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The Relative Abundance of Mountain Pine Beetle Fungal Associates Through the Beetle Life Cycle in Pine Trees

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Abstract The mountain pine beetle (MPB) is a native bark beetle of western North America that attacks pine tree species, particularly lodgepole pine. It is closely associated with the ophiostomatoid ascomycetes *Grosmannia clavigera*, *Leptographium longiclavatum*, *Ophiostoma montium*, and *Ceratocystiopsis* sp.1, with which it is symbiotically associated. To develop a better understanding of interactions between beetles, fungi, and host trees, we used target-specific DNA primers with qPCR to assess the changes in fungal associate abundance over the stages of the MPB life cycle that occur in galleries under the bark of pine trees. Multivariate analysis of covariance identified statistically significant changes in the relative abundance of the fungi over the life cycle of the MPB. Univariate analysis of covariance identified a statistically significant increase in the abundance of *Ceratocystiopsis* sp.1 through the beetle life cycle, and pair-wise analysis showed that this increase occurs after the larval stage. In contrast, the abundance of *O. montium* and *Leptographium* species (*G. clavigera*, *L. longiclavatum*) did not change significantly through the MPB life cycle. From these results, the only fungus showing a significant increase in relative abundance has not been formally described and has been largely ignored by other MPB studies. Although our results were from only one site, in previous studies we have shown that the fungi described were all present in at least ten sites in British Columbia. We suggest that the role of *Ceratocystiopsis* sp.1 in the MPB system should be explored, particularly its potential as a source of nutrients for teneral adults.

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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 [1]. 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 microorganisms, are complex and vary from beneficial to antagonistic [2, 3]. Here we focus on the native western North American bark beetle, *Dendroctonus ponderosae* (mountain pine beetle, MPB). The MPB has infested over 17.5 million hectares of pine (*Pinus*) forest in interior British Columbia (BC), Canada, in an epidemic that began in 1994 and has since spread into all suitable habitats in BC [4]. Large-scale outbreaks of MPB are also present in western Alberta, Canada and in some of the western states in the USA [5].

The MPB life cycle is largely driven by local climate and typically is univoltine, having one brood per year. Depending on the climate, populations can also be semivoltine (more than one year per brood) or have two broods per year (parents will emerge from a tree and attack another tree in the same season). During an epidemic, mass attack of trees by adult beetles typically occurs between July and early August. While overcoming the host trees' chemical

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defenses, the adult beetles create vertical galleries in the phloem, propagating fungal spores that are carried externally or internally (maxillary mycangium or gut) by both sexes. The beetles mate and lay eggs along the walls of the gallery. Upon hatching, the larvae create feeding galleries perpendicular to the parents' main gallery. Typically, the larvae over-winter as third or fourth instars and pupate in late spring. The young adult beetles feed on microorganisms present in the galleries, before emerging from their host trees, spreading into new hosts during the summer and repeating the annual life cycle [5–8].

The MPB-associated ascomycetes *Grosmannia clavigera*, *Leptographium longiclavatum*, and *Ophiostoma montium* belong to the Ophiostomatales. They are the first to colonize beetle galleries. As they enter the adjacent phloem and sapwood host tissues, these fungi produce the polyketide pigment melanin, which causes a blue or gray discoloration of the wood of the host tree [9]. While *G. clavigera* and *L. longiclavatum* have been shown to kill trees in the absence of the beetle, the pathogenicity of *O. montium* is uncertain [10, 11]. These three species have been isolated from MPB-infested sapwood, MPB body surfaces, mycangia, and adult and larval galleries [9, 12–17]. Two additional, slower growing fungal species have also been commonly isolated from MPB and their galleries: the ophiostomatoid *Ceratocystiopsis* sp.1 and the basidiomycete *Entomocorticium* sp. [9, 18]. The four Ophiostomatales species, *G. clavigera*, *L. longiclavatum*, *O. montium*, and *Ceratocystiopsis* sp.1, have not been isolated from other bark beetles and, therefore, appear to be specifically associated with MPB [9, 19]. The specificity of the association of *Entomocorticium* with the MPB is uncertain. The genus has been observed on various beetle species, but a thorough taxonomic analysis has not been performed [20].

