Genetic and genomic evidence of niche partitioning and adaptive radiation in mountain pine beetle fungal symbionts

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Key words: *Grosmannia*, *Leptographium*, *Ophiostoma*, *Dendroctonus*, genotype environment association, multipartite symbionts, pine pathogens, tree disease, outbreak, blue stain fungi

Running title: *Population dynamics of MPB fungal symbionts*

Abstract (250 words)

Bark beetles form multipartite symbiotic associations with blue stain fungi (Ophiostomatales, Ascomycota). These symbionts play an important role during the beetle's life cycle by providing nutritional supplementation, overcoming tree defenses and modifying host tissues to favor brood development. The maintenance of stable multipartite symbioses with seemingly less competitive symbionts in similar habitats is of fundamental interest to ecology and evolution. We tested the hypothesis that the coexistence of three fungal species associated with the mountain pine beetle is the result of niche partitioning and adaptive radiation using SNP genotyping coupled with genotype-environment association analysis and phenotypic characterization of growth rate under different temperatures. We found that genetic variation and population structure within each species is best explained by distinct spatial and environmental variables. We observed both common (temperature seasonality and the host species) and distinct (drought, cold stress, precipitation) environmental and spatial factors that shaped the genomes of these fungi resulting with contrasting outcomes. Phenotypic intraspecific variations in *Grosmannia clavigera* and

Leptographium longiclavatum, together with high heritability, suggest potential for adaptive selection in these species. By contrast, *Ophiostoma montium* displayed narrower intraspecific variation but greater tolerance to extreme high temperatures. Our study highlights unique phenotypic and genotypic characteristics in these symbionts that are consistent with our hypothesis. By maintaining this multipartite relationship, the beetles increase the likelihood of obtaining the benefits afforded by the fungi and reduce the risk of being left aposymbiotic. Complementarity among species could facilitate colonization of new habitats and survival under adverse conditions.

INTRODUCTION

Ecological niche theory predicts that closely related species with similar resource requirements will compete for shared resources, leading to competitive exclusion of one or more species (Gause 1934). This implies that the coexistence of sibling species in the same habitat is predicated upon niche partitioning. Niche differences may be engendered by subtle differences in the functional requirements of each species (Chesson 2000; Amarasekare 2003). Evidence of niche partitioning has been well documented in plants (Thorpe *et al*. 2011), birds (MacArthur 1958), and algae (Cardinale 2011). Recent advances in molecular sequencing have revealed evidence of temporal and fine scale niche partitioning among fungi and microorganisms (Feau *et al*. 2012; Taylor *et al*. 2014; Pirondi *et al*. 2015). This raises fundamental questions about the factors leading to niche partitioning and coexistence of closely related fungi and other microorganisms.

Insects are well known for their symbiotic relationships with microorganisms (Feldhaar 2011; Aylward *et al*. 2012; Frago *et al.* 2012, Ohkuma *et al.* 2015). These stable relationships provide insects with mechanisms to exploit previously inaccessible ecological niches (Douglas 2015). Bark beetles have symbiotic relationships with many kinds of microbes, including bacteria, single celled yeasts, and filamentous fungi (Six & Wingfield 2011; Popa *et al.* 2012; Six 2012, 2013). Ophiostomatoid fungi (Ophiostomatales, Ascomycota), also known as blue stain fungi, are a key group of bark beetle symbionts. Blue stain fungi play an important role in enhancing the fitness of bark beetles, by improving access to nutritional resources, overcoming host tree defenses, and modifying host-tree microclimates to favor brood development (Ayres *et al.* 2000; Bentz & Six 2006; Bleiker & Six 2007; Cook *et al.* 2010; Goodsman *et al.* 2012; DiGuistini *et al.* 2007, 2011; Wang *et al.* 2013; Six & Wingfield, 2011; Krokene & Solheim, 1996). The mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) and its fungal symbionts are one example of a highly successful insect-fungal symbiotic system. Over the past 20 years populations of MPB and its associated symbionts have exploded and caused large-scale pine mortality in western North America. The recent MPB outbreak is an order of magnitude larger in area and severity than all previous outbreaks and has converted the forest from carbon sink to carbon source (Kurz *et al.* 2008).

The MPB symbiosis is multipartite and comprises at least nine fungal species from five genera (Lee *et al.* 2006a). *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield (i.e. *Grosmannia* sp. *sensu* Alamouti *et al*. 2011, hereafter referred to as *G. clavigera*), *Leptographium longiclavatum* Lee, Kim and Breuil, and *Ophiostoma montium* (Rumbold) von. Arx. are intimately associated with the MPB (Lee *et al*. 2006a). They are found

in brood galleries, on larvae, the exoskeleton of adults, and in mycangia, specialized body structures that evolved to transport fungal spores (Whitney & Farris 1970). During the early phase of a beetle infestation these fungi likely compete for limited, but readily available nutrients in the phloem of host pine trees (Bleiker & Six 2007, 2009a,b). Such competition should result in exclusion of less competitive fungi over time; however, the multipartite existence of these three fungi with MPB has remained apparently stable over evolutionary time (Six & Bentz 2007).

