primer set.

Each primer set was tested on 10 isolates of the target species, including the holotype, as well as on one or two isolates of other ophiostomatoid fungi and other fungi that were occasionally found in the MPB microbial community (Hausner et al. 2003; Six et al. 2003; Kim et al. 2005; Lee et al. 2006a; Zipfel et al. 2006; Massoumi Alamouti et al. 2009). In total, 76 isolates were tested in our screening analysis (Table 2.1). Identities of the isolates were confirmed morphologically and through DNA sequencing.

PCR reactions were carried out following standard methods (Kim et al. 2004). Table 2.2 shows the annealing temperatures of the primers. All reactions included general primer positive controls, LROR (Bunyard et al. 1994) and LR3 (Vilgalys and Hester 1990) and no template negative controls in order to ensure that negative bands were due to specificity of the primers and not to poor DNA quality. All reactions were repeated to ensure consistency. In order to ensure that the specific primers amplify their targets, we sequenced the amplicons for two representatives of each species from pure culture as well as from DNA extracted from MPBs. Purified PCR products were sent to Macrogen Inc. in Seoul, South Korea for sequencing (using the BigDyeTM terminator kit and run on ABI 3730XL). A specific primer set was considered satisfactory if it produced a PCR amplicon only from all the isolates of its respective species.
The minimum DNA concentration that each primer set could detect was determined using serially diluted target fungal DNA in the PCR reaction. Initial DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE).

2.2.4 DNA extraction from pure cultures, MPBs, MPB galleries and fungal inoculated lodgepole pine phloem

We extracted the DNA of fungal species (Table 2.1) grown on 1% OMEA. Cultures were harvested with a blunt scalpel when mycelia had covered approximately one quarter of a standard 100×15mm Petri dish. DNA was extracted by adding the mycelia to a lysing matrix A tube (MP Biomedicals Solon OH) with 500µl of TES buffer (100mM Tris, 100mM EDTA and 2% SDS). The tubes were shaken in a Mixer/Mill 8000 (SPEX CertiPrep Metuchen NJ) for seven minutes. Then 0.2% lysing enzyme (Trichoderma harzianum, SIGMA St. Louis MO) was added to each of the tubes and incubated at 40°C for 15 minutes, with intermittent mixing. 250µl of 3M sodium acetate was added to the tubes that were then vortexed and placed in a -80°C freezer for at least 15 min. The tubes were thawed, vortexed and centrifuged for 15 min at 14,000 rpm. The lysate was removed and the precipitate was washed twice with phenol:chloroform:isoamyl alcohol (25:24:1). Finally, DNA was precipitated in isopropanol (90%) and sodium acetate (10%), washed with 70% ethanol and then resuspended in distilled water.

DNA was extracted from the same beetles from which we isolated the fungi. We added 50µl of 10X concentrated TES buffer to the crushed beetles in Tween 20 and transferred the whole mixture into a lysing matrix A tube. The tubes were shaken for 30s in a Mixer/Mill 8000 chamber that had been cooled to -20°C. The remainder of the procedure was identical to that of DNA extraction from pure cultures.

The same procedure was used to extract DNA directly from MPB galleries and inoculated phloem. The samples were maintained at -20°C until they were processed. The frozen samples were freeze-dried over-night in microfuge tubes with punctured lids. They were transferred to lysing matrix A tubes and shaken for five minutes in a Mixer/Mill 8000 chamber that had been
cooled to -20°C. Once removed, 500µl of TES buffer and 0.2% lysing enzyme were added to the tubes, which were then incubated at 40°C for 15 minutes. 143µl of 5M NaCl and 100µl of 10% CTAB were added and the tubes were incubated for 10 minutes at 65°C. The remainder of the procedure was identical to that of the DNA extraction from pure cultures.

2.3 Results

2.3.1 Primer design and testing

We designed three sets of target-specific primers from ribosomal DNA (Figure 2.1) for each of the three groups of ophiostomatoid fungi that occupy different ecological niches in the MPB system. The set of Lepto primers amplify species in the Leptographium clade, including the pathogens G. clavigera and L. longiclavatum, as well as G. aurea, L. pyrinum, G. robusta and L. terebrantis that are usually not associated with MPB. The Omon primers amplify O. montium. The CopMPB primers amplify Ceratocystiopsis sp.1.

Since we could not differentiate L. longiclavatum and G. clavigera with rDNA primers, we designed specific primer sets to differentiate the two species using a gene of unknown function. These two specific primer sets, Llongi and Gclavi differentially amplify L. longiclavatum and G. clavigera, respectively. The primer sequences and the conditions for the amplification are shown (Table 2.2). The Gclavi primer set detected only the G.clavigera isolates and none of the other closely related species. The Llongi primer set reacted with L. longiclavatum but not with G. clavigera or most species from the Leptographium clade; the one exception was L. terebrantis, which is rarely associated with the early MPB colonization of the trees.

Primers were designed to amplify regions of approximately 200 bases. The annealing temperatures were selected between 60 and 65°C (Table 2.2).

2.3.2 Comparing fungal isolation on media with DNA detection techniques

We optimized the DNA extraction protocols for pure cultures and microbial communities associated with MPB bodies and galleries. Extraction from pure cultures and beetles were completed in less than four hours, while for MPB galleries and fungal inoculated phloem
extractions took six hours after the samples had been freeze-dried. In contrast, the fungal isolation technique required on average two to three weeks before a final identification could be confirmed using either DNA sequencing or the specific DNA primer sets developed in this work.

On inoculated phloem, *G. clavigera* was detected within and outside the edge of the lesions using the Gclavi primer set. Similar data were obtained with fungal isolation on 1% OMEA; *G. clavigera* was present inside and outside the lesion edge. Both methods gave consistent results.

Overall, *Cop. sp.1* and yeasts were abundant in our adult beetle samples. We isolated *Cop. sp.1* three times more frequently than the other filamentous fungi. As well, the beetle body isolation produced eight to ten times more yeast colonies than filamentous fungi. Yeasts were also isolated from every beetle gallery sample. We only sampled a single point from each gallery, which is not representative of the whole gallery and does not allow for quantification.

On MPB bodies, *Cop sp.1* was detected on all samples with both methods. For the other fungi associated with MPB we observed some variation in detecting *G. clavigera, L. longiclavatum* and *O. montium* between the two methods. From the BC MPB galleries, we were successful at detecting *Cop. sp* with the DNA detection method but not with the isolation method. With the isolation method, the other fungal species were more frequently detected. Finally, for the MPB gallery samples from California, while the two methods gave overall similar results, species varied somewhat between samples from adjacent galleries. We detected neither *Cop sp.1* nor *L. longiclavatum*, in the California samples using either method; however, these two species have not been reported in MPB galleries sampled in the US (Table 2.3).