MPB and its vectored fungi form symbiotic relationships, which have been shown in some cases to be mutualistic [3, 8, 15]. The fungi depend on their association with the beetles for transport and dissemination into new host trees for access to fresh nutrients. The fungi can influence beetle fitness in several ways. Fungal colonization of trees has been reported as a prerequisite for successful bark beetle brood development in pines [18, 21]. Fungi grow in the phloem and sapwood of the host trees relatively faster when trees have been mass attacked by MPBs, compared to when they are artificially inoculated (i.e., the sapwood can be completely colonized by staining fungi 4 weeks after initial beetle attack); this results in a substantial decrease of the moisture content to a level that may be more suitable for successful beetle brood development [22]. Adult beetles and their larvae feed on the phloem, which is rich in carbon but low in sterols, nitrogen, vitamins, and other growth factors [15]. However, sterols are essential for the normal growth, molting, and reproduction of many beetles [23–25]. Larvae and new adults may benefit from the fungal production of ergosterol, which has been shown to have positive impacts on the weight, size, and normal development of the beetles [23–25]. It has also been shown that phloem with MPB fungal associates contains more nitrogen than phloem without [26]. Thus, fungi in the pupal chamber and galleries provide an important source of nutrients (sterol and nitrogen) for maturing young beetles [27].

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. Microscopic observation, isolation, and growth of fungi have commonly been used to suggest that beetles feed selectively on specific fungi or to compare phloem and sapwood colonization by different fungi [26, 28]. However, these methods are not necessarily accurate; they are biased toward the fast-growing species and do not differentiate similar species [26, 28]. Determining the presence and abundance of a fungal symbiont at any particular time during the bark beetle's life cycle requires molecular methods such as target-specific diagnostic PCR or quantitative (qPCR). These methods use target-specific DNA primers; they are simple and sensitive and can detect both fast- and slow-growing species independent of their growth rates [29]. They have been used extensively for detecting and quantifying microorganisms in environmental samples [29–32]. However, to our knowledge, there is only one report of qPCR to quantify ophiostomatoid spores associated with bark beetles [33].

The goals of this study were to identify and compare the relative abundance of MPB fungal associates through different stages of the beetle's life cycle. Such a quantitative assessment may generate insights into the ecological roles of individual fungi or their communities. In this research, we used qPCR with target-specific primers to detect and assess the relative abundance of four fungal species associated with MPB: *G. clavigera*, *L. longiclavatum*, *O. montium*, and *Ceratocystiopsis* sp.1. We hypothesized that the faster growing pathogenic fungi would be more abundant in stages of the MPB life cycle associated with host attack and host colonization, while the less pathogenic and slower growing fungi may increase in abundance throughout the following stages of the life cycle that involve larval development or in the stage of teneral adults feeding on fungi in preparation for their flight. Changes in the relative abundance of a fungus in the gallery at a particular life stage suggest that this fungus may be a factor in beetle fitness at that stage; testing this possibility is an area for future work.

Materials and Methods

Site and Experimental Setup

We used lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) trees from a Similkameen very dry cool Montane

Spruce variant stand (MSxk1) located at the Bear Forest Service Road near Merritt, BC, at an elevation of approximately 1,400 m. The stand was populated primarily with lodgepole pine, alpine fir (*Abies lasiocarpia*), and hybrid spruce (*Picea glauca* × *Picea engelmannii*). 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 temperatures (Supplementary Fig. 1) and a low overall number of degree-days, resulting in a local MPB population with a semivoltine life cycle and an immigrant beetle population with a univoltine life cycle. We determined that the immigrant population was univoltine because no local emergence was occurring while new trees were being attacked in July. Based on data from HOBO data loggers (Onset Computer, Bourne, MA, USA), from July 15, 2009 to July 15, 2010, a total of 798 degree-days above 5.5 °C were recorded; this falls short of the 833 degree-days required for the MPB to be univoltine [34]. To ensure consistent MPB mass attack, we baited 30 trees with Mountain Pine Beetle Tree Bait (Contech Enterprises, Delta, BC, Canada). A bait pouch was attached to the north side of the tree at approximately 2 m high on July 15, 2009 and removed 3 weeks later as the MPB attack ceased. By baiting the trees and obtaining mass attack in July, we were able to ensure that the beetles in our sample trees did follow a univoltine life cycle.

