**Fine-scale genetic structure and relatedness in fungi associated with the mountain pine beetle**

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>Canadian Journal of Forest Research</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjfr-2018-0418.R2</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>21-Feb-2019</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Tsui, Clement; Sidra Medicine; University of British Columbia, Department of Forest and Conservation Science Beauseigle, Stephanie; Biopterre Ojeda, Dario; Norsk Institutt for Biookonomi Stjordal Rice, Adrianne; University of Alberta, Biological Sciences Cooke, Janice; University of Alberta Sperling, Felix; University of Alberta, Biological Sciences Roe, Amanda; Great Lakes Forestry Centre Hamelin, Richard; University of British Columbia</td>
</tr>
<tr>
<td>Keyword:</td>
<td>Symbiosis, Ophiostomatoid fungi, Blue-stain fungi, Population genetics, Mountain Pine Beetle</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>MPB TRIA-Net</td>
</tr>
</tbody>
</table>

https://mc06.manuscriptcentral.com/cjfr-pubs
Fine-scale genetic diversity and relatedness in fungi associated with the mountain pine beetle

*Clement K.-M. Tsui\textsuperscript{a,b}, *Stéphanie Beauseigle\textsuperscript{c}, Dario I. Ojeda Alayon\textsuperscript{c,d}, Adrianne V. Rice\textsuperscript{f}, Janice E.K. Cooke\textsuperscript{f}, Felix A. H. Sperling\textsuperscript{f}, Amanda D. Roe\textsuperscript{e}, Richard C. Hamelin\textsuperscript{c,g}

\textsuperscript{a}Department of Pathology, Sidra Medicine, Doha, Qatar
\textsuperscript{b}Department of Pathology and Laboratory Medicine, Weill Cornell Medicine - Qatar, Doha, Qatar
\textsuperscript{c}Department of Forest and Conservation Sciences, University of British Columbia, Vancouver, BC, Canada
\textsuperscript{d}Norwegian Institute of Bioeconomy Research (NIBIO), Department of Forest Genetics and Biodiversity, Høgskoleveien 8, 1433 Ås, Norway
\textsuperscript{e}Canadian Forest Service - Natural Resources Canada, Great Lakes Forestry Centre, Sault Ste. Marie, ON, Canada
\textsuperscript{f}Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada
\textsuperscript{g}Département des sciences du bois et de la forêt, Université Laval, Québec, QC, Canada

*Clement Tsui and Stephanie Beauseigle have joint first authorship

**Corresponding Author:** Richard C. Hamelin (email: richard.hamelin@ubc.ca)
Department of Forest and Conservation Sciences, University of British Columbia, Vancouver, BC, Canada; Département des sciences du bois et de la forêt, Université Laval, Québec, QC, Canada

https://mc06.manuscriptcentral.com/cjfr-pubs
Abstract

The mountain pine beetle forms beneficial symbiotic associations with fungi. Here we explored the fine-scale spatial genetic structure of three of those fungi using single nucleotide polymorphism. We found that single mated pairs of beetles not only carry multiple fungal species, but also multiple genotypes of each species into their galleries. We observed genetic diversity at a fine spatial scale. Most of the diversity was found within and among galleries with non-significant diversity among trees. We observed clonal propagation almost exclusively within galleries. *Ophiostoma montium* possessed larger expected number of multilocus genotypes and lower linkage disequilibrium than *Grosmannia clavigera* and *Leptographium longiclavatum*. More than 80% of fungal samples were genetically unrelated, a result that parallels what has been observed in the beetles. The proportion of genetically-related samples within galleries was higher in *O. montium* (40%) than in *G. clavigera* (20%) or *L. longiclavatum* (6%), likely the consequence of within-gallery sexual recombination in *O. montium*. The underlying genetic diversity reported here and the differences among fungal species could enable the symbiont community to quickly respond to new environmental conditions or changes in the host, enhancing the maintenance of this multipartite relationship and allowing the MPB to colonize new habitats.