How can less competitive symbionts coexist with more competitive species? Niche partitioning is one plausible explanation. These fungal species may occupy slightly distinct niches and play different roles within this multipartite symbiosis, providing niche space for stable, long-term coexistence. There is evidence supporting this hypothesis. First, fungal species differ in key life history traits, such as pathogenicity (Reid *et al.* 1967; Yamaoka *et al.* 1990, 1995; Solheim & Krokene 1998; Lee *et al.* 2006b), tolerance or adaptation to temperature (Solheim & Krokene 1998; Rice *et al.* 2008; Addison *et al.* 2013; Moore & Six 2015; Six & Bentz, 2007), and resistance to variation in host water potential (Bleiker & Six 2009b). Second, fungi colonize the beetle galleries and surrounding tree tissues at different times or beetle lifecycle stages. These temporal differences may result in temporal niche partitioning or use of different carbon sources (Adams & Six, 2007; Bleiker & Six, 2009a,b; Six & Wingfield, 2011). Third, fungal associates vary in their spatial and temporal distribution on the landscape (Rice & Langor 2009; Roe *et al.* 2011a), as well as in their population structure and genetic diversity (Roe *et al.* 2011b; Tsui *et al.* 2012, 2014). MPB is found across a broad, ever expanding range, and this variability may be indicative of different adaptive responses in each of the three associated fungi.

Our aim in the present study was to compare and contrast the inter- and intraspecific population genetic and phenotypic characteristics of three common MPB-fungal associates (*G. clavigera*, *L. longiclavatum*, and *O. montium*) collected over a wide geographic and environmental range. We assessed whether the adaptation of fungal symbionts to a wide range of environmental conditions would result in species-specific signatures of natural selection. We conducted landscape genomic analyses to detect loci involved in local adaptation and identify environmental variables driving this selection. Our data support the hypothesis of niche adaptation and demonstrate that the fungal symbionts show differential response to environmental drivers. Each species possesses distinct genetic and phenotypic characteristics that could enhance complementarity and permit coexistence. This provides a likely benefit to beetle fitness by ensuring the presence of fungal symbionts under a wide range of environmental conditions.

MATERIALS AND METHODS

Population sampling, culture collection and DNA extraction

Grosmannia clavigera, *L. longiclavatum* and *O. montium* fungal samples were isolated from MPB and infected pine trees from central and western North America (Table S1). We isolated over 1350 samples from the three fungal species collected from 35 locations in Canada and the USA, including 17 locations that were shared among the three species. The final dataset comprised 898 samples with genotypic data, including 414 *G. clavigera* samples from 32 sites, 264 *L. longiclavatum* samples from 24 sites, and 220 *O. montium* samples from 22 sites (Table S1). Samples were maintained on malt extract agar (MEA) and their DNA was extracted according to Roe *et al.* (2011a). Samples were initially identified to species based on morphology

and corroborated by PCR amplification of rDNA and other protein coding gene fragments using species-specific primers coupled with Sanger sequencing (Khadempour *et al.* 2010) (data not shown).

SNP panel design and genotyping

We developed three species-specific genotyping arrays at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada) using the Sequenom iPLEX Gold SNP genotyping technology (http://gqinnovationcenter.com). Arrays consisted of four panels, each containing up to 36 SNPs. For *L. longiclavatum* we used our previously developed array (Ojeda *et al.* 2013) and followed a similar strategy for SNP discovery and validation for the other two species – *G. clavigera* and *O. montium*. Briefly, genes were selected according to their differential expression profiles in *G. clavigera* while grown under different media conditions, including media enriched with terpenoid products (DiGuistini *et al.* 2011). Gene sequences were functionally annotated with Blast2GO (Conesa et al. 2005), following an Interproscan analysis and a TBLASTx search against the NCBI Reference Sequence database (RefSeq: http://www.ncbi.nlm.nih.gov/refseq/) to ensure that our selected SNPs represented a wide array of gene categories and had shared functions across the three focal species (Table S2). SNP discovery in the selected genes was carried out in the orthologs identified among the three species to facilitate cross-species comparisons. For *G. clavigera,* we used whole genome re-sequencing data from six isolates and previously available sequences (Alamouti *et al.* 2014). For *O. montium*, SNPs were selected within these gene regions by using the re-sequencing data of 36 isolates (data not shown). Samples or loci with poor or incomplete genotyping data were removed from the

final dataset (http://dx.doi.org/10.5061/dryad.mt16j); in total, 129, 147 and 59 SNPs were obtained for *G. clavigera*, *L. longiclavatum*, and *O. montium*, respectively.

Genetic diversity and population structure of three fungal symbionts

Genetic polymorphism was assessed and compared for the three species by calculating genetic diversity (expected heterozygosity; H_{exp}), and number of multilocus genotypes (MLG) with POPPR version 2.2.1 in R (Kamvar *et al.* 2014), following removal of loci with missing data (>5%) and missing genotypes (>1%). We estimated linkage disequilibrium as a proxy for recombination by calculating the index of association (I_A) and $\bar{r}_d$, a measure that is not dependant on the number of loci (Agapow & Burt, 2001). To implement this analysis, we used the clonecorrect function in POPPR to remove individuals with identical SNP profiles within a sampling population. To assess whether or not linkage disequilibrium was independent among species, we compared the index of association for the three fungal species by plotting the I_A and $\bar{r}_d$ values for each species pair and calculating Pearson correlation coefficient. Population differentiation statistics were estimated from Euclidean distances among all pairs of multilocus haplotypes using the hierarchical analysis of molecular variance (AMOVA) implemented in the Arlequin software (version 3.5) (Excoffier & Lischer, 2010). The variance components and Φ-statistic values were tested statistically by a nonparametric randomization test with 1000 repetitions. Additionally, Nei's unbiased genetic distance (Nei's F_{st}-values; Nei 1978) was calculated among all pairs of populations with GenAlEx6 (Peakall & Smouse 2006). T-REX (Boc *et al*. 2012) was then used to reconstruct a Neighbor-joining tree from the pairwise F_{st}-distance matrix.