### 2.4 Discussion

In this work, we successfully designed and tested three sets of rDNA primers that we used to differentiate fungal species grown in artificial media and directly from bodies of MPBs. While our rDNA primers were able to distinguish species from genera *Ceratocystiopsis, Ophiostoma* and *Grosmannia*, the *Leptographium* primers cross-reacted with different species closely related to the two MPB-associated fungal pathogens: *G. clavigera* and *L. longiclavatum*. We selected the rDNA region for the design of primers because rDNA genes are present in high copy numbers
and are easily amplified in fungi. As well, they have been the most commonly targeted genes for clarifying ophiostomatoid systematics and phylogeny (Hausner et al. 1993; Jacobs and Wingfield 2001; Zipfel et al. 2006). While they provide valuable information at the genus and sometimes at the species levels, they do not effectively distinguish closely related species like G. clavigera, L. longiclavatum and L. terebrantis (Six et al. 2003; Lim et al. 2004).

The O. montium primer set is specific for this species. Previous work by the UBC research group showed that O. montium and O. ips were often misidentified, even for specimens from culture collections (Kim et al. 2003; Massoumi Alamouti et al. 2009). The two species are very similar morphologically and both are vectored by bark beetles and colonize pine trees. However, O. montium seems more specifically associated with MPB, while O. ips is distributed worldwide and does not appear specifically associated with a particular beetle (Zhou et al. 2007). In previous work, the UBC research group differentiated the two species by growing them at different temperatures and by amplifying their β-tubulin genes. O. montium had a single amplicon of 607 bp while O. ips had two amplicons of 776 and 876 bp (Kim et al. 2003). In the current work, we designed the O. montium primers in the ITS region of the rDNA, since β-tubulin is sometimes difficult to amplify, especially when the DNA is of low concentration and of lower quality, which is often the case for ecological samples (Arbeli and Fuentes 2007).

The Cop. sp.1 primer set, CopMPB, was also very efficient in differentiating MPB-associated species from the closely related Cop. ranaculosa and Cop. brevicomis (Plattner et al. 2009). These three species have been isolated from bark beetles or trees infested by Dendroctonus frontalis, D. valens and D. brevicomis, respectively. The red turpentine beetle, D. valens and the western pine beetle (D. brevicomis) inhabit Pinus ponderosa and have been found in BC. The southern pine beetle (D. frontalis) seems to inhabit mainly loblolly (Pinus taeda L.) and shortleaf (Pinus echinata Mill.) pines (Davidson and Prentice 1967). It is important to note that very little work has been done on the fungi associated with these beetles. The CopMPB primers differentiated the MPB species from the more distantly related Cop. manitobensis and Cop. manitobensis-like from Ips perturbatus, which were isolated in Canada (Massoumi Alamouti et al. 2007). All of the species found in Canada (MPB associates, Cop. manitobensis and
manitobensis-like) grow slowly on 2% OMEA and form white colonies. They also produce similar conidia from their Hyalorhinocladiella anamorphs (Plattner et al. 2009).

To differentiate the MPB associated Leptographium species, we developed two more specific primer sets Llongi and Gclavi from a protein-coding gene with an unknown function (DiGuistini et al. 2007). While the Gclavi primer set exclusively amplifies G. clavigera, the Llongi primer set is effective at differentiating L. longiclavatum from G. clavigera but not from L. terebrantis. It is important to note that we rarely isolated L. terebrantis from trees that have recently been attacked by MPB; rather, we occasionally found it in trees that have been infested for a year or more (Kim et al. 2005). Furthermore, from the work of the UBC research group others, it seems that the L. terebrantis species reported in the literature will need to be re-assessed, as we recently found that some of the species assigned as L. terebrantis for MPB and a few other beetles were genetically different from the L. terebrantis holotype isolated from D. terebrans (Six & Massoumi Alamouti unpublished data). Since the complete genome of G. clavigera has now been sequenced (DiGuistini et al. 2009) and the UBC research group is comparing some of its genes with closely related species, it may be possible in the future to design primers to further differentiate L. longiclavatum from the Leptographium terebrantis complex.

From our sampling from different hosts and sites, we can conclude that PCR and isolation methods detect fungi in a range of ecological niches, and each method has strengths and weaknesses. The methods gave comparable results when a single species was dominant, either in the case of Cop sp.1 being naturally abundant on MPB bodies or G. clavigera that was the only fungus present as it was inoculated in trees. Both methods are efficient under these circumstances.

However, the methods performed differently when the fungi were less abundant or when other micro-organisms (e.g. yeasts) dominated the sample. This was evident in the BC MPB galleries where we did not detect Cop sp.1 using isolation but did with the DNA detection method. The effects of competition between the fungi on media hamper the isolation technique in its ability to detect slower-growing or less abundant species. This could explain why Cop. sp.1 was only recently reported as an MPB associate (Plattner et al. 2009). In contrast, on the MPB bodies, the
PCR method detected *Leptographium* with the Lepto rDNA primer set, but neither *G. clavigera* nor *L. longiclavatum* with their species-specific primers. Because in earlier experiments, while we optimized our DNA extraction method, we were able to detect these species with the specific primers on a pair of beetles (data not shown), these two species were likely much less abundant on the MPBs used in this work, making them difficult to detect with primers that target single copy genes. Among other variables that might affect the DNA extraction method is the cell wall composition of the targeted fungi. Cell walls of staining fungi that contain chitin, cellulose and melanin are difficult to disrupt, in contrast to spores and mycelium of non-staining species (e.g. *Cop. sp.1*) (data not shown). Our results suggest two issues. First, the abundance and the cell wall composition of the targeted fungi are important variables in the direct DNA extraction. Second, neither PCR nor isolation methods will always give a true representation of this ecosystem’s microflora.

For the beetle galleries, we used adjacent samples of phloem for isolation and PCR methods. Differences in the diversity and abundance of the fungi between the two locations may explain why results varied between the methods. Despite this uncertainty, both detection methods worked well for detecting the fungi associated with the MPB and the PCR method requires less time and expertise.