Keywords: beetle gallery, population structure, beetle symbiont, relatedness, pathogen, pine

Introduction

The mountain pine beetle (MPB; *Dendroctonus ponderosae*) is a forest pest that is experiencing a large-scale outbreak since the 1990s that resulted in the mortality of millions of pines in western North America (Safranyik and Carroll 2006; Kurz et al. 2008). These beetles have developed a mutualistic association with certain fungi that they carry on their
exoskeleton and in mycangia, special structures evolved to harvest and transport spores (Whitney and Farris 1970). Three ascomycetous fungi are consistently associated with MPB: *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., *Leptographium longiclavatum* S.W. Lee, J.J. Kim & C. Breuil and *Ophiostoma montium* (Rumbold) Arx (Ophiostomataceae) (Solheim 1995; Six and Klepzig 2004; Lee et al. 2005; Zipfel et al. 2006). These fungi are known to play important roles in the MPB life cycle by providing supplementary nutrition to the beetle larvae, helping to overcome tree defences and modifying host tissues to favour brood development (Raffa and Berryman 1983; Paine et al. 1997). MPB attack trees en masse to overcome their defenses. During the attack, adult beetles penetrate through the bark and females build vertical galleries in the phloem tissues where they lay eggs; during this phase of the attack they also introduce fungi into the galleries (Six and Wingfield 2011). As the fungi develop and spread throughout the phloem and sapwood, they interrupt the flow of water to the tree’s crown and reduce the tree’s flow of pitch, thus aiding the beetles in overcoming the tree’s defences. In exchange, the fungi gain access to host tissues that would be otherwise inaccessible. The ability of *G. clavigera* and *L. longiclavatum* to cause lesions within the phloem tissue and to kill trees has been demonstrated following artificial inoculations (Yamaoka et al. 1995; Lee et al. 2006a); by contrast, *O. montium* is either less aggressive or non-pathogenic. The combined action of beetles and fungi results in a general breakdown of the tree’s vital function, such as water transport, ultimately resulting in tree death.

The nature of the interaction among the beetles and the fungi is complex and involves a multipartite association. The relationship has been studied using various approaches including population genetics and genomics. The population structure of the three fungal symbionts and their beetle vector has been observed to be largely congruent, which supports the hypothesis of a close relationship among the organisms of this multipartite association.
(James et al. 2011; Tsui et al. 2012, 2014; Ojeda Alayon et al. 2017). However, small
differences in spatial and temporal distribution (Roe et al. 2011a), population structure, and
genetic diversity (Roe et al. 2011b; Tsui et al. 2012, 2014; Ojeda Alayon et al. 2017) suggest
that these fungi could occupy slightly distinct ecological niches and play different roles in
this multipartite symbiosis.

In spite of this rich landscape-level knowledge of the population structure of the MPB
symbionts, there are gaps in our knowledge of their genetic composition at a fine-scale; this
could be important since dispersing MPB can move over both long and short distances. Some
of the unanswered questions are: 1) how is the genetic diversity distributed at a very fine
spatial scale and is it similar in the three fungal symbionts? 2) do multiple genotypes of a
single species occupy a single gallery or do single clonal lineages dominate via competitive
exclusion? 3) are fungi within galleries more genetically related than among galleries? The
aim of this research was to answer these questions by comparing and contrasting the fine-
scale genetic structure of three common MPB–fungal associates sampled hierarchically
within a stand using single nucleotide polymorphism (SNP) panels developed for each
species (Ojeda Alayon et al. 2017). This study will help further our understanding of the
ecological factors that regulate the complex interactions among the MPB and its fungal
symbionts. The data may provide important information for understanding MPB dispersal
and developing more effective and sensitive ways to control their movement.

Materials and Methods

*Isolates, culture conditions and DNA extraction*

Fungi were isolated from an MPB infested lodgepole pine stand in Fairview, Alberta,
Canada (latitude 56.28° N, longitude 118.31° W). We felled five trees infested with MPB and
cut each tree into five to six sections 0.5m-1.0m in length. We randomly selected two
sections of each tree and brought them back to the laboratory. We removed the bark of each section and exhaustively sampled every beetle (adults and larvae) found within each complete gallery; all the beetle adults and larvae within the galleries were sampled using sterilized forceps. Fungi were isolated from the larvae or the adults, and the wood (5 mm² chips) adjacent to the beetle in the gallery tunnel by wiping the beetles or wood sample on 2% malt extract agar (MEA; 20 g malt extract (Difco Laboratories, Detroit, Michigan), 1L H2O) covered with a cellophane sheet (Lee et al. 2005). Fungi were allowed to grow and colonies displaying Ophiostomatacae morphology were transferred to new plates to generate pure cultures, followed by single hyphal tip transfer to obtain a single individual per plate. In total, we collected 155 samples from the three fungal species (Table S1, Supporting information). Each culture was maintained on MEA, and genomic DNA was extracted using the CTAB/phenol-chloroform method (Lee et al. 2007; Roe et al. 2011b; Tsui et al. 2012; Ojeda Alayon et al. 2017). Taxonomic identification was performed first by using morphological features (Zipfel et al. 2006) followed by polymerase chain reaction (PCR) amplification of the ribosomal RNA (rRNA) region (about 300 bp) using species-specific oligonucleotide primers (synthesized by Integrated DNA Technologies, Inc.) (Khadempour et al. 2010, 2012) to confirm species identity.