The population structure of the three fungal symbionts was determined using the Bayesian clustering software STRUCTURE 2.3.4 (Pritchard *et al.* 2000) with a number of *K*-clusters set from 1 to 10. Each *K*-run was replicated 10 times after a burn-in period of 100 000 generations followed by 800 000 generations to check for convergence of likelihood values. The model allowed individuals to have mixed ancestry (admixture) and correlated allele frequencies; spatial sampling location of each population was used as input prior and the admixture model in which the fraction of ancestry from each cluster was estimated for each individual. Optimal *K* value was estimated by computing ΔK computed with STRUCTURE HARVESTER Web Version 0.6.94 (Evanno *et al.* 2005).

To compare patterns of population genetic structure across species, we calculated the significance of congruence among genetic distance matrices with CADM using the R package ape version 3.0-11 (Legendre & Lapointe 2004; Paradis *et al.* 2004). The test applied in CADM is considered an extension of the Mantel test and allows comparison of more than two distance matrices (Legendre & Lapointe 2004). Geographic distance among sampling locations was also included to identify potential spatial autocorrelation.

Landscape genomics

We explored the association between genomic and environmental variables by conducting genotype-environment association (GEA) analyses. Bioclimatic variables from the Worldclim database were obtained using the R package 'raster' (Hijmans & Van Etten, 2013) (script available upon request); only a subset of 10 variables related to temperature and rainfall extremes (BIO5, BIO6, BIO10, BIO11, BIO13, BIO14, BIO16 and BIO17), and seasonality of

precipitation and temperature (BIO3 and BIO15) that were likely to convey conditions of physiological stress for bark beetles and pine trees were considered (Godefroid *et al*. 2016; Campbell *et al*. 2007). To remove collinearity among these climatic variables and with altitude, longitude and latitude, we applied a principal components analysis (PCA) that reduced the dataset to three main principal components retaining 93.0% of the original variation (Table S3). For the GEA analyses, we used PC1, PC2, and PC3 that were positively related to drought, cold and precipitation stress and seasonality of precipitation and temperature variables (BIO3 and BIO15), as well as elevation, longitude, and latitude (data not shown).

We used the Bayesian approach implemented in BAYENV to identify allele frequencies correlated with these environmental variables (Coop *et al*. 2010). We first generated a covariance matrix for each of the three species-SNP datasets by averaging covariances matrices generated every 5 000th iteration over a total of 500 000 iterations. Each of the five environmental parameters defined above (PC1 to 3, BIO3 and BIO15) was standardized by subtracting the mean and dividing by the standard deviation (SD) across sampling locations as suggested in Coop *et al*. (2010). BAYENV was then run separately with 100 000 iterations for each SNP in the full species-SNP sets with the five environmental parameters. To ensure that the results were not sensitive to stochastic errors, three independent runs with different random seeds were run. For each variable SNPs into the top 5% and 1% quantile of the Log_{10} Bayes Factor (BF) distribution were considered as potential outliers.

To further examine the genotype-environmental associations and account for the joint action of selection and demography across the genome (Forester *et al*. 2016), we used multidimensional multilocus analyses to reduce the data into a lower dimensional space and tease

apart the environmental and spatial contributions shaping the fungal genomic profiles. We used redundancy analysis (RDA) to discover environmental drivers of selection and identify outlier loci. This approach reduces the likelihood of type I error, which may occur when performing multiple pairwise tests (Forester *et al*. 2016). The RDA applies linear regression to multivariate response data in order to maximize the proportion of the response variable that is explained by the predictor (constraining) variables. Combinations of the genetic data (the response variable) were modeled as a function of the environmental variables (the predictors). Distance-based Moran's eigenvector maps (dbMEMs) were calculated from the coordinates of the samples and used as predictors in the RDA. Forward selection was used to reduce the number of dbMEMs (Blanchet *et al*. 2008). To select the best model, we used forward selection with adjusted regression coefficients of multiple determination (R2adj) and *P*-value (*P*-value for inclusion < 0.01) with the ordiR2step function (Oksanen *et al*. 2015). We report R2adj for the global RDA as well as the variance partitioning to estimate the variance explained by the spatial variables only or by environmental variables representing temperature, precipitation and hosts. Significance of the individual variables was tested using partial RDA. These analyses used the pcnm, rda, ordiR2step and varpart functions in the vegan package for R (Oksanen *et al*. 2015). Once the best model was selected for each fungal species, we identified loci that fell outside of the 99.9% quantile for each of the first three axes of the RDA. To assess congruence of selection pressure in different fungal species, we derived the locus scores for loci common in the three fungi for the first three axes and performed a correlation analysis.

Phenotypic characterization of fungal growth under a temperature gradient

We characterized the effect of temperature on growth rate for each of the three fungal symbionts at six temperatures (from 5 to 30°C with steps of 5°C). For each of the three genetic groups observed in previous studies (Tsui *et al*. 2012, 2014), we selected three locations where all three fungal symbionts were obtained from the phloem of the same host pine (*P. contorta*) or from beetles and/or larvae collected from galleries of *P. contorta*. For each fungal species in the three genetic groups, we selected 6 single-spore isolates, for a total of 48 isolates (16 *G. clavigera*, 18 *L. longiclavatum* and 14 *O. montium*) (Tsui *et al.* 2012, 2014). We conducted supplementary growth experiments at 15, 20 and 25°C with 64 additional fungal isolates (21 *G. clavigera*, 22 *L. longiclavatum* and 21 *O. montium*).