Three baited trees were randomly selected and felled at four beetle life stages: eggs, larvae, pupae, and teneral adults. They were cut on July 24 and August 7, 2009 for the first two stages and July 5 and 28, 2010 for the two last stages. One 1.5-m bolt was cut from each tree, at 30 cm off the forest floor. To prevent moisture loss, the bolt ends were sealed with silicone, wrapped in heavy plastic, and transported to the laboratory where they were kept at 4 °C until they were processed within the following week.

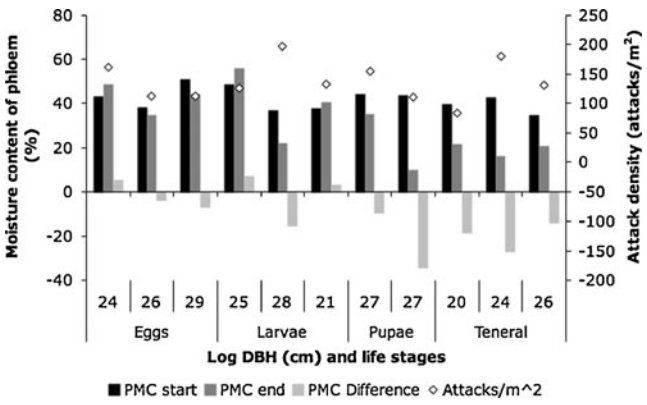

Figure 1 Summary data for sampled trees, including phloem moisture content (PMC) at the beginning of the study (*black*) and at time of harvesting (*dark gray*) and the difference between the two values (*light gray*), attack density (*diamond*), life stage, and diameter at breast height (DBH). These data show the variability between the trees used and why a hierarchal statistical analysis was necessary to account for this variation

Sampling

Mountain pine beetle entry and exit holes were marked and dated with thumbtacks during the entire period of the MPB attack in 2009. Surveys were carried out daily during the initial attack and once every 2 to 4 days as the MPB attack frequency declined. This information was used to determine attack densities (Fig. 1). For each tree, moisture measurements were taken from a 2.5×2.5-cm piece of phloem and sapwood removed from the north side of the tree at approximately 30 cm above the ground before baiting the tree and again at the time of harvesting, once the logs were brought to the laboratory, within 12 h of harvesting. Sapwood moisture content was taken from a layer just below the phloem, with a depth of a few millimeters (Fig. 1).

When possible, bark was peeled off in large pieces that contained the complete, intact galleries; samples that included both the phloem lining the gallery and the phloem directly adjacent to it were taken using a 7-mm diameter cork borer (Fig. 2). We targeted particular life stages in the galleries. The samples were taken at the locations where the beetles, larvae, or pupae were active. Sampling was done on two levels: trees and samples within trees. For most life stages, we obtained 10 independent samples from each of three independent trees. Independent in this case means that we only sampled once from a particular parental gallery. It was not possible to obtain 10 independent samples from each tree for the pupal and teneral adult stages where we obtained a total of 22 samples from two trees and 21 samples from three trees, respectively. At these two stages, due to low moisture content, it was difficult to isolate large, intact pieces of bark. We also observed relatively low overwintering success, which resulted in a low number of beetle progeny present in the pupal and teneral adult samples. All samples were frozen in microcentrifuge tubes at −20 °C until they were processed for DNA extraction.

DNA Extraction

Gallery samples in 1.5 ml microcentrifuge tubes were freeze-dried overnight prior to DNA extraction. DNA was extracted using the protocol described in Khadempour et al. [35]. Briefly, the lyophilized samples were homogenized for 60 s using a FastPrep Automated Homogenizer (MP Biomedicals, Solon, OH, USA, speed setting 6). TES buffer and lysing enzymes were added to each tube to complete cell lysis. This was followed by one CTAB and two phenol/chloroform purification steps. DNA was precipitated from the solution using a mixture of sodium acetate and