**SNP panel design and genotyping**

Three species-specific genotyping arrays had been developed using the Sequenom Iplex Gold technology (Ojeda et al. 2014). These arrays consisted of four panels, each containing up to 36 SNPs. The workflow for gene selection and SNP discovery and validation are described in (Ojeda et al. 2014, Ojeda Alayon et al. 2017). DNA from each sample was genotyped at McGill University and the Génome Québec Innovation Centre.

**Genetic diversity and population structure of three fungal symbionts**
Population genetic analyses were performed in *poppr* in R (Kamvar et al. 2014) and *GenAlEx*6 (Peakall and Smouse 2012). We removed loci with >5% of missing data in each species’ dataset using the *missingno* function in *poppr*. We clone-corrected each species dataset prior to frequency-based analyses using the *clonecorrect* function in *poppr*. The number of multi-locus genotypes (MLG), Shannon-Wiener index of diversity (H), and expected heterozygosity were calculated in *poppr*. To account for the difference in the number of fungal isolates of each fungal species obtained in the different trees, we calculated the expected MLGs (eMLGs) using the lowest sample size with a rarefaction method using the function *rarecurve* implemented in the R package *vegan*. This provides an estimate of the number of MLGs expected if the sample size had been the same for all three species.

MLGs were collapsed with the *mlg.filter* function in *poppr* by setting a cutoff of 2. The index of association (*I_A*), a measure of linkage disequilibrium that is used to measure clonality in organisms with asexual cycles (Agapow and Burt 2001), was calculated using *poppr*. *I_A* is zero in populations at equilibrium and increases with linkage disequilibrium. In addition, we calculated rbarD, a linkage disequilibrium index that is adjusted for differences in the number of loci sampled (Agapow and Burt 2001). The statistical significance of these measures was evaluated by comparing the observed values to a distribution of the values generated with 1000 random permutations of the dataset. A minimum spanning network for each fungal species was generated in *poppr* with the *popp.msn* function using the pairwise distance matrix generated with the *diss.dist* function (Kamvar et al. 2014).

We performed an analysis of molecular variance (AMOVA) to partition the data into different stratifications, treating individual trees as populations and tree sections (galleries) as subpopulations. We estimated the degree of differentiation within and between population division by measuring the components of variation (Excoffier et al. 1992); the statistical
significance was assessed by comparing the observed measures to a distribution generated by 1000 random permutations of the sample matrices. The AMOVA was first conducted on the entire dataset and then on the clone-corrected subset.

Since all the samples were collected within a small spatial distance, we estimated the relatedness among all pairwise unique MLGs using the Lynch and Ritland (LR) estimator (Lynch and Ritland 1999) using the coancestry function implemented in the R package related (Pew et al. 2015). We obtained the 95% confidence interval of the LR relatedness measure. We categorized pairs of samples as unrelated (with lowest 95% confidence interval LR values greater than zero) and related (with 95% confidence interval LR values overlapping zero). We averaged and compared relatedness for each species and compared the proportion of pairwise samples that were related within and between galleries.