In conclusion, we showed that PCR was able to detect the targeted ophiostomatoid fungi from DNA extracted from pure cultures, MPBs and their associated microbial communities. This method can detect species without requiring that fungi be isolated from their substrates, and can be completed for a large number of samples in one day. The method can be used to detect fungi in imported and exported wood and we anticipate that it will be effective in field surveys that characterize fungal diversity at diverse geographic scales.
### 2.5 Tables and Figures

**Table 2.1 List of isolates**

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Location</th>
<th>Identified by</th>
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<td>J-J. Kim</td>
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<td>PY8-1</td>
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Table 2.1 List of isolates (cont’d)

**MPB-associated ophiostomatoid fungi**

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Location</th>
<th>Identified by</th>
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<td>J-J. Kim</td>
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<tr>
<td></td>
<td>DPSMWC2</td>
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<td>C. Breuil</td>
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<tr>
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<td>Kamloops, BC</td>
<td>C. Breuil</td>
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<td></td>
<td>866 A EG1-L23</td>
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**Other ophiostomatoid fungi**

<table>
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<th>Location</th>
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<td><em>D. brevicomis</em></td>
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<td><em>Ceratocystis manitobensis</em></td>
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<td><em>P. resinosa</em></td>
<td>MB, Canada</td>
<td>J. Reid</td>
</tr>
<tr>
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<td>UM237</td>
<td><em>P. resinosa</em></td>
<td>MB, Canada</td>
<td>J. Reid</td>
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<tr>
<td><em>Ceratocystis minima</em></td>
<td>UM 235</td>
<td><em>P. banksiana</em></td>
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<td>UM 85</td>
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Table 2.1 List of isolates (cont’d)

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<tr>
<th>Species</th>
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<tr>
<td></td>
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<td>Gallery of <em>Ips cembrae</em></td>
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<td>Y. Yamaoka</td>
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<td>Ceratocystiopsis minuta-bicolor</td>
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<td>UAMH 9551</td>
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<td>Ceratocystiopsis ranaculosa</td>
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<td>Grosmannia aurea</td>
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<td>Grosmannia robusta</td>
<td>CMW668</td>
<td><em>Picea abies</em></td>
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<td><em>Ips integer</em> gallery</td>
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<td>C.T. Rumbold</td>
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Table 2.1 List of isolates (cont’d)

<table>
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<tr>
<th>Other ophiostomatoid fungi</th>
<th>Species</th>
<th>Isolate</th>
<th>Host/substrate</th>
<th>Location</th>
<th>Identified by</th>
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<td><em>Abies</em> sp.</td>
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<td>Cranbrook, BC</td>
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Cultures were from American Type Culture Collection (ATCC), Centraalbureau voor Schimmelcultures (CBS, Netherlands), Reid’s culture collection at University of Manitoba (UM), University of Alberta Microfungus Herbarium (UAMH), Yamaoka’s culture collection, Japan (YCC) and Breuil at University of British Columbia. AB – Alberta, BC, British Columbia, MB – Manitoba, SK – Saskatchewan, D – *Dendroctonus*, P. – *Pinus*, MPB – mountain pine beetle.
Table 2.2 Sequences and summary information for each target-specific primer set

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Annealing temperature</th>
<th>Amplicon length</th>
<th>Min. DNA detection (ng/ml)</th>
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<td>OmonR</td>
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<td>219bp</td>
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<tr>
<td>LeptoF</td>
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<td>195bp</td>
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<tr>
<td>LeptoR</td>
<td>CGCCAGAGCATCTCTCTCCA</td>
<td>63°C</td>
<td>195bp</td>
<td>4x10⁻⁵</td>
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<tr>
<td>CopMPBF</td>
<td>AGTCTTAACGAGCGTCTGAGTAGGA</td>
<td>64°C</td>
<td>227bp</td>
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<tr>
<td>CopMPBR</td>
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<tr>
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<td>263bp</td>
<td>4x10⁻⁵</td>
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Table 2.3 Targeted fungi detected from MPBs and MPB galleries using either isolation on 1% OMEA or PCR specific primers on DNA extracted from selected samples

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<th>BC MPB Galleries</th>
<th>CA MPB Galleries</th>
<th>MPB Bodies</th>
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<td>Isolation¹</td>
<td>PCR²</td>
<td>Isolation¹</td>
</tr>
<tr>
<td><strong>O. montium</strong></td>
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<td>3</td>
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<tr>
<td><strong>Cop. sp.1</strong></td>
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<tr>
<td><strong>Leptographium</strong></td>
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<td>5</td>
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<tr>
<td><strong>G. clavigera</strong></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>L. longiclavatum</strong></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total samples taken</strong></td>
<td><strong>10</strong></td>
<td><strong>13</strong></td>
</tr>
</tbody>
</table>

¹ Micro-organisms isolated on 1% MEA and further identified by morphology and by PCR using specific primers.
² PCR method: DNA was extracted from selected samples and amplified with specific primers.
* Each sample included two adult beetles

MPB, mountain pine beetle; BC, British Columbia; CA, California

Values represent the total number of samples for each substrate in which each fungus was detected.
Figure 2.1 Schematic diagram indicating the relative position of the target-specific primers on the rDNA.

Figure 2.2 Agarose gel images of PCR amplicons obtained with target-specific primers: A, B and C are results obtained with primer sets Lepto, Omon and CopMPB, respectively, on DNA extracted from pure culture. D shows the PCR amplicons obtained with target-specific primer sets on DNA extracted from two MPBs and their associated microbial communities.
Chapter 3: The relative abundance of mountain pine beetle fungal associates are shown to change through the beetles’ life cycles

3.1 Introduction

Bark beetles have been found in conifer hosts since at least the Mesozoic era and are among the most damaging forest pests in North America (Seybold et al. 2000). These beetles are often associated with an assemblage of fungi, bacteria, mites and nematodes. Vectored species range from those that are specific to a single beetle species to generalists that are shared by many beetle species. Interactions between the beetles and their associated organisms, or between the vectored micro-organisms, are complex and vary from beneficial to antagonistic. Here we will focus on the native western North American bark beetle, *Dendroctonus ponderosae* Hopkins (mountain pine beetle, MPB). The MPB has infested over 17.5 million hectares of pine (*Pinus* sp.) forest in interior British Columbia (BC), Canada, in an epidemic that began in 1994 and spread to all suitable habitats in BC (BC Ministry of Forests, 2011a). MPB large-scale outbreaks are also present in western Alberta, Canada, and in some of the western states in US (Safranyik and Carroll 2006).

The MPB life cycle is largely driven by local climate, and typically is univoltine having one brood per year. During an epidemic, univoltine beetles mass attack trees between July and early August. When MPBs overcome tree preformed chemical defenses, the adults create vertical galleries in the phloem, propagating fungal propagules (mainly spores) that are carried externally or internally (gut or maxillary mycangia) by both sexes. The beetles mate and lay eggs along the walls of the gallery. The eggs hatch into larvae that create galleries perpendicular to the parents’ main gallery. Typically, the larvae over-winter as third or fourth instar larvae and pupate in late spring. Before emerging, young adult beetles feed on micro-organisms present in the gallery, and then fly to attack and colonize new trees early July to early September (Whitney 1971; Bentz et al. 1991; Paine et al. 1997; Safranyik and Carroll 2006).