All fungal isolates were acclimatized at room temperature for one week on 25 ml MEA media following long-term storage at 4ºC. Mycelial plugs were transferred to a new Petri dish containing 25 ml of MEA and maintained in an incubator in the dark for each of the six temperatures tested. Growth (in mm) was measured each day in four directions and averaged. For temperature 5°C, 10°C and 30°C, growth rates were obtained by calculating the area under the curve after seven days using the trapeze method using the pracma package (Borchers, 2016). For temperature between 15°C and 25°C, rapid growth of many isolates brought the fungus to the edge of the plate before day seven, preventing further growth measurements. Instead, a linear regression was performed and the resulting equation used to calculate the area under the curve. To assess whether different parameters affecting growth rate we used an ANOVA and linear mixed model implemented in the R lme4 package (Bates *et al.* 2015). For heritability calculations, the variance observed in the population of each species in the 15, 20 and 25°C

temperature experiment was analyzed for its components. The variance component of the samples was divided by the total variance.

RESULTS

Population structure of three fungal symbionts of the mountain pine beetle

The population structure analyses revealed very similar clustering patterns in the three fungi, but also some differences in distribution of the genetic diversity among populations within species. The global CADM test rejected the null hypothesis of incongruence among all the matrices (Kendall's coefficient of performance among distance matrices, W = 0.81; Friedman's $\chi 2$ statistic = 435.7, $P < 0.001$), and the posterior results indicate that all matrices were congruent to each other. All genetic distance matrices were congruent with the geographic distance as well, suggesting significant spatial correlation and isolation-by-distance (Table S4). Pairwise F_{st} analysis and NJ tree reconstruction identified three main population clusters based loosely on geography or epidemiological events common to the three symbionts (Fig. 1; Fig. S1). Cluster Group I comprised genetically similar populations from sites in new epidemic areas that had not been attacked by the MPB prior to 2006 and were located north of 52°N latitude (with the exception of Valemount for *L. longiclavatum* and Peachland for *G. clavigera*). Cluster Group II was composed of populations in BC and Alberta from within the natural historic distribution range of the MPB, mostly between 48°N and 51°N latitudes. Cluster Group III comprises populations from the USA located south of 48°N (with the exception of the Black Hills, SD, for *G. clavigera*). This pattern was more pronounced in *L. longiclavatum* and *O. montium,* where higher levels of genetic differentiation were observed among the groups (F_{st} = 0.15 to 0.34 in *L.*

longiclavatum and 0.11 to 0.20 in *O. montium vs.* 0.04 to 0.11 in *G. clavigera*; Fig. 1). Bayesian clustering revealed notable geographic structuring in the three fungi with northern-southern partitioning in populations at the optimum *K* of 2. Increasing *K*-values at *K* = 3 and 4 supported the groupings obtained with the pairwise F_{st} analysis (Fig. S2).

The level and distribution of genetic diversity differed among the three fungi. Populations of *G. clavigera* had the lowest genetic diversity (H_{exp} = 0.27 in *G. clavigera vs.* 0.35 in *L. longiclavatum* and 0.31 in *O. montium*; Table 1). An analysis of molecular variance (AMOVA) indicated that most of the genetic diversity in *G. clavigera* was attributed within populations (87.8%), leaving only 6.9% among groups and 5.4% among populations within groups. By contrast, *L. longiclavatum* had the lowest proportion of genetic diversity within populations (70.1%), but the highest diversity among groups (23.1%). *Ophiostoma montium* had the highest diversity among populations within groups (10.81%) and intermediate diversity within populations (74.4%) and among groups (14.35%).

Extent of sexual reproduction in mountain pine beetle fungal symbionts

We assessed and compared clonality and linkage disequilibrium among the fungal species and found different patterns (Table 1). The clonal fraction was the lowest in *O. montium* (3.5%) and the highest in *L. longiclavatum* (19.1%). Significant linkage disequilibrium was observed in all fungal species, as indicated by I_A and $\bar{r}_d$ values significantly greater than zero (Table 1). However, linkage disequilibrium was more pronounced in *G. clavigera* (I_A=1.29, $\bar{r}_d$=0.014) and *L. longiclavatum* (I_A=4.17, $\bar{r}_d$=0.032), than in *O. montium* (I_A=0.40, $\bar{r}_d$=0.009) (Table 1). Linkage disequilibrium was strongly correlated between populations of *G. clavigera* and *L. longiclavatum*

(Pearson correlation coefficient = 0.575, *P*=0.02; Fig. S3A and B); no correlation was observed between *G. clavigera* or *L. longiclavatum* and *O. montium* (*P*>0.05; Fig. S3C to F).