**Results**

We isolated representatives of each of the three fungal species from every gallery in every tree, with the exception of a single gallery where *G. clavigera* was not isolated (Table S1). In total, 155 fungal samples from two galleries in each of five trees were genotyped. Although we performed an exhaustive sampling, isolating all Ophiostomatoid fungi from all beetles collected in all galleries, there was some variation in the abundance of the three fungal species. The most abundant species isolated from the galleries was *G. clavigera* (n=65), followed by *O. montium* (n=62) and *L. longiclavatum* (n=28) (Table 1). All three fungi were cultured from insect larvae, adults, and from phloem and sapwood adjacent to egg and larval galleries (Table S1). We genotyped every individual and, following removal of loci with missing data, obtained a total of 54 (*O. montium*), 58 (*L. longiclavatum*) and 57 (*G. clavigerum*) loci with bi-allelic SNPs (Table 1).
We observed genetic diversity at a fine spatial scale in all three fungi as well as the presence of a few dominant clonal lineages in some galleries. We found 23, 15 and 37 multilocus genotypes (MLGs) for *G. clavigera*, *L. longiclavatum* and *O. montium*, respectively in five trees within a single stand (Table 1). There were multiple unique MLGs in each tree and in all galleries where N>1 (Table 1, Fig.1). Within each species, most MLGs were rare and were found only once (Fig. 1). However, some MLGs were found in high frequency, the likely result of clonal propagation. We noted that in each fungal species some MLGs dominated in some trees and some galleries in an exclusive pattern. For example, *G. clavigera* MLG #37 dominated tree gallery #2 in tree # 2 with 21 individuals; in that same gallery *L. longiclavatum* and *O. montium* were represented each by a single individual belonging to one MLG (Figs. 1 and 2). Gallery #1 in tree # 5 was dominated by *O. montium* MLG #25 with 7 individuals; in that gallery, the other species were represented by MLGs with single individuals (Figs. 1 and 2).

The minimum spanning network analysis (MSN) revealed that the relationship among MLGs did not reflect the sample origin. MLGs within the same tree and gallery did not generally cluster together in the haplotype network (Fig. 2). *G. clavigera* MLGs # 4, 13, 15, 20, *L. longiclavatum* MLGs # 8 and 15 and *O. montium* MLGs # 10, 22, 40, 44 and 49 were all found in the gallery #2 of tree #3; but they are not clustered and are sometimes on opposite sides of the network (Fig. 2). The MSN analysis revealed reticulation, in particular in *G. clavigera* and *O. montium*, an indication of recombination among the MLGs. The clonal fraction, calculated as 1-[# MLG / Total N] was 40% in *O. montium*, 46% in *L. longiclavatum* and 65% in *G. clavigera*. Clonal propagation (identified by the presence of multiple individuals with the same MLG) was only observed within galleries, with the exception of *G. clavigera* MLG #13 and *L. longiclavatum* MLG # 4 that were found in two different trees (Figs. 1a, 1b, 2a, 2b). Most MLGs that were found more than once were present in multiple
substrates sampled (Fig. S1, Table S1). The most abundant, *G. clavigera* MLG # 37 was found in larvae, adults and wood tissues (Fig. S1).

The observed number of MLGs and the Shannon-Wiener index of diversity (H) were highest in *O. montium*, followed by *G. clavigera* and *L. longiclavatum* (Table 1). However, the absolute number of MLGs can be biased by the uneven abundance of the fungi. To account for this difference, we calculated the expected number of MLGs estimated based on rarefaction using the species with the lowest abundance (*L. longiclavatum*, with 28 samples).

The eMLG was still highest in *O. montium* (eMLG=20.4), followed by *L. longiclavatum* (eMLG=15.0) and *G. clavigera* (eMLG=13.6). Expected heterozygosity (calculated on the clone-corrected dataset) was highest in *L. longiclavatum* ($H_{\text{exp}}=0.36$), followed by *O. montium* ($H_{\text{exp}}=0.32$) and *G. clavigera* ($H_{\text{exp}}=0.29$) (Table 1). $I_A$ and $rbarD$ were the highest in *L. longiclavatum*, followed by *G. clavigera*, indicating some linkage disequilibrium even after eliminating clone-mates from the analysis, and significantly larger than zero (Table 1, Fig. S3). By contrast, $I_A$ and $rbarD$ were low in *O. montium* and not statistically different from zero (Table 1, Fig. S3).

The analysis of molecular variance (AMOVA) revealed that genetic diversity was present at a fine scale within galleries. In the analysis using all samples, including clones, the proportion of genetic variation between samples within galleries ranged from 54% to 73% of the total for the three fungi and was highly significant (p<0.01; Table 2). The proportion of genetic variation between galleries within trees ranged from 29 to 44%, yielding $\Phi_g$ of 0.450 in *G. clavigera*, 0.438 in *L. longiclavatum* and 0.282 in *O. montium*; these values were all highly significant (p<0.01; Table 2). The proportion of variation and $\Phi_g$ between trees ranged from -2.05% to 1.71%, yielding $\Phi_t$ between -0.020 and 0.017; none of those values were statistically significant in the three fungal species (p>0.05; Table 2). The analysis of the clone-corrected dataset revealed that all of the variation observed was found between samples.
within galleries for *G. clavigera* and *L. longiclavatum* (Table 2); the variation between galleries within trees was 0.18% and 4.96%, yielding $\Phi_g$ of 0.002 and 0.047, respectively in *L. longiclavatum* and *G. clavigera*; these values were not significant ($p>0.05$; Table 2). Most of the variation in *O. montium* was also found between samples within galleries, yet 10.55% of the variation, yielding $\Phi_g$ of 0.104, was observed between galleries within trees ($p<0.05$; Table 2).