The ascomycetes *Grosmannia clavigera*, *Leptographium longiclavatum*, and *Ophiostoma montium* are included in the ophiostomatoid group. They are the first to colonize beetle galleries
and the adjacent phloem and sapwood tissues, and produce a pigment, melanin, which causes a blue to black wood discoloration (Lee et al. 2006a). While the first two have been shown to kill trees in the absence of the beetle, the pathogenicity of *O. montium* is uncertain (Yamaoka et al. 1995). These three species have been isolated from infested sapwood, MPB body surfaces, mycangia, and adult and larval galleries (Rumbold 1941; Robinson 1962; Whitney and Farris 1970; Six 2003a; b; Kim et al. 2005; Lee et al. 2006a). Two additional slower growing species have been more commonly isolated from MPB and their galleries: the ophiostomatoid *Ceratocystiopsis* sp.1 (*Cop*. sp.1) and the basidiomycete *Entomocorticium* sp. (Lee et al. 2006a; Plattner et al. 2009). These four ophiostomatoid species have not been isolated from other bark beetles, and so appear to be specifically associated with MPB (Lee et al. 2006a). The specificity of *Entomocorticium* sp. is uncertain. The genus has been observed on various beetles but a thorough phylogenetic analysis has not been performed on these isolates, to determine whether the genus consists of several species associated with different bark beetles (Klepzig and Wilkens 1997; Hsiau and Harrington 2003).

MPB and its vectored fungi have a symbiotic or mutualistic relationship (Paine et al. 1997; Six 2003a; Harrington 2005). The fungi benefit from their association with the beetles by being transported and disseminated into new trees where they have access to fresh nutrients. Fungi contribute to beetle fitness in many ways. Fungal colonization of trees has been reported as a prerequisite for successful bark beetle brood development in pine (Christiansen 1985; Klepzig et al. 1991; Paine et al. 1997). Fungi grow in the phloem and sapwood of trees relatively faster when trees have been mass attacked by MPBs (i.e. the sapwood can be completely colonized by staining fungi four weeks after the initial attack); this results in a substantial decrease of the moisture content to a level that may be more suitable for successful beetle brood development (Reid 1961; Webb and Franklin 1978; Wagner et al. 1979). Beetles entering a tree feed on the phloem, which is rich in carbon but poor in sterols, vitamins and other growth factors (Norris and Baker 1967; Six 2003a). However, sterols are essential for the normal growth, molting and reproduction of the beetles (Clayton 1964). Larvae and new adults may benefit from fungal ergosterol, which has been shown to have positive impacts on the weight, size and normal development of the beetles (Clayton 1964; Norris et al. 1969; Barras 1973; Morales-Ramos et al. 1997; Hsiau and Harrington 2003).
Thus, fungi in the pupal chamber and galleries are a good source of nutrients (sterol and nitrogen) for maturing young beetles that do not benefit from fresh phloem (Ayres et al. 2000).

It is important to note that fungal identity, frequency and biomass are often assessed by a variety of methodologies and the data generated can lead to conflicting statements. Often observation and isolation have been used to show that beetles feed only on specific fungi or to compare phloem and sapwood colonization by different fungi (Bleiker and Six 2007; Adams and Six 2007). These methods are not necessarily accurate; they are biased toward the fast-growing species, and do not differentiate similar species (Harrington 1987; Kim et al. 2003; Lee et al. 2003; Lim et al. 2005; Khadempour et al. 2010b). Determining where and at which frequency a fungal symbiont is present at a particular bark beetle’s life cycle will require using molecular methods like PCR or qPCR. These methods use target-specific primers; they are simple, sensitive and can detect slow-growing species (Luchi et al. 2005). They have been used extensively for detecting and quantifying micro-organisms in environmental samples (Bahnweg et al. 2000; Winton et al. 2002; Filion et al. 2003; Schena et al. 2004; Luchi et al. 2005; Karlsson et al. 2007; van Doorn et al. 2007). However, to our knowledge, qPCR has been used only once to quantify ophiostomatoid spores associated with bark beetles (Schweigkofler et al. 2005).

The objective of this study is to identify and compare the relative abundance of MPB fungal associates through different phases of the beetle’s life cycle, in order to generate insights into the ecological roles played by the fungi. In this research, we used qPCR with target-specific primers to detect and assess the relative abundance of four fungal species: \textit{G. clavigera}, \textit{L. longiclavatum}, \textit{O. montium} and \textit{Cop.} sp.1. We hypothesized that the faster growing pathogenic fungi would be more abundant in early phases of the MPB life cycle, while the less pathogenic and slower growing fungi would increase in abundance either throughout the life cycle or in the last phase, when the teneral adults feed in preparation for their flight.
3.2 Materials and methods

3.2.1 Site and experimental set up

We used lodgepole pine (Pinus contorta Dougl. var. latifolia Engelm.) trees from a Similkameen very dry cool Montane Spruce variant stand (MSxk1) (BC Ministry of Forests, 2011b) located at the Bear Service Road near Merritt, BC. The stand was populated primarily with lodgepole pine, alpine fir (Abies lasiocarpa (Hook.) Nutt.) and hybrid spruce (Picea alba (Ait.) Link x Picea engelmannii Parry ex Engelm.). Most pine trees were over 100 years old (average age of 156 years, from 330 cored trees, unpublished data) and the stand was surrounded by clear-cut logging operations. This stand had highly variable summer temperatures and a low overall number of degree-days, resulting in local MPB populations with semivoltine life cycles. Based on data from HOBO data loggers (Onset Computer, Bourne, MA), there were 798 degree-days above 5.5°C from July 15, 2009 to July 15, 2010; this falls short of the 833 degree-days required for the MPB to be univoltine (Carroll et al. 2003). Further, these populations do not necessarily mass attack the trees at the beginning of July. To ensure homogeneous MPB mass attack necessary for the work reported here, we baited 30 trees with Mountain Pine Beetle Tree Bait (Contech Enterprises, Delta BC). A bait pouch was attached to the north side of the tree at approximately 2m high.

Three baited trees were randomly selected and felled at four beetle life phases: eggs, larvae, pupae and teneral adults. They were cut on July 24, and August 7, 2009 for the first two phases and July 5 and 28, 2010, for the two last phases. Bolts of 1.5m in length were cut from the felled trees. To prevent moisture loss, the bolt ends were sealed with silicone then the bolts were wrapped in heavy plastic and transported to the lab where they were refrigerated at 4°C until they were processed within the following week.

3.2.2 Sampling

Mountain pine beetle entry and exit holes were marked and dated with thumbtacks during all the period of the MPB attacks in 2009 (Figure 3.1). This survey was carried out every day during the initial attack and once every several days as the MPB attack frequency declined. For each tree, moisture measurements were taken from 2.5 by 2.5 cm piece of phloem and sapwood removed.
from the north side of the tree, ~ 30 cm above the ground before baiting the tree and again at the time of harvesting.