Landscape genomics

The RDA conducted for the three fungal species were significant ($P < 0.001$) with adjusted R-square values more than two times higher for *O. montium* and *L. longiclavatum* (0.210 and 0.184, respectively) than for *G. clavigera* (0.085) (Table 2). The first three axes of the RDA accounted for 72%, 90%, and 83% of the genetic variation in *G. clavigera*, *L. longiclavatum* and *O. montium*, respectively. Two environmental variables, temperature seasonality (BIO3) and host tree species, were selected in the best model for all three fungi (Table 2; Fig. 2). However, different models were obtained by our forward selection procedure for the three fungi. The best model for *G. clavigera* was dominated by spatial variables (MEM1-5 and MEM27), with two environmental variables, temperature and precipitation seasonality (BIO3 and BIO15) and the host tree variable also contributing to the model (Table 2, Fig. 2). By contrast, only environmental variables related to temperature, precipitation, and host trees were significant contributors to the models for *O. montium* and *L. longiclavatum*. To further explore the contribution of the environmental variables to these models, we performed partial RDA. Groups of variables representing temperature, precipitation and hosts were all significant when other variables were subtracted (Table 2). Environmental variables related to temperature had the highest adjusted R-square values for *L. longiclavatum* and *O. montium* and were strongly loaded along axis 1 (for *L. longiclavatum*) and axes 1 and 3 (for *O. montium*) (Table 2; Fig. 2). PC3, a synthetic variable that measures cold stress, was only significant in the model for *O. montium* (Table 2; Fig. 2). We assessed whether SNPs in genes that were common in more than one

species were subjected to the same selection pressure by performing a correlation test between the RDA score values of loci that are shared between species. The correlation coefficients were not significantly different from zero for the score values between any two species comparisons for the first three axes of the RDA (results not shown).

A total of 23 SNPs (seven in *G. clavigera*, seven in *L. longiclavatum* and nine in *O. montium*) were identified as outliers in the RDA (Table 3). Although RDA and BAYENV use different methods to identify outlier loci, 11 of the 23 loci identified as outliers by RDA were also outliers in BAYENV. Nine of those loci had Bayes factor values larger than the top 5% and 1% quantiles (Table 3). BAYENV-outlier loci were generally correlated with different environmental variables for the three fungal species. PC2 and BIO3 were correlated to SNPs in *G. clavigerum and O. montium*, whereas PC3 was correlated with a SNP in *G. clavigera* (Table 3); PC1 and BIO15 were associated with SNPs in *O. montium* only (Table 3).

We annotated the genes associated with environmental patterns and found shared functional gene ontology categories between species as well as gene categories unique to each species. Functions related to gene expression and regulation such as chromatin silencing and rRNA processing and translation were shared in *G. clavigera* and *L. longiclavatum*. Chitin hydrolysis and chitin synthase genes, potentially involved in mechanisms of fungal cell wall modification, were shared by *L. longiclavatum* and *O. montium* (Table 3). Genes related to secondary metabolism and detoxification of secondary metabolites (i.e. mono-oxygenases, dehydrogenases and transmembrane transporters) were shared by *G. clavigera* and *O. montium*. The remaining gene ontology categories for each species were different, indicating that different categories of genes and pathways were under selection in each species (Table 3).

Phenotypic characterization of fungal growth under a temperature gradient

We compared growth of subsets of isolates from three fungal species at six temperatures ranging from 5°C to 30°C. The optimal growth temperature for each species was 25°C. At all temperatures except 30°C, *G. clavigera* had the highest growth rate, followed by *L. longiclavatum,* and *O. montium* ($P < 0.001$). This ranking was reversed at 30°C with *O. montium* growing the fastest, followed by *L. longiclavatum* and *G. clavigera* (Fig. 3).

We compared growth of isolates representing genetic groups (Groups I, II, III) within each species. Growth was different among genetic groups in all three species (ANOVA F-value = 14.49 for *G. clavigera*, 15.54 for *L. longiclavatum* and 19.69 for *O. montium*, $P < 0.001$). However, there was a significant interaction between temperature and genetic group for *G. clavigera* ($F = 2.13$, $P < 0.05$), but not for *L. longiclavatum* or *O. montium*. Growth patterns suggest differential temperature adaptation among groups of the three fungi. *G. clavigera* from Groups II and III grew faster than those from Group I ($P < 0.001$). By contrast, *L. longiclavatum* from Group II had the fastest growth, followed by Group I and Group III ($P <0.01$). *O. montium* from Group II grew faster than those from Groups I and III ($P < 0.01$). Growth variation among isolates was highly heritable, with estimates ranging between 75% to 90% in all three symbionts.

Ophiostoma montium was the most tolerant to high temperatures, with 86% of the isolates (12/14) able to grow at 30° C, compared to 28% (5/18) for *L. longiclavatum* and 12.5% (2/16) for *G. clavigera*. Interestingly, *G. clavigera* and *L. longiclavatum* isolates that could successfully grow at 30°C achieved on average close to 100% of their 20-25°C growth (Fig. S4). We found an

interaction between species and temperature in the 5°C-25°C range. When the 30°C data point had been added, there was a very noticeable increase of the strength of this interaction, confirming the very different response of each symbiont to high temperatures.

DISCUSSION

Blue stain fungi are important symbionts in multipartite associations with bark beetles; this association is believed to be mutualistic as both beetles and fungi derive benefits. Fungi gain transport and access to ephemeral hosts and beetles are provided with nutritional supplements and beneficial modification of their host environment. However, the presence of multiple fungal species occupying an apparently similar habitat is intriguing (Bleiker & Six, 2009a; Moore & Six, 2015). The stable coexistence of multiple mutualists can be facilitated by different ecological adaptations and life strategies that minimize competitive exclusion. Our study provides evidence that the three fungal symbionts of the MPB have developed species-specific adaptations that could favor the coexistence of these species within the MPB-tree ecosystem.