We measured LR relatedness values and obtained the 95% confidence interval (CI) for all pairwise comparisons of samples for each species. The proportion of unrelated individuals was highest in *O. montium* (97.4%), followed by *G. clavigera* (93.8%) and *L. longiclavatum* (83.3%) (Fig. 3A). The average LR relatedness was not different within and among galleries (Fig. S4). However, the proportion of related samples within galleries was much higher in *O. montium* (40%) than in *G. clavigera* (20%) or *L. longiclavatum* (6%) (Fig. 3B; Table S2).

**Discussion**

The consistent finding that mated pairs of adult beetles not only carry multiple fungal species, but also multiple genotypes of each species into their galleries supports the hypothesis that the beetles transport a diverse fungal community both at the intraspecific and interspecific levels. We observed genetic diversity in all three fungal symbionts even at this fine spatial scale. In fact, the proportion of genetic variation was highest within MPB galleries and after clone-correction we observed panmictic populations ($\Phi_s$ values not different from zero) within the site in all three fungal species. Other studies have shown that more than one fungal genotype can be isolated from a single MPB adult or larva (Lee et al. 2006b; Rice and Langor 2009). Similar observations were reported for another
Ophiostomatoid fungus, *Raffaelea quercivora*, where multiple genotypes were detected in the galleries of the ambrosia beetle *Platypus quercivorus* in oak trees in Japan (Takahashi et al. 2015). Takahashi et al. (2015) suggested that the *R. quercivora* conidia of various genotypes in the mycangia of female beetles were unloaded and inoculated repeatedly onto the gallery wall at different spots, rather than once. This would allow the fungi to expand their mycelium locally and increase the gallery area occupied.

Yet, in our study most MLGs were rare or unique. The level of diversity observed for *L. longiclavatum* was particularly surprising, given that the sexual stage of this fungus has never been observed (Lee et al. 2005). The adaptive significance of this underlying genetic diversity should not be underestimated. Since selection is dependent upon genetic variation, the high level of genetic diversity at such a fine spatial scale could enable the symbiont community to quickly respond to changes in environmental conditions or in the tree host. In fact, phenotypic variation for growth at different temperatures was highly heritable in these fungi and provides the potential for selection (Ojeda Alayon et al. 2017). The clonal propagation observed in all three fungi could generate selective sweeps if a particular MLG became selectively advantaged.

Genetic variation was observed within galleries in all three fungi but the amount of genetic variation was species-dependent. The lower clonal proportion and the linkage equilibrium observed in *O. montium* compared to *G. clavigera* and *L. longiclavatum* are consistent with what has been reported in population genetic analyses with much broader sampling across Western North America (Tsui et al. 2012, 2014; Ojeda Alayon et al. 2017). The differences in genetic variability among species could be explained by mutation rate, population history or mode of reproduction. Fungi have a mixed mating system with both asexual and sexual cycles; the frequency of sexual and asexual reproduction is likely to vary.
in these fungi and probably impacts the genetic variability. Sexual fruiting bodies are rarely observed in *G. clavigera* and *L. longiclavatum*, but asexual conidia and conidiophores are abundant in the galleries. *O. montium* differs from *G. clavigera* and *L. longiclavatum* since it produces sexual fruiting bodies abundantly in beetle galleries (Whitney 1971, Tsui et al. 2013). The wide variation in the clonal fraction and linkage disequilibrium values measured in our study is consistent with these observations.