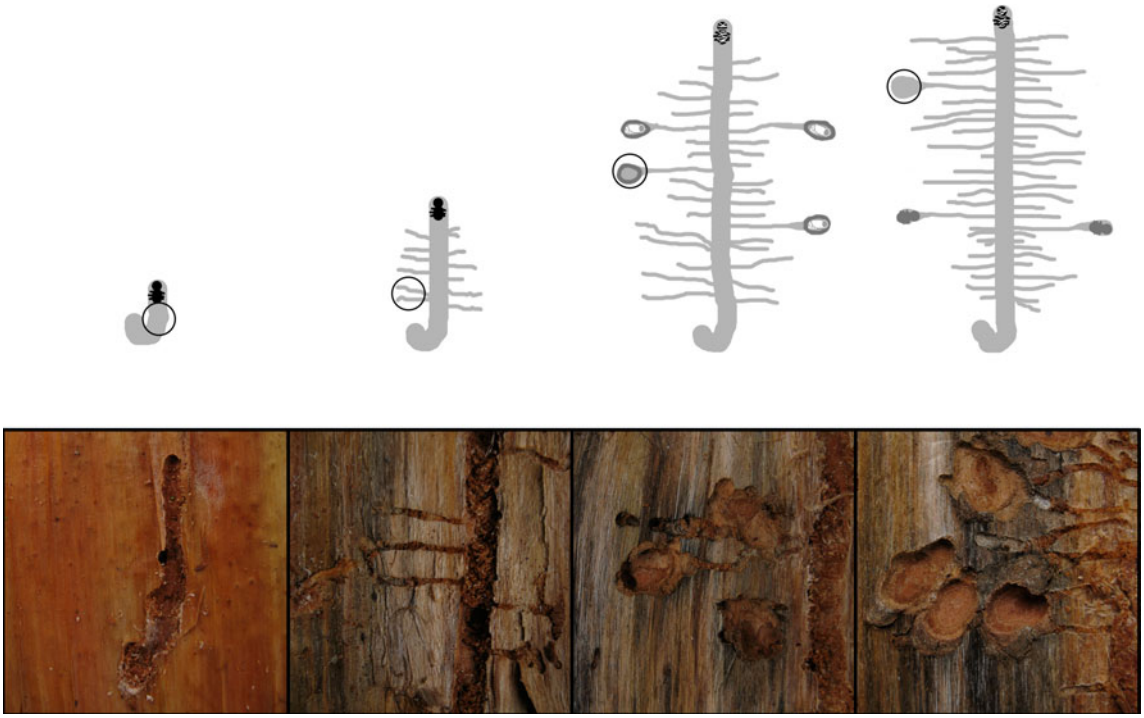

Figure 2 Schematic diagram of sampling locations, with images of beetle galleries at respective life stages: **a** eggs, **b** larvae, **c** pupae, and **d** teneral adults. The samples for DNA extraction were from the galleries and surrounding phloem. Samples were taken where the targeted life stage was active

isopropanol; DNA pellets were washed with ethanol and re-suspended in distilled water.

Quantitative PCR and Target-Specific Primers

All qPCR reactions were performed using the CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with Sso Fast Eva Green Supermix (Bio-Rad). 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 melting curve analysis ranging from 65 to 95 °C. Each reaction tube contained 10 μl of Supermix, 0.5 μl of each of the forward and reverse primers, 5 μl of undiluted DNA, and 4 μl of distilled water.

Each reaction was duplicated and the reciprocal of the mean threshold cycle (C_t) value was used as a measure of the relative abundance of each fungus. The primers used were Omon, Lepto, Llongi, and CopMPB as previously described in Khadempour et al. [35]; they amplify *O. montium*, MPB-associated *Leptographium* (i.e., *G. clavigera* and *L. longiclavatum*), *L. longiclavatum*, and the MPB-associated *Ceratocystiopsis* sp.1, respectively [35]. We added a plant-specific primer set: 28KJ (GGCGGTAAATTCCGTCC) and 28B (CGTCCGTGTTTCAAGACG) [36]. We tested the phloem of non-infected lodgepole pine trees to ensure that our fungal target-specific primers did not amplify tree DNA. Although the primers had been thoroughly tested for specificity in previous work [35, 36], we retested and confirmed their specificity against closely related species with the new reaction mixture and qPCR program.