Differences in tolerance to environmental conditions, in particular temperature, can affect the presence and relative prevalence of fungal partners by influencing growth, sporulation, and resource capture (Six & Bentz, 2007; Rice *et al*., 2008; Bleiker & Six, 2009b; Moore & Six, 2015). In fact, a temperature-based model found that temperature variability was a stabilizing mechanism for the mountain pine beetle–fungi mutualism (Addison *et al.* 2013). Our phenotypic analysis showed extensive intraspecific variation to temperature response in *G. clavigera* and *L. longiclavatum.* Fungal populations from different genetic groups varied in growth rates at different temperatures and this trait had high heritability, suggesting potential for further

adaptation in those species. By contrast, *O. montium* displayed much narrower intraspecific variation, but more tolerance to extreme high temperature (Addison *et al.* 2013; Moore & Six 2015). Field observations support these results. A gradual increase in abundance of *L. longiclavatum* was observed with increasing latitude while a symmetrical and opposite rate of change was observed in *G. clavigera* (Roe *et al.* 2011a). Prevalence of *O. montium* on field-collected beetles was positively correlated with the number of high temperature days in the season while *G. clavigera* showed the opposite trend (Six & Bentz 2007). High thermal tolerance in *O. montium* may offer additional niche space to this fungus that would contribute to its maintenance in the MPB system (Roe *et al.* 2011a).

Heterogeneity in environmental conditions across large spatial ranges can lead to local selective pressures that act to maximize adaptation to regional conditions (Volis *et al*. 2005). Our genotype-environment association analysis supports the hypothesis that the fungal associates occupy slightly different niches and could play different roles within this system (Bleiker & Six 2009a; Roe *et al*. 2011b). We found that both common (temperature seasonality and the host species) and distinct (drought, cold stress, precipitation) environmental and spatial factors shaped the genomes of these fungi, with contrasting outcomes. The MPB and its symbionts have a distribution that spans 20 degrees of latitude and are exposed to a wide variety of environments. This environmental heterogeneity likely resulted in different adaptive responses in each fungal species. Temperature is clearly an important factor of the beetle-fungi complex association. Fungal growth response to temperature is variable, both at the species and individual levels (Moore & Six 2015; Rice *et al.* 2008; Six & Bentz 2007). In addition to temperature, several distinct metabolic and cellular processes were correlated with environmental variables that we

tested. This suggests that there are likely some additional, yet unidentified, factors involved in adaptation of each fungus.

Host tree species was a significant component of our genotype-environment association models for all three fungal species. Adaptation to a pine host has resulted in divergence among *G. clavigera* lineages (Alamouti *et al.* 2011). Host defence is one of the main hurdles encountered by the fungi during colonization of pine species. Terpenoid compounds are important components of the conifer host defense. Terpenoids are toxic to bark beetles and inhibit the growth of beetle-associated fungi (Raffa & Smalley 1995). The bark beetle-fungi system evolved key mechanisms to overcome host tree defenses and capture nutrition resources from an ephemeral source (Ayres *et al*. 2000; Six & Bentz, 2007, Alamouti *et al.* 2014; Wadke *et al.* 2016). Functional genomic and transcriptomic analyses have revealed that *G. clavigera* can detoxify tree defense compounds, including terpenoids, and use them as carbon sources (DiGuistini *et al*. 2011, Wang *et al*. 2013, 2014). For example, two outlier genes identified in our analyses (Glean_647 and Glean_3226) were previously shown to be strongly induced in the presence of tree defense compounds (+)-limonene and monoterpenes. Furthermore, a deletion mutant of Glean_3226 was unable to grow in the presence of (+)-limonene when provided as a sole carbon source (Wang *et al.* 2014). Like *G. clavigera*, *L. longiclavatum* also has the capacity to colonize pine sapwood (Lee *et al*. 2006b), so we were not surprised to find outlier genes involved in detoxification pathways in this species. By contrast, *O. montium* is considered the least pathogenic of these fungi (Reid *et al.* 1967; Yamaoka *et al*. 1995; Solheim & Krokene, 1998) and yet we found outlier genes putatively involved in secondary metabolism processes and in detoxification of pine terpenoid defence compounds. While interpretation of individual outlier loci should be done with

care, the presence of these outliers supports the hypothesis that *O. montium* could also be an invader, or possesses the adaptive potential to become one (Six & Paine 1998, 1999; Bleiker & Six 2009a). We speculate that the genes responsible for detoxification are undergoing selection in *O. montium* and could increase its survival in a toxic host environment, thereby generating similar adaptation as the pathogenic species.