Still, even though the sexual stage is rarely observed in *G. clavigera* and *L. longiclavatum* sexual reproduction must occur. Most of the pairs of samples in our relatedness comparisons were statistically unrelated (with the exception of clone-mates). This is not unexpected given the heterothallic mating system in these fungi which requires different mating type alleles for sexual reproduction (Tsui et al. 2013). Both mating type loci were found in these fungi and the alleles were in equilibrium overall and in most populations tested (Digustini et al, 2011; Tsui et al. 2013). Sexual reproduction is also supported by the large number of MLGs we consistently observed in these fungi (this report, Tsui et al. 2012, 2014). One explanation for the lack of relatedness among these fungi is that they could be vectored by unrelated beetles. Polygamy and absence of fine-scale spatial genetic structure were reported in the bark beetles sampled in the same trees and genotyped using SNPs distributed throughout the genome (Janes et al. 2016). The contribution and combination of different genotypes from multiple source locations may have led to genetic homogenization in the MPB (Janes et al. 2016). The proportion of the beetles that were unrelated (89.54%) is very similar to that observed in the fungi (83-97%) and unrelated beetles were also detected within galleries. Janes et al. (2016) suggested that brood parasitism was one reason for this pattern of relatedness in MPB. If true, this fine-scale movement and placement of unrelated beetles could contribute to the homogenization of fungal genotypes and species we observed.
in the stand. It is yet one more parallel between the beetles and fungi that we observed previously (James et al. 2011).

Competition for resources and space is critical for organisms that specialize on transient resources, such as weakened and dying trees (Goodsman et al. 2017). Competition has been demonstrated experimentally within the MPB-symbiont system and could play a role in shaping the composition of fungal populations at a fine spatial scale (Adams et al. 2008; Bleiker and Six 2009; Moore and Six 2015). Interspecific interactions could change fungal community composition and functioning and grazing could affect the outcome of these interactions (Boddy 2000). Most mycophagous fauna display distinct feeding preferences and preferential grazing could impose selective pressures on saprotrophic communities, resulting in shifts in fungal succession and community composition (Crowther et al. 2012).

Intraspecific competition could also be a crucial factor controlling the genetic composition within fungal species. Competitive exclusion conditional on genotypic characteristics was demonstrated among strains of a plant pathogen (Koskella et al. 2006). We can speculate that the presence of multiple fungus genotypes that we observed within galleries sets the stage for competitive or antagonist interactions among individual fungal strains within each species. Our experiments were not designed to test for competition, but the pattern of exclusive dominance of MLGs in trees and galleries that we observed could indicate competitive exclusion. An investigation of Microbotryum, a group of plant pathogenic fungi that cause anther-smut on Silene, showed that multiple-genotype infections are common and that the level of antagonism was positively correlated with genetic distance between competitors; thus intraspecific competitive exclusion tends to occur between less related strains (Koskella et al. 2006) (López-Villavicencio et al. 2007). The low levels of relatedness within a gallery observed in G. clavigera and L. longiclavatum was proposed to
be due to their intrinsic low genetic variability (Alamouti et al. 2011; Massoumi Alamouti et al. 2014). We report abundant genetic variability and low relatedness within galleries in *G. clavigera* and *L. longiclavatum*. The higher level of genetic similarity within galleries in *O. montium* and the significant $\Phi_g$ could be explained by inbreeding caused by mating among siblings within galleries or within trees; an alternative explanation is that competitive exclusion of dissimilar genotypes is responsible. Additional experiments would need to be conducted before reaching a conclusion.

Temporal variation in fungal population dynamics is another potential cause of variation in MLG frequency among galleries and trees. *Grosmannia 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 (egg, larvae, pupae, adults) (Khadempour et al. 2012). Fungal species prevalence may change over the course of a beetle life cycle due to the functional differences in each species. *Grosmannia clavigera* and *L. longiclavatum* are pathogens so they can colonize the phloem more rapidly than *O. montium* that is either non-pathogenic or only weakly pathogenic and has been considered a ‘hitchhiker’ in this symbiosis (Six and Paine 1998). The MLGs that occur with a high frequency may have greater fitness than the rare MLGs. Intra-specific variation in traits that can impact survival, fitness or growth, has been demonstrated previously. Growth rates variation among *G. clavigera* isolates in low oxygen environments was shown *in vitro* (Plattner et al. 2008). Phloem and sapwood moisture content and temperature also influenced fungal growth (Six and Paine 1998; Lee et al. 2006b; Plattner et al. 2008; Bleiker and Six 2009; Ojeda Alayon et al. 2017). Variation in these traits could affect tissue colonization and fitness at a fine scale. Alternatively, it is possible that the most abundant MLGs are not more fit but just happen to have been isolated more often due to chance.
This study was conducted at a single site to allow dissection of the trees and the exhaustive sampling of the various stages of the beetles and the three fungal symbionts (Janes et al. 2016). Given the low level of genetic structure observed in these fungal symbionts at the landscape level (Lee et al. 2007, Ojeda et al. 2014, Ojeda Alayon 2017, Roe et al. 2011b, Tsui et al., 2012, Tsui et al., 2014), we believe that this study design was appropriate. But future studies could be conducted in additional sites to reveal if the fine-structure diversity observed here is widespread. Another caveat is that we sampled SNPs that may or may not be neutral. Our overall interpretation of relatedness and diversity (e.g., number of MLG, LRM) should not be affected when averaged over the number of loci sampled. Future studies using whole genome sequencing or genotyping-by-sequencing will be necessary to assess whether or not the SNPs used in this study are representative of the fungal genomes.