Statistical Analysis

We performed *a priori* power analysis to determine the number of samples that would likely detect a biologically significant result in changes in fungal abundance, based on the work of Schweigkofler et al. [33]. The reciprocal C_t values from each of the four primer sets were used as four *y*-variables, as a measure of fungal relative abundance. 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 [1, 2]; untransformed reciprocal plant C_t was used as a covariate along with beetle life stage as a class variable. These four stages were eggs, larvae, pupae, and teneral adults. Because of the hierarchical structure of the sample data, two error terms were included: logs within life stage and samples within logs. For the MANCOVA tests, the logs within life stage was used as the error term, whereas the samples within logs provided within log variation only [37]. 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 α=0.05 and a normalized-rank transformation of each *y*-variable [38] 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 [39]. All tests were performed using SAS® 9.2 Statistical Software Package (SAS Institute Inc., Cary, NC, USA).

Results

General Site Observations

Described here are a number of general observations of site parameters, which were beyond the control of the experiments, but which might be relevant for the interpretation of some of the results. The study site was surveyed annually for 4 years prior to the experiments. During those years, the local MPB beetle population did not show consistent univoltine life cycles. Specifically, due to the low, variable summer temperatures in 2009, the local beetle population showed a semivoltine life cycle. In addition, due to the massive spread of the MPB epidemic at the time of the experiment, beetles from surrounding regions invaded the study site with attack of trees in July, before the local population began their flight. We used pheromone baits to orchestrate a synchronous univoltine mass attack similar to natural attack scenarios. We also observed that the overwintering success of beetle offspring for the duration of this study was relatively low. This may be explained by the increased presence of parasites and secondary beetles during the late phase of the MPB epidemic, or by suboptimal conditions for bark beetle development in trees that were baited and attacked only late in the epidemic and may not have been the preferred host trees under natural attack. Furthermore, the density of attack on baited trees was relatively high, between 89 and 197 attacks/m^2 (Fig. 1), which is substantially above the optimal density of 40–80 attacks/m^2 [40]. High attack density may have affected offspring survival. Between the time of baiting and the last sampling time point, the moisture content of the phloem changed most strongly for the pupal and teneral stages, while loss of phloem moisture was much less during the egg laying and larval stages (Fig. 1).

Assessment of the Abundance of Fungal Associates Using qPCR

Before determining the relative abundance of the target fungi in the gallery samples, we optimized the qPCR reaction. The Sso Fast EvaGreen Supermix used for the qPCR reactions contains a double-stranded DNA binding dye and a high fidelity polymerase; the mixture is optimized to give a fast reaction that can decrease the impact of inhibitors that may be present in the DNA extracts. Except for the *G. clavigera* and *L. longiclavatum*-specific primers, the target-specific PCR primers that we used in qPCR were developed from the ribosomal DNA of the fungal species present on or in MPB bodies and in beetle galleries [35]. We confirmed the specificity of the Omon, Lepto, Llongi, and CopMPB primers in the qPCR reaction. While the *G. clavigera* (Gclavi) primer set was specific in PCR, allowing us to differentiate *G. clavigera* from *L. longiclavatum*, it was non-specific in qPCR. We hypothesized that the non-specificity of *G. clavigera* primers in the qPCR was due to the high GC content of the gene selected. Thus, for the qPCR work, we excluded the *G. clavigera* primer set and used instead the Lepto primer set that reacts with *G. clavigera* and *L. longiclavatum*, two closely related species with leptographium anamorphs [35].

To further verify the efficiency and the specificity of each set of fungal primers, we generated three types of standard curves by serial DNA dilution. First, we tested efficiency by generating a standard DNA curve for each of the fungal species with their target primers. Supplementary Fig. 2 shows that the efficiency of the primers was approximately 100 %, i.e., the PCR product was doubling in each reaction cycle. Next, we assessed whether the specific primers could detect their targets as effectively in the presence of non-target DNA, e.g., DNA from the host tree or from other fungal species. The threshold cycles for detecting known concentrations of target fungal DNA were insensitive to additions of DNA extracted from tree phloem and non-target fungal DNA. This indicates that the primers were specific and detected their targets efficiently in complex DNA mixtures (Supplementary Fig. 3).

Target-specific primers were used for quantifying the relative abundance of the four different Ophiostomatales colonizing the beetle galleries during the different stages of its life cycle. To ensure consistent results, given higher concentrations of tree DNA than DNA from targeted fungi in the gallery samples, and traces of tree defense chemicals like phenolics or terpenoids in the reaction, it was necessary to use a relatively large amount (5 μl) of undiluted DNA.