One striking result of our analysis is the dominance of spatial variables in the genotype-environment association model for *G. clavigera,* and their absence in *L. longiclavatum* and *O. montium*. We also observed much lower genetic differentiation among *G. clavigera* populations compared to the other fungi, which is intriguing especially given the importance of spatial variables in the *G. clavigera* model. One explanation for these differences is the reproduction modes employed by these fungi. *L. longiclavatum* has no known sexual stage and also showed the highest linkage disequilibrium and clonality, suggesting that this species lacks a mechanism for genetic exchange (Maynard-Smith *et al*. 1993). Linkage disequilibrium values were lower in *G. clavigera* compared to *L. longiclavatum,* but still differed significantly from zero. *G. clavigera* primarily reproduces asexually and its sexual structures are rarely observed. *G. clavigera* and *L. longiclavatum* are heterothallic, but despite the rarity or absence of sexual reproduction in these two species the opposite mating types required for sexual reproduction were found with a ratio of 1:1 in their populations (Tsui *et al*. 2013). This suggests that sexual reproduction must have occurred in the past or takes place sporadically even in these two species with high linkage disequilibrium. Following sporadic recombination, clonal lineages or inbred lines could expand locally and inflate genetic differentiation, creating the genetic patterns observed in *G. clavigera* and *L. longiclavatum*. In contrast, the sexual stage is frequently observed in *O. montium* and

linkage disequilibrium was low. Interestingly, linkage disequilibrium values were highly correlated among populations of *G. clavigera* and *L. longiclavatum*, but not among populations of *G. clavigera* and *O. montium* or *L. longiclavatum* and *O. montium*. It is plausible that similar demographic factors or environmental cues affect the production of the sexual stage in *G. clavigera* and *L. longiclavatum*, but differ in *O. montium*. Given that *L. longiclavatum* and *O. montium* both have the most differentiated population structure, but show contrasting modes of sexual reproduction, it is difficult to invoke sexual reproduction alone to explain these species level differences.

Blue stain fungi are considered essential partners in the MPB system, but their relative contributions to beetle fitness may vary. An alternative explanation for the spatial and environmental differences observed among the three species is that *G. clavigera* could be more beneficial to the beetle than the other fungi. *G. clavigera* is better adapted for transport on the beetle and it appears to be completely dependent on the insect for dissemination and host ingress (Six & Paine 1998; Six 2003). Both *G. clavigera* and *L. longiclavatum* produce long conidia within beetle galleries that may be more easily grazed by the beetles and carried preferentially in the mycangia rather than on the exoskeleton (Lee *et al.* 2006a). But *G. clavigera* is more aggressive than *L. longiclavatum* and *O. montium*, producing larger lesions (Lee *et al.* 2006b) and growing faster on artificial media. By contrast, *O. montium* is more frequently found on the beetle exoskeletons than the other two fungi (Lee *et al.* 2006a) and it is either a saprophyte or a weak pathogen as it is a less efficient colonizer of phloem compared to *G. clavigera* (Bleiker & Six 2009a,b; Cook *et al*. 2010). Indeed, *O. montium* has been hypothesized to be a more recent

invader (Six & Paine 1998, 1999; Bleiker & Six 2009a) or even an opportunistic hitchhiker in this multipartite system (Cook *et al.* 2010).

Beetles themselves may alter the dynamics of the fungal community. MPB may preferentially select *G. clavigera* strains, reflecting differences in nutritional benefit among the fungal species. Beetles feeding on *G. clavigera* showed the highest levels of fitness, and those feeding on *O. montium* were less fit potentially due to its lower efficiency in concentrating nitrogen (Bleiker & Six, 2009b; Cook *et al.* 2010). But, beetles with *O. montium* still fared better than those feeding on a diet that lacked fungal symbionts. Beetles reared in bolts of lodgepole pine inoculated with *G. clavigera* produced more progeny and had higher adult emergence than those inoculated with *O. montium* (Six & Paine 1998). A preference for *G. clavigera* could explain the lower genetic differentiation among populations (via increased gene flow) and the increased genetic differentiation in *O. montium* and *L. longiclavatum*, because of the stochasticity expected with the fluctuation in environmental conditions that would favor their selection. The increased adaptive amplitude of *O. montium* and *L. longiclavatum* compared to *G. clavigera*, highlighted in this study, would result in accidental transport and successful colonization, especially under extreme conditions or conditions that are less favorable to *G. clavigera*, allowing them to persist in association with the beetle. It is noteworthy that the beetles themselves show very similar range-wide population genetic structure (Batista *et al.* 2016), suggesting the possibility of some degree of coadaptation on a larger geographic scale (James et al. 2011).

Perhaps there is a trade-off for the beetles. They may derive higher benefits from associating with *G. clavigera*, but by transporting and exploiting a number of fungal associates with different and complementary characteristics, the beetles reduce the risk of being left

aposymbiotic and increase the likelihood of obtaining a full complement of benefits from the multipartite symbiosis (Six & Bentz, 2007). Maintaining a multipartite relationship could be instrumental in allowing the mountain pine beetle to colonize new habitats, survive in highly variable climatic regimes, and withstand adverse environmental conditions.

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 thank C. Breuil, Y. El-Kassaby (University of British Columbia) and A.V. Rice (University of Alberta) for their valuable discussion and collection and processing of samples. We also acknowledge M. Bryman (University of Alberta) for project management, and L. Khadempour, L. Farfan, S. Beauseigle, P. Herath, S. Cervantes, J. Burke for technical assistance as well as J.F. Mao, J. Klápště and C. Cullingham for advice in data analysis.

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Data Accessibility

Growth, environmental and genotypic data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.mt16j

Author Contributions

R.C.H., D.I.O., J.E.K.C., A.C. and F.A.H.S. designed the research, C.K.B., A.D.R. and D.I.O. collected the samples, D.I.O, C.K.M.T and Y.Z. conducted the genotyping and phenotyping, D.I.O, R.C.H., N.F., A.C., B.D., Y.Z., S.M.A, C.K.M.T and A.D.R. analyzed the data. All authors interpreted the data and wrote the manuscript.