From each log, we measured the phloem and sapwood moisture content and the density of MPB attacks (Figure 3.2). When possible, bark was peeled in continuous pieces that contained the galleries; samples that included both the phloem lining the gallery and the phloem directly adjacent to it, were removed using a 7mm diameter cork borer (Figure 3.3). Sampling was done on two levels, trees and samples within trees. Our objective was to obtain 10 independent samples from each of the three trees at each life phase of the beetles. However, this was not possible for the pupal and teneral adult phases where we obtained a total of 22 samples from two logs and 21 samples from three logs, respectively. At these two phases, because the moisture content was low it was difficult to obtain continuous pieces of bark. As well, the beetle progeny at the larvae phase were not very successful; there were very few that were able to successfully overwinter and reach the last life phases. All samples were frozen in micro-centrifuge tubes at -20°C until they were processed for DNA extraction.

3.2.3 DNA extraction

Gallery samples in 1.5ml micro-centrifuge tubes were freeze-dried overnight prior to DNA extraction. DNA was extracted using the protocol from Khadempour et al. (2010). Briefly, the lyophilized samples were homogenized using the FastPrep Automated Homogenizer (MP Biomedicals, Solon OH) for 60s at speed 6. Then, TES buffer and lysing enzymes were added to each tube to complete cell breakages. This was followed by one CTAB and two phenol/chloroform purification steps. The DNA was precipitated from the solution using a mixture of sodium acetate and isopropanol; DNA pellets were washed with ethanol and re-suspended in distilled water.

3.2.4 Quantitative PCR and target-specific primers

All qPCR reactions were performed using the CFX384 Real-Time PCR Detection System (BioRad, Hercules CA) with Sso Fast Eva Green Supermix (BioRad, Hercules CA). Real time PCR conditions were 2 min at 95°C followed by 39 cycles at 95°C for 5 s then 66.6°C for 5 s.
This was followed by a melt curve analysis ranging from 65 to 95°C. Each reaction tube contained 10µl of Supermix, 0.5µl of each of the forward and reverse primers, 5µl of undiluted DNA and 4µl of distilled water.

Each reaction was duplicated and the reciprocal of the mean threshold cycle (Ct) value was used as a measure of the relative abundance of each fungus. The primers used were Omon, Lepto, Llongi and CopMPB; they amplify *O. montium*, MPB-associated *Leptographium* (i.e. *G. clavigera* and *L. loniclavatum*), *L. longiclavatum* and the MPB-associated *Cop*. sp.1, respectively (Khadempour et al. 2010b). We excluded the Gclavi primers because they were not specific when used in qPCR. We added a plant specific primer set: 28KJ (GGCGGTAAATTCCGTCC) and 28B (CGTCCGTGTTTCAAGCG) (Cullings 1992). We tested the phloem of non-infected lodgepole pine trees to ensure that our specific primers did not amplify tree DNA. Although the primers had been thoroughly tested for specificity in previous work (Cullings 1992; Khadempour et al. 2010b), we retested their specificity against closely related species with the new reaction mixture and qPCR program.

### 3.2.5 Analysis

We performed *a priori* power analysis to determine the number of samples that would likely detect a biologically significant result based on the work Schweigkofler et al. (2005). The reciprocal Ct values from each of the four primer sets were used as four y-variables. In order to meet the assumptions of normality and equal variance, the values were rank-transformed. Transformed data were used in all statistical tests but untransformed data are presented in graphs.

Multivariate analysis of covariance (MANCOVA) was used since the four y-variables were expected to be related (Tabachnick and Fidell 2000); untransformed reciprocal plant Ct was used as a covariate along with life phase as a class variable. Because of the hierarchical structure of the sample data, two error terms were included: logs within life phase, and samples within logs. For the MANCOVA tests, the logs within life phase was used as the error term, whereas the samples
within logs provided within log variation only (West et al. 2006). Analysis of covariance (ANCOVA) was then used to test each y-variable when differences in the vector of y-variables were noted using MANCOVA. Finally, t-tests were used to test for differences among pairs of life stages where differences in a y-variable were noted. For the MANCOVA a significance level of alpha=0.05 and a normalized-rank transformation of each y-variable (Blom 1958) was used. For subsequent univariate ANCOVA and t-tests, this alpha was divided by the number of y-variables (four) and pairs of means (six pairs), respectively, following a Bonferonni correction (Holm 1979). All tests were performed using SAS® 9.2 Statistical Software Package (SAS Institute Inc., Cary, NC).

3.3 Results

3.3.1 Site observations and measurements

Due to the low, variable summer temperatures, the local beetles in the stand had a semivoltine lifecycle. This site had been surveyed every year for four years before the start of this study. Records (David Jack, UBC, unpublished data) indicate that the local beetle population rarely had a univoltine life cycle, but that, due to the widespread epidemic, beetles from the surrounding regions would fly in and attack trees in July, before the local population began their flight. We used pheromone baits to mitigate this problem and create a synchronous mass attack similar to the one created by the beetles’ natural attack. However, there were some unexpected issues. This experiment took place after the peak of the epidemic, as the MPB began to exhaust its supply of suitable host trees but also as populations of parasites and secondary beetles increased. Thus, at the later phases of the beetle life cycle, the new brood appeared less successful than previously observed in the epidemic. The baited trees had remained in this heavily infested stand for several years and had not been selected by the beetles. As such, it is believed that they provided sub-optimal growing conditions for the beetles, contributing to the low brood productivity. Furthermore, the attack density that resulted from the tree baiting was much higher than the optimal density of 40-80 attacks/m².

The attack density of the trees ranged between 89 and 500 attacks/m² with a median attack
density of 130 attacks/m² (Figure 3.2). Between the baiting time and the last sampling time, the moisture content of the phloem changed most markedly for the pupal and teneral phases whereas during the egg laying and larval phases, there was relatively little loss in moisture content (Figure 3.2).

3.3.2 qPCR
Target-specific primers (Omon, Lepto, Llongi and CopMPB) were used for quantifying the relative abundance of the four different ophiostomatoid species colonizing the beetle galleries through its life cycle. Reaction conditions were changed from Khadempour et al. (2010) to be appropriate for the new qPCR reaction. To detect and quantify the different fungi in the DNA extracted from gallery samples, we used the Sso Fast EvaGreen Supermix in combination with our target-specific primers. The Sso Fast EveGreen Supermix contained a dye with similar properties to SYBR Green; they are double stranded DNA binding fluorescent dyes. However, the Sso Fast EveGreen Supermix contained a high fidelity polymerase and the mixture is optimized to give a fast reaction that could decrease the impact of potential inhibitors present in the DNA extracts. Because the concentration of the fungal targeted DNA was very low in contrast to tree DNA in our samples, and because there were potential traces of induced tree chemicals (e.g. phenolic or terpenoids) in the reaction, it was necessary to use 5µl of undiluted DNA to ensure consistent results. All of the fungal target-specific primers from Khadempour et al. (2010) were able to detect and quantify their specific target, except for the primers targeting *G. clavigera* that showed non-specific products and were consequently excluded from this study. The melt curves did not show any indication of non-specific reaction or excessive primer-dimers. The plant specific primers 28KJ and 28B, specifically detected the tree DNA in the sample and served as a control for DNA extraction efficiency.

qPCR quantification can be either absolute or relative. With absolute quantification, it is possible to attribute a certain cycle threshold in the reaction with the number of gene copies (Heid et al. 1996) representing the number of spores, cells or amount of biomass (Gamper et al. 2008) in a sample. Relative quantification is effective when comparing the response of one sample to
another, particularly when the absolute amount of material is not critical; it is often used in gene expression studies, where up- or down-regulation of a gene is more important than the absolute copies of RNA present in the reactions (VanGuilder et al. 2008).