Our study of the fine scale genetic structure and variability of MPB fungal symbionts revealed high variability, low relatedness within beetle galleries, and a panmictic population structure within a site. These results indicate that frequent movement of the fungal symbionts carried by beetles from various sources creates a large gene pool that selection can shape. This ultimately generates the potential for the fungi to co-exist, adapt to specific niche and contribute to the current MPB expansion across large geographic areas. This may be important for managing the MPB in expanding and marginal areas.

Acknowledgements

Funding for this research was provided by Genome Canada, Genome BC, Genome Alberta and the Government of Alberta (AAET/AFRI-859-G07) in support of the Tria I and Tria II Projects (http://www.thetriaproject.ca) and NSERC Tria-net (NETGP-434810-12). We acknowledge Ben Lai, Padmini Herath, Lina Farfan, Ting Pu, Sandra Cervantes-Arango,
Allan Carroll, Yousry El-Kassaby (Forest and Conservation Sciences, UBC) and Forest Health Officers of the Alberta Sustainable Resource development fund for advice and technical assistance.
References


Figure legends

Figure 1. Multilocus genotype (MLG) counts of (A) *Grosmannia clavigera* (B) *Leptographium longiclavatum* and (C) *Ophiostoma montium* sampled in the galleries or on mountain pine beetle larvae or adults sampled in two galleries on five lodgepole pine trees. An asterisk (*) indicates where the same MLG was found in different trees.

Figure 2. Minimum spanning network generated for samples of (A) *Grosmannia clavigera*, (B) *Leptographium longiclavatum*, and (C) *Ophiostoma montium*. The abundance of the multilocus genotype (MLG) in the network is represented by color codes for each tree and gallery. The size of the circles is proportional to the abundance of the MLG. Line weight represents the degree of relatedness among the MLGs, with thicker lines representing closely related MLGs and thinner lines representing more distantly related MLGs.

Figure 3. Comparison of relatedness in fungal symbionts of the mountain pine beetle. (A) Proportion of pairwise sample comparison with Lynch and Ritland relatedness measures greater (related) or not different from (unrelated) zero for each species. (B) Proportion of the related individuals that were found within or between beetle galleries in each species.
Table 1. Population statistics at single nucleotide polymorphisms for three fungal associates of the mountain pine beetle

**Grosmannia clavigera**

<table>
<thead>
<tr>
<th>Tree</th>
<th>N</th>
<th>MLG</th>
<th>H^4</th>
<th>H_{exp}^5</th>
<th>I_A^6</th>
<th>rbarD^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>5</td>
<td>1.61</td>
<td>0.27</td>
<td>3.14</td>
<td>0.112</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>4</td>
<td>1.39</td>
<td>0.25</td>
<td>-0.47</td>
<td>-0.019</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>7</td>
<td>1.95</td>
<td>0.29</td>
<td>0.98</td>
<td>0.029</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0.69</td>
<td>0.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>6</td>
<td>1.79</td>
<td>0.27</td>
<td>0.83</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>65</td>
<td>23</td>
<td>3.12</td>
<td>0.29</td>
<td><strong>0.85</strong></td>
<td><strong>0.019</strong></td>
</tr>
</tbody>
</table>

**Leptographium longiclavatum**

<table>
<thead>
<tr>
<th>Tree</th>
<th>N</th>
<th>MLG</th>
<th>H^4</th>
<th>H_{exp}^5</th>
<th>I_A^6</th>
<th>rbarD^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.69</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1.10</td>
<td>0.37</td>
<td>-0.44</td>
<td>-0.013</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>5</td>
<td>1.61</td>
<td>0.38</td>
<td>0.74</td>
<td>0.018</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>4</td>
<td>1.39</td>
<td>0.30</td>
<td>3.33</td>
<td>0.108</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0.69</td>
<td>0.29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>28</td>
<td>15</td>
<td>2.69</td>
<td>0.36</td>
<td><strong>4.22</strong></td>
<td><strong>0.087</strong></td>
</tr>
</tbody>
</table>