Statistical Analysis

Using MANCOVA, we observed a significant difference in the combined relative abundance of the four fungal species through the life cycle of the beetle, indicating that the relative abundance of the fungi considered together, changed through the beetle life cycle (Table 1). With ANCOVA, for each of the fungal species, only *Ceratocystiopsis* sp.1 showed a significant

Table 1 MANCOVA test criteria and *F* approximations for the hypothesis of no overall life stage effect

Statistic	Value	*F* value	Num *DF*	Den *DF*	Pr > *F*
Wilks' lambda	0.019303	3.12	12	10.875	0.0352
Pillai's trace	1.514418	1.53	12	18	0.2016
Hotelling–Lawley trace	26.12989	8.71	12	4	0.0252
Roy's greatest root[a]	25.27600	37.91	4	6	0.0002

[a] *F* statistic for Roy's greatest root is an upper bound

change over the beetle life cycle (P=0.0038) (Fig. 3). The other fungi did not show a significant difference in relative abundance at different life stages, although they all showed a trend toward an increase (Fig. 3). Pairwise tests of *Ceratocystiopsis* sp.1 means between different life stages showed a significant difference between the eggs and pupal life stages (P=0.0018). Other life stage comparisons were not significantly different but had P values just above the α=0.002 threshold, indicating that there was a trend toward change throughout the life stages.

Discussion

In the early stages of its life cycle, the MPB may maintain specific microflora that prevent 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) [16, 17]. For the fast-growing sap-staining fungi, our results on the relative fungal abundance during the life cycle of the beetle are consistent with the literature [28, 41]; *G. clavigera* and *L. longiclavatum* colonize the phloem rapidly and are followed by the less pathogenic *O. montium*. *G. clavigera* and *L. longiclavatum* were most abundant in the teneral adult stage, while the abundance of *O. montium* was approximately constant during the four stages. Consistent with our results, species of the genus *Leptographium* are known to be pioneer MPB-vectored fungi that colonize the phloem and sapwood during the 2 to 3 weeks following a massive MPB attack [42]. However, we also showed that the slow-growing species *Ceratocystiopsis* sp.1, while present at low abundance in