Schweigkofler (2005) used qPCR to quantify the number of fungal spores on beetle bodies. In order to create a standard curve for their quantification, they extracted DNA from beetles mixed with a known serial dilution of spores. They compared their unknown Ct values from their samples with the values from the standard curve. This was not possible in this study because we were measuring DNA from mixtures of spores and mycelia. The mycelia were tightly associated with the phloem cells, and even if we had attempted to add spores or mycelia to phloem samples before DNA extraction, this would not have been accurate for generating a standard curve. Among the potential issues, we noted that DNA extraction efficiency from spores differs from mycelia in a species and between species and the variation could be higher in phloem tissues that are not homogenous and contain a variety of chemicals. Relative quantification is still very informative. In the study ecosystem, it was less important to determine the exact amount of each fungus in a sample, but the relative frequency of each fungal species was key to give us some insights on the potential roles of these fungi at different phases of the beetle life cycle.

3.3.3 Statistical analysis

Using MANCOVA we observed a significant difference in the combined relative abundance of the four fungal species through the lifecycle of the beetle, indicating that the relative abundance of the fungi considered together, changed through the beetle lifecycle (Table 3.1). With ANCOVA for each of the fungal species, only Cop. sp.1 showed a significant change over the beetle lifecycle (P = 0.0038) (Figure 3.4). The other fungi did not show a significant difference in relative abundance at different life phases, although they all showed a trend toward an increase (Figure 3.4). Pairwise tests of Cop. sp.1 means between different life phases showed a significant difference between the first and third life phases (P = 0.0018). Other life phase comparisons were not significantly different but had P values just above the \( \alpha = 0.002 \) threshold, indicating that there was a trend toward change throughout the life phases.
3.4 Discussion

In the early phases of its life cycle, the MPB seems to maintain a very specific microflora that prevents competition by other fungi that are occasionally found in galleries or wood, and are likely fungal associates of other cohabiting beetles (e.g., Ips and ambrosia beetles) (Six 2003b; Kim et al. 2005). Our results on the relative frequencies of the ophiostomatoid fungi during the life cycle of the beetle are consistent with results in the literature (Adams and Six 2007; Bleiker and Six 2009); pathogenic and staining associates like G. clavigera and L. longiclavatum colonize the phloem rapidly and are followed by the less pathogenic O. montium as the beetle builds its gallery. Leptographium associates showed its highest abundance in the teneral adult phase, while the frequency of O. montium was approximately constant during the four phases. In concordance with our results, species of the genus Leptographium are known as the pioneer MPB-vectored fungi colonizing the sapwood during the two to three weeks following a massive MPB attack (Lee 2006). However, we also showed that the slow-growing species Cop. sp.1, while present at low frequency in the beetle gallery at the early infestation, became statistically more abundant during the pupal phase of the beetle life cycle. It is important to note that only a few researchers have reported this fungus, which has been often misidentified or ignored (Harrington 2005; Plattner et al. 2009). This is likely because the fungal associates of the MPB have been studied largely in relation to their effect on the tree. Since Cop. sp.1 is not pathogenic and does not grow beyond the beetle galleries, it may not have been considered important in this ecosystem. As well, it grows slowly compared to the staining fungi, making its detection difficult using fungal isolation.

These results demonstrate that qPCR can be more effective than fungal isolation for quantifying changes in relative frequencies of specific fungi through the beetle life cycle in the host tree. Traditionally, culture isolation on malt extract agar has been used to determine which fungi are present in/on MPB, its gallery and in the tree phloem (Adams and Six 2007) or to assess the colonization of phloem by staining fungi (i.e. G. clavigera and O. montium) at different phases of the beetle life cycle (Bleiker and Six 2009). However, these fungal isolations were limited
spatially, and identification was done using morphological characterization that does not accurately differentiate the fungal species that morphologically and genetically resemble each other (e.g. *G. clavigera* from *L. longiclavatum*). In previous work, the specificity of the primers was verified and fungal isolation was compared with detection by PCR (Khadempour et al. 2010b); here, we used these specific primers in qPCR. qPCR is widely used in microbial ecology and has a number of advantages. While PCR determines the presence or absence of a species by producing more copies of specific DNA region, qPCR quantifies the number of copies of a segment of DNA in a sample. Using qPCR with primers that target several species from the same sample allows us to determine the relative fungal abundance in ecological samples. Here, we assessed the relative abundance of the combined *Leptographium* species (*G. clavigera* and *L. longiclavatum*), *L. longiclavatum*, *O. montium*, and *Cop.* sp.1.

Our target-specific PCR or qPCR primers were developed from the ribosomal DNA of the fungal species present on/in MPB body and in beetle gallery (Khadempour et al. 2010b). In the current work, we showed that the *G. clavigera* primers that we used for differentiating *G. clavigera* from *L. longiclavatum* were not specific in the qPCR and produced non-specific products. The GClavi primer set was designed from gene regions that contain a high GC content, making it difficult to design specific primers. The high GC content combined with the necessity to use high fidelity polymerases for environmental samples that may contain trace amounts of tree chemicals (e.g. phenolics and terpenoids), resulted in the primer set being less specific with the qPCR conditions used. The Lepto primer pair was developed from rDNA that amplify the *Leptographium* species associated with MPB. This primer set reacts with *G. clavigera*, and *L. longiclavatum*. Since *G. clavigera* and *L. longiclavatum* have very similar genetics and physiological traits, we expect that they have similar ecological niches and roles, consistent with the Lepto and Longi primer sets producing similar results throughout the lifecycle of the beetle.