**Ophiostoma montium**

<table>
<thead>
<tr>
<th>Tree</th>
<th>N</th>
<th>MLG</th>
<th>H^4</th>
<th>H_{exp}^5</th>
<th>I_A^6</th>
<th>rbarD^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>11</td>
<td>2.40</td>
<td>0.32</td>
<td>0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1.61</td>
<td>0.28</td>
<td>0.56</td>
<td>0.033</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>10</td>
<td>2.30</td>
<td>0.26</td>
<td>0.20</td>
<td>0.010</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>8</td>
<td>2.08</td>
<td>0.30</td>
<td>1.12</td>
<td>0.056</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1.10</td>
<td>0.26</td>
<td>-0.50</td>
<td>-0.038</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>62</td>
<td>37</td>
<td>3.61</td>
<td>0.32</td>
<td>0.05</td>
<td>0.002</td>
</tr>
</tbody>
</table>

1The number of bi-allelic single nucleotide polymorphisms (SNP) after eliminating loci with more than 5% missing data is: 57 (**G. clavigera**), 58 (**L. longiclavatum**) and 54 (**O. montium**); for the expected heterozygosity, loci that were fixed were excluded from the calculation

2Number of fungal samples isolated in each gallery

3Number of unique observed multilocus genotype (MLG)

4Shannon-Wiener Index of MLG diversity

5Expected heterozygosity calculated with the clone-corrected data

6Index of association and rbarD, two measures of linkage disequilibrium, calculated on the clone-corrected dataset

7Values in bold are significantly different from zero (p<0.05)
### Table 2. Analysis of molecular variance for three fungal associates of the mountain pine beetle genotyped at SNP loci

<table>
<thead>
<tr>
<th>Components of variation</th>
<th>( G. \ clavigera )</th>
<th>( L. \ longiclavatum )</th>
<th>( O. \ montium )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% variation</td>
<td>( \Phi )-value</td>
<td>% variation</td>
</tr>
<tr>
<td>All samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between trees</td>
<td>1.71</td>
<td>0.017</td>
<td>0.78</td>
</tr>
<tr>
<td>Between galleries within trees</td>
<td>44.20</td>
<td>0.450**</td>
<td>43.48</td>
</tr>
<tr>
<td>Between samples within galleries</td>
<td>54.09</td>
<td>1.000**</td>
<td>55.74</td>
</tr>
<tr>
<td>Clone-corrected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between trees</td>
<td>-6.23</td>
<td>-0.062</td>
<td>-4.00</td>
</tr>
<tr>
<td>Between galleries within trees</td>
<td>4.96</td>
<td>0.047</td>
<td>0.18</td>
</tr>
<tr>
<td>Between samples within galleries</td>
<td>101.27</td>
<td>1.000**</td>
<td>103.81</td>
</tr>
</tbody>
</table>

**\( \Phi \)-values** are the \( F_{st} \) analogs \( \Phi_{st} \) (between trees), \( \Phi_{gt} \) (between galleries within trees) and \( \Phi_{sg} \) (between samples within galleries). Significance of the values was tested by comparison of the observed values with a distribution obtained with 1000 permutations of the dataset; **\( p<0.01 \); *\( p<0.05 \).
A. *G. clavigera*

B. *L. longiclavatum*

C. *O. montium*
A) 

<table>
<thead>
<tr>
<th>Species</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grosmannia clavigera</td>
<td>0.00</td>
</tr>
<tr>
<td>Leptographium longiclavatum</td>
<td>0.25</td>
</tr>
<tr>
<td>Ophiostoma montium</td>
<td>0.50</td>
</tr>
</tbody>
</table>

B) 

<table>
<thead>
<tr>
<th>Relation</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Related</td>
<td>0.75</td>
</tr>
<tr>
<td>Unrelated</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between galleries</td>
<td>0.50</td>
</tr>
<tr>
<td>Within galleries</td>
<td>0.50</td>
</tr>
</tbody>
</table>

https://mc06.manuscriptcentral.com/cjfr-pubs