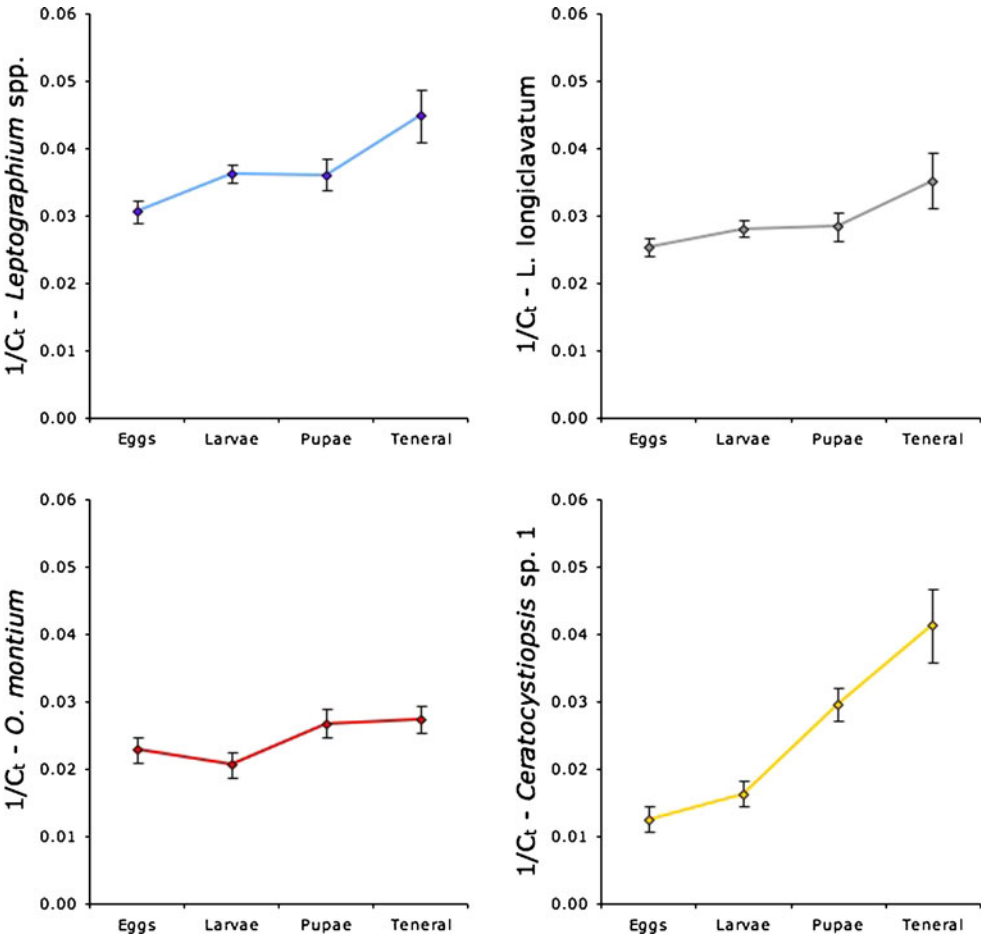

Figure 3 Untransformed data showing relative abundance of target fungi changing throughout the MPB life cycle. Only *Ceratocystiopsis* sp.1 shows a significant change while *Leptographium* and *L. longiclavatum* both show trends toward increasing. *Error bars* show a 95 % confidence interval. $1/C_t$ is the reciprocal of the cycle threshold and is a measure of the abundance of the target fungi

the beetle gallery during early infestation, became statistically more abundant during the pupal stage of the beetle life cycle. Because this fungus has largely been ignored or reported under different names in previous work, below we review what is known for this species.

Surveys of MPB-associated fungi carried out in British Columbia during the past decade by the Breuil laboratory at UBC isolated a slow-growing *Ceratocystiopsis* species frequently from MPBs, beetle progeny (larvae, pupae, and young adults), and beetle galleries, but only occasionally from sapwood surfaces of MPB-infested lodgepole pine [9, 17, 19]. Also, this species was not found on other beetles cohabiting MPB-infested lodgepole pine. In an early work, we reported this species as *Ophiostoma minutum* [17] or *O. minutum*-like [9]. This *Ceratocystiopsis* species produces a *Hylalorhinocladiella* anamorph with oblong conidia and a teleomorph with perithecia and falcate ascospores. Perithecia were occasionally found in beetle galleries, but were not produced in the laboratory despite multiple attempts. This species has also been reported as *Ceratocystiopsis minuta* and *O. minutum* in other studies [13, 43]; further, *C. minuta* was reported from five different bark beetles infesting nine tree species in six continents [18]. Detailed phylogenetic analyses with additional *Ceratocystiopsis* species showed that these *Ophiostoma*/*Ceratocystiopsis minuta* isolates represented multiple species [18] and that the MPB associates surveyed were not the *C. minuta* found in Europe and Japan, but a different species that is closely related to *Ceratocystiopsis ranaculosa* and *Ceratocystiopsis manitobensis* [18]. Given this, in more recent work, we have reported *Ceratocystiopsis* from MPB as *Cop*. sp.1 (*Ceratocystiopsis* sp.1) (for more information on *Ceratocystiopsis* sp.1, refer to supplementary materials).

Our results show that qPCR can be effective 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, galleries, and tree phloem [28] and to assess the colonization of phloem by staining fungi (i.e., *G. clavigera* and *O. montium*) at different stages of the beetle life cycle [41]. However, identification using morphology may not accurately differentiate closely related fungal species (e.g., *G. clavigera* from *L. longiclavatum*). In a previous work, we verified primer specificity and compared traditional fungal isolation to detection by PCR [35]. Here, we used these specific primers in qPCR. Our PCR primers developed from rDNA (Omon, Lepto, and CopMPB) were sensitive and specific in qPCR, as expected when multiple copy genes are used to develop primers [44, 45]. rDNA regions are often used for developing molecular diagnostics and have been used for analyzing phylogenetic relationships in a wide range of fungal taxa [46]. However, as we report here for *G. clavigera* and *L. longiclavatum*, rDNA can be unsuitable for differentiating closely related species; for such cases, primer design should use other conserved regions of the genome, or other genes. In our work, we used a peroxisomal coenzyme A gene (GenBank Accession number EE729832), that, when sequenced, differentiated *G. clavigera* and *L. longiclavatum* [47]. However, for qPCR, only the *L. longiclavatum* primer set was specific, while the *G. clavigera* set was non-specific. The Llongi primers were designed on a 21-bp insertion that was not present in *G. clavigera*, while the Gclav primers were designed on a high GC region in the same gene. We note that the qPCR curves for the Llongi and Lepto primers were nearly identical (Fig. 3) and that we have isolated both *G. clavigera* and *L. longiclavatum* from this stand. Given this, we anticipate that the life cycle abundance profiles for *G. clavigera* and *L. longiclavatum* were similar; if *G. clavigera* and *L. longiclavatum* had different abundance profiles, the Lepto and Llongi curves would have differed. As more genomes are sequenced in the near future, designing effective primers for such work should become easier.