While all the fungi described here are ophiostomatoids, they show preference for certain tree tissues and their relative abundances differ during the life cycle of the beetle and likely could assist or benefit the MPB differently. It is important to note that when researchers reported
different results and contradictory statements on the role of fungi, they often compare different hosts (e.g. lodgepole pine versus ponderosa pine) of different ages in various geographic areas (e.g. southern California versus northern Canada) and with variable beetle populations. Different opinions regarding MPB ecology result from generalization from these variable studies. For example, the role of fungi in reducing the level of host defences that the beetles have to overcome is controversial (Nelson 1934; Berryman 1972; Paine et al. 1997; Six and Wingfield 2011). In addition, many studies have been carried out in logs that may not accurately represent the complex interactions occurring in trees. Recently, Boone et al. (2011) have shown that beetle population density and total monoterpenes (induced or constitutive) were important in tree defence. While fungi utilize sugars from the phloem that could contribute to producing induced defences (e.g. synthesis of terpenoids), fungi likely colonize phloem and sapwood too slowly to contribute to the initial overwhelming of tree defences, which occurs within a few days (Six and Wingfield 2011). However, fungi may contribute to establishing the beetle progeny and may play a role after the initial beetle attack or in the localized environment of their galleries by modifying or reducing toxic tree defence chemicals (Paine et al. 1997). Recently, it was shown that G. clavigera can overcome the toxic effect of monoterpenes and grow with monoterpenes as a single carbon source (DiGuistini et al. 2011). It was also shown that other MPB fungal associates, including the slow growing Cop. sp.1, tolerate or grow in the presence of monoterpenes (unpublished results).

Consistent with the literature, we showed that the phloem moisture content decreases with time. The fast-growing Leptographium colonizers G. clavigera and L. longiclavatum are known to grow at high moisture content with low oxygen (Lee et al. 2006a; Kim et al. 2008). Such conditions prevail in trees when MPBs enter, build their galleries, and spread their fungal associates. In BC, the two species found most frequently in and on beetles and in colonized wood are the Leptographium species and then O. montium (Solheim 1995; Plattner et al. 2008). When these are well established, they decrease phloem and sapwood moisture contents and, in combination with the beetle, block the transport of nutrients and water, typically resulting in tree death (Amman et al. 1990; Paine et al. 1997). Reid (1961) showed that brood establishment and colonization by staining fungi are associated with a sharp decrease in the moisture content of
attacked trees. We also observed a decrease in moisture at the pupal phase of the life cycle. Since the pioneer staining fungi can colonize the galleries and phloem quickly, the prevalence of the staining fungi increases only marginally over the lifecycle of the beetle in the tree.

The role of fungi as nutrients or supplements to the MPB diet is also widely discussed in the literature. Early papers reported that larvae develop into normal adults in pine phloem that contains no fungi, and that fungi may be a supplement rather than a requirement for beetle development. However, these early experiments were carried out in logs, rather than living trees, used small numbers of beetles, and were not comparable with MPB mass attacks (Whitney 1971). Six and Paine (1998) indicated that successful brood development and higher production of progeny require fungi. Bentz and Six (2006) showed that fungal ergosterol substantially increases in infested trees, and suggested that this sterol could benefit MPB progeny. Furthermore, tree phloem and sapwood have low nitrogen contents (0.1% to 0.05%) (Abraham et al. 1998; Bleiker and Six 2007) and beetle-associated fungi could be an additional source of nitrogen. We showed that the slow-growing Cop. sp.1, which was mainly isolated from galleries, increased substantially during the pupal and teneral adult phases. The sudden increase in this fungal population may be related to the presence of the meconium (i.e. waste products exuded by larvae before pupation) that coats the pupal chamber wall. This coating is high in nitrogen (Jack, unpublished data) and may stimulate fungal growth and increase fungal biomass. These fungi may be preferred and grazed by the young teneral adults. It is important to note that Cop. sp.1 does not produce melanin. This cell wall pigment does not contribute to the nutritional value of fungi, but protects fungi against environmental stress (Butler and Day 1998). Further, this fungus does not seem to be pathogenic to pine trees. Before emerging, new adult beetles need to feed on phloem and fungi before their flight, and also have to contact fungi that have grown from the spores introduced by the parents, whose hyphae and spores now coat the pupal chamber and galleries (Six 2003a). Whether Cop. sp.1 requires sources of nitrogen other than the phloem to produce a high biomass, and whether it is an important source of nutrients for the beetle progeny remains to be confirmed.
3.5 Tables and figures

Table 3.1 MANCOVA test criteria and F approximations for the hypothesis of no overall life phase effect

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<th>DEN DF</th>
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<td>4</td>
<td>6</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

\(^1\)F Statistic for Roy's Greatest Root is an upper bound.

Figure 3.1 A log with thumb tacks indicating location of MPB attacks.
Figure 3.2 Summary data for sampled trees, including phloem moisture content (PMC) at the beginning of the study (dark purple), at time of harvesting (violet) and the difference between the two values (blue), attack density (○), life stage and diameter at breast height (DBH).
Figure 3.3 Schematic diagram of sampling locations, with images of beetle galleries at respective life stages, (A) eggs, (B) larvae, (C) pupae and (D) teneral adults.
Figure 3.4 Untransformed data showing relative abundance of target fungi changing throughout the MPB lifecycle. Only Cop. sp.1 shows a significant change while Leptographium and L. longiclavatum both show trends toward increasing. Error bars show a 95% confidence interval.
**Chapter 4: Conclusions**

The novel work presented in this thesis improves our knowledge about the relationships between the different ophiostomatooid fungi that are associated with the mountain pine beetle (MPB). The MPB ecosystem provides an excellent opportunity to study the interactions of a bark beetle with its fungal symbionts and its tree host. The symbionts include *Grosmannia clavigera*, *Leptographium longiclavatum*, *Ophiostoma montium* and *Ceratocystis* sp.1 (*Cop.* sp.1). During the last two decades, considerable work has been published on the potential role(s) of fungi in the MPB ecosystem, especially for the two most frequently isolated staining fungi, *G. clavigera* and *O. montium*; this work addressed fungal classification, identification, colonization and pathogenicity, and the nutritional value of the fungi for the beetle. However, some of these studies have been limited by the methodology used and because at least two other fungal associates were not considered. In this thesis, I addressed the inefficiencies of previous sampling methodologies by using a new method for detecting and identifying four fungal species (Chapter 2). This method could be easily applied to other fungal genera or other microbes (e.g. yeasts and bacteria). Then, I used this method to conduct a systematic study on the abundance of these four fungal species during the lifecycle of the beetle (Chapter 3).