FIGURES

Figure 1. Pairwise genetic differentiation (Nei's unbiased F_{st}-values) among genetic groups and populations of *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium*;

below diagonal are Nei's unbiased F_{st}-values and above diagonal F_{st} *P*-values estimated from 1000 permutations of haplotypes between populations.

Figure 2. Biplot scores from the redundancy analysis (RDA) using SNPs as the dependant variable and environmental variables as the predictors. Variables are described in Table S3; MEMS are distance-based Moran's eigenvector maps calculated from the coordinates of the samples. The best model was selected for each fungus by forward selection based on Adj-R-square values and significance of the contribution ($P < 0.01$). The first three axes of the RDA accounted for cumulative values of 72%, 90%, and 83% of the total genetic variation in *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium*, respectively. The red crosses represent the locus scores and are the coordinates of each locus in the ordination space. The black circles represent the individual sample scores. The blue crosses are non-numerical factor scores and represent tree host species. The biplot arrows represent correlation of scores and environmental variables retained in the model.

Figure 3. Growth rate for 48 isolates of the three fungal symbionts (16 *G. clavigera*, 18 *L. longiclavatum* and 14 *O. montium*) at six temperatures (from 5 to 30°C with steps of 5°C). The whiskers represent the 95% confidence interval of the mean.

TABLES

Table 1. Measures of genetic diversity and recombination in three species of mountain pine beetle fungal symbionts from three genetic groups (see Table S1).

Table 2. RDA models inferred for *Grosmannia clavigera, Leptographium longiclavatum* and *Ophiostoma montium*.

Table 3. *Grosmannia clavigera, Leptographium longiclavatum* and *Ophiostoma montium* SNP loci showing evidence of association with environmental variables as indicated by RDA. BAYENV $\log_{10}$ Bayes Factor are indicated for these loci.

SUPPORTING INFORMATION

Table S1. Table S1. Collections of *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium*.

Table S2. SNP sets for *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium*.

Table S3. Rotated component matrix (Varimax rotation with Kaiser normalization) extracted following a PCA on environmental variables related to temperature, precipitations and seasonality of temperature and precipitation. Values in the tables correspond to the load of variation at each component for every variable. The three first components of the PCA explained 93.0% of the total variance observed.

Table S4. Congruence among genetic distance matrices of three fungi and geographic distance among sampling locations

Figure S1. Neighbor-Joining trees based on F_{st}-pairwise distance matrices obtained for *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium*. Median latitude (in °N) is given for each population.

Figure S2. Distribution of genetic structure clusters among populations of *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* under the estimations from *K* = 2 to *K* = 4 clusters from STRUCTURE.

Figure S3. Pairwise correlations (Pearson's correlation) among populations of *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* for genetic recombination indexes (I_A and $\bar{r}_d$).

Figure S4. Growth of fungal isolates of *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* from three genetic groups (see Table S1) at 30°C.

Table 1. Measures of genetic diversity and recombination in three species of mountain pine beetle fungal symbionts based on SNP genotyping.

Fungal Species	Genetic groups[1]	N	MLG[2]	Clonality (%)[3]	H_{exp}	I_A	$\bar{r}_d$	*P*-value
G. clavigera	Group 1	171	140	18.13%	0.268	1.336	0.015	0.001
	Group 2	95	91	4.21%	0.255	1.377	0.016	0.001
	Group 3	110	95	13.64%	0.212	1.675	0.023	0.001
	Total	376	326	13.30%	0.277	1.292	0.014	0.001
L. longiclavatum	Group 1	127	94	25.98%	0.297	4.515	0.037	0.001
	Group 2	77	69	10.39%	0.312	3.448	0.028	0.001
	Group 3	53	48	9.43%	0.230	5.417	0.055	0.001
	Total	257	208	19.07%	0.348	4.170	0.032	0.001
O. montium	Group 1	91	88	3.30%	0.285	0.264	0.006	0.001
	Group 2	46	45	2.17%	0.283	0.067	0.002	0.225
	Group 3	61	58	4.92%	0.254	1.012	0.030	0.001
	Total	198	191	3.54%	0.310	0.403	0.009	0.001

[1]Defined based on pairwise F_{st} analyses; see Fig. 1; [2]Multilocus genotypes; [3]Proportion of the isolates that shared identical SNP profiles.

Table 2. Redundancy analysis models inferred for *Grosmannia clavigera, Leptographium longiclavatum* and *Ophiostoma montium*. The best model was selected for each fungus by forward selection based on Adj-R-square values and significance of the contribution ($P < 0.01$).

Species	Analysis	Variable in selected model[a]				
		Spatial	Temperature	Precipitation	Host	R-square[b]
G. clavigera	**Global RDA**	**MEM1 to 5 + MEM27**	**BIO3**	**BIO15**	**Tree species**	**0.085*****
	Partial RDA	MEM1 to 5 + MEM27				0.025***
	Partial RDA		BIO3			0.004***
	Partial RDA			BIO15		0.002**
	Partial RDA				Tree species	0.004***
L. longiclavatum	**Global RDA**		**BIO3 + elevation**	**PC1+ PC2**	**Tree species**	**0.184*****
	Partial RDA		BIO3 + elevation			0.052***
	Partial RDA			PC1+ PC2		0.018***
	Partial RDA				Tree species	0.033***
O. montium	**Global RDA**		**BIO3 + PC3**	**BIO15 + PC1 + PC2**	**Tree species**	**0.210*****
	Partial RDA		BIO3 + PC3			0.056***
	Partial RDA			BIO15