PCR and qPCR are widely used in microbial ecology because they have a number of advantages over conventional culturing techniques. PCR quantification can be either absolute or relative. Absolute quantification associates a reaction cycle threshold with gene copy number [48], which can represent biomass, or the number of spores or cells [49] in a sample. For example, Schweigkofler et al. [33] 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. We could not use a similar approach in our work because we were measuring DNA from mixtures of spores and mycelia. Both tightly bound fungal and phloem cells, and the presence of chemicals (e.g., terpenes and phenolics), can affect DNA extraction; further, we noted that DNA extraction efficiency differs between spores and mycelia, both within a species and between species. Thus, we used relative qPCR quantification, which is still very informative when comparing samples, in order to determine the relative abundance of fungi in samples in the ecosystem that we studied. Our ability to compare our results to previous work is constrained; we applied qPCR, and two of the four species that we profiled were not included in earlier published studies. While for qPCR, we only have data for one site, in our previous work we isolated all four fungal species at 10 BC sites, confirming that they are part of the MPB ecosystem in Canada [9, 17, 19].

All the fungi described here belong to the Ophiostomatales and are disseminated in the pine tree as the MPB mines its gallery in the tree. Three of the BC species most frequently isolated in and on beetles and in wood are the pioneer

colonizers *G. clavigera*, *L. longiclavatum*, and *O. montium* [50]. The *Leptographium* colonizers *G. clavigera* and *L. longiclavatum* are known to grow at high moisture content with low oxygen [9]. Such conditions prevail in trees when MPBs enter and build their galleries. When these are well established, they decrease phloem and sapwood moisture content. We also observed a decrease in the phloem moisture at the pupal stage of the life cycle. Since these pioneer fungi can colonize the galleries and phloem quickly, their abundance increases only marginally over the life cycle of the beetle in the tree. These fungi may contribute to the establishment of the beetle progeny, and they have been reported as nutrients or supplements to the MPB diet [21, 26].

We showed that the slow-growing *Ceratocystiopsis* sp.1, which was mainly isolated from galleries, increased substantially during the pupal and teneral adult stages. The sudden increase in this fungus may be related to the presence of the meconium (i.e., waste products exuded by larvae before pupation) that coats the pupal chamber walls. This coating is high in nitrogen (Jack, unpublished data) and may stimulate fungal growth and increase fungal biomass. *Ceratocystiopsis* sp.1 may be a preferred food for young teneral adults. It is important to note that this fungus does not produce melanin, a cell wall pigment that protects fungi against environmental stress [51]; this may make the fungus more palatable than the other fungi in the system.

In this work, we profiled how relative abundance of four fungal species changed through the beetle life cycle at one site in BC. It has been shown that *G. clavigera*, *O. montium*, and *L. longiclavatum* can be isolated from MPB mycangia and are MPB symbionts. Although *Ceratocystiopsis* sp.1 has not yet been shown to benefit the beetle, it is associated only with MPB and has been isolated from no other co-inhabiting beetles (e.g., *Ips* or ambrosia beetles). *Ceratocystiopsis* sp.1 has the potential to be a significant MPB symbiont and should be formally described. The relative fungal abundance in different life stages may contribute to beetle fitness. The potential benefits from these four fungal species should be tested at different beetle life stages, e.g., following methods described by Six and Paine [21]. Such changes may be due to each species filling a micro-niche and having a specific role within the MPB system and may reflect differences in environmental conditions like temperature, moisture content, and nutrient abundance in parts of the ecosystem [52]. The clarification of such factors remains for future work.

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