Chapter 2 addresses the inefficiency of the methodology for sampling the fungal associates of the mountain pine beetle, and provides a solution through a simple DNA extraction and PCR reaction. In previous studies, fungi were detected and differentiated through the traditional isolation and morphological identification or sometimes through DNA sequencing. Both of these methods are time consuming. Further, morphological identification is inaccurate, especially for closely related fungal species with a *Leptographium* anamorph, or asexual phase. *Cop.* sp.1 was often not isolated, because it is a slow-growing species present in the beetle gallery and absent in the phloem or sapwood of the host tree. *L. longiclavatum* has a *Leptographium* anamorph similar to *G. clavigera* and, therefore, they are likely grouped together instead of being studied as two unique species. The three staining fungi, *G. clavigera*, *L. longiclavatum* and *O. montium*, contain melanin in their cell walls in addition to cellulose and chitin. These extra melanin and cellulose components make DNA extraction more difficult and often traces of melanin can affect the *Taq* polymerase in PCR. I developed and optimized a method for extracting DNA from these species in pure culture or environmental samples. I also developed specific target primers that I used to
detect the fungi in DNA samples from the beetle and its gallery and from inoculated phloem. The primers Omon, CopMPB, Gclavi, Llongi and Lepto were used in detecting *O. montium*, *Cop. sp.1*, *G. clavigera*, *L. longiclavatum* and all pine-associated *Leptographium* spp. (including *G. clavigera* and *L. longiclavatum*), respectively. While I used ribosomal DNA sequences for designing target-specific primers for the Omon, CopMPB and Lepto primer sets, this was not possible for the *G. clavigera* and *L. longiclavatum* because their DNA sequences are too similar in this region. Instead, to design Glavi and Llongi, I used a gene that was recently sequenced as part of the sequencing of the *G. clavigera* genome. With the exception of the Lepto primer set, all the other primer sets were species-specific. They were tested against a panel of 74 isolates of ophiostomatoid fungi and other fungi that were isolated from the MPB ecosystem. I was able to use these specific primers to quickly and easily identify cultures of fungi, which is faster than sequencing and more accurate than morphological identification. I was also able to detect these fungi in the phloem removed from MPB galleries and on or in the MPB bodies.

Once I had optimized DNA extraction and developed specific primers, I combined these methods with quantitative PCR to conduct an ecological study (Chapter 3). My objective was to assess the relative abundance of four ophiostomatoid fungi throughout the lifecycle of the MPB, and to investigate whether the changes in relative abundance of these four fungi could give some insights on the roles played by these fungal species in the beetle ecosystem. Similar studies used isolation and morphological identification to measure the abundance of only two fungal species: *G. clavigera* and *O. montium*. Because of these reasons, two potentially important fungi, *L. longiclavatum* and *Cop. sp.1* were not considered in these studies. Previous work indicated that these four species are specifically associated with the MPB. It was established that *G. clavigera* and *L. longiclavatum* are pathogenic to trees, meaning that they are able to kill a healthy tree if inoculated at sufficient densities. *O. montium* has been shown to be a weak pathogen, while *Cop. sp.1* is not considered as pathogenic, since it grows only in the beetle gallery and does not colonize the host tree tissues. Because the MPB-associated *Leptographium* species are able to kill living trees when inoculated at a certain density in the absence of the beetle, it has been suggested that these fungi play an active role in overwhelming tree defences in the initial MPB mass attack of the pine trees. However, more recent research along with my research suggested that the staining fungi do not grow fast enough to kill the tree during the initial entrance and
building of the main gallery by the beetle (~ a week). It is likely that the staining fungi that colonize the phloem and the sapwood during the 2 to 4 weeks following an initial MPB mass attack, modify the tree environment into a more favourable habitat for the progeny of the MPB. Further, all the associated MPB fungi might act more locally and participate in the decrease of moisture content, as well as in detoxifying chemicals (e.g. monoterpenes) in the gallery to allow for brood development. Then, I would expect that the fast growing fungi, *G. clavigera* and *L. longiclavatum* would be immediately abundant but then not increase much through the life cycle of the beetle. It has also been suggested that *G. clavigera* and *O. montium* provide a nutritional benefit for the beetle as the larvae and adults consume their spores and mycelium. Although no work has been done on the ecology of *Cop. sp.*1, I expect that this species could be a better nutrient source for the teneral beetles because its growth is restricted to the galleries and it is not melanized and so it cells are easily broken (unpublished data). Therefore, I anticipated that this species would be more abundant during the pupal and teneral phases of the MPB life cycle. To verify our initial hypothesis I used the primer sets developed in Chapter 2, in combination with qPCR, to track the relative abundance of the target fungi throughout the lifecycle of the MPB. To carry out the study, I sampled beetle galleries at four life stages: eggs, larvae, pupae and teneral adults. I extracted DNA from the gallery phloem and used the target-specific primers with qPCR to measure the relative abundance of the fungi. From the logs that were sampled, I measured moisture content and beetle attack density. Using multivariate analysis, I found an overall difference between the life stages, in terms of the relative abundance of all the fungi. When all the fungi were analyzed individually, I found a significant difference for *Cop. sp.*1. The sample data indicated that *Leptographium* in general and *L. longiclavatum* specifically showed a trend of increase through the life phases, although differences in life stages were not statistically significant. *O. montium* did not change much from the first life phase to the last. Finally, pairwise comparisons of the relative abundance of *Cop. sp.*1 between the different life phases, showed a significant difference between the pupal phase and the first two phases. Once they emerge from pupation, teneral adults eat nutrients available in the pupal chamber walls before maturing and leaving the tree. The sharp increase in *Cop. sp.*1 at the pupal phase could be associated with the higher nitrogen content observed in the lining of the pupal gallery chamber and this observation would support the hypothesis that *Cop. sp.*1 serves primarily as a nutritional source for the MPB.
Use of a combination of target specific primers and qPCR is a powerful tool to directly analyze environmental samples. Future work could include similar surveys in different stands to determine whether stands had an impact on changes of relative abundance of these species over the MPB lifecycle. As well, the work could be extended to the landscape to determine whether geographic variation affected the relative abundance of the target species. Finally, it would be important to compare galleries from beetles in epidemic, endemic and incipient phases; I expect that beetle populations will not have to overcome tree defences in an endemic population, since beetles attack mainly weakened trees and this may change the role that the fungi play in the system.

It is surprising that the only fungus showing a significant increase in relative abundance has not been formally described and has been largely ignored by other MPB studies. The physiology of this fungal species should be further studied. The UBC research lab has shown that Cop sp.1 can tolerate and use monoterpenes as a carbon source; however, it would be also important to characterize the nitrogen requirement of this fungal species, since its sudden abundance corresponds to nitrogen increases in the pupal chamber. qPCR is also an appropriate tool to detect and quantify this fungus in environmental samples. It is important that researchers assess the whole communities and not just an individual species or a few fungi. The qPCR method that I used here was limited to four main associates of the MPB. However, it is impractical to design target-specific primers for all the organisms that been described in association with the beetle. Instead, a more inclusive methodology such as metagenomics could be used to track the progression of the associated microflora of the MPB throughout its lifecycle. Currently, the costs associated with this methodology are too high to perform on a large scale, but the costs are decreasing making metagenomics more practical and relevant for studying microbial communities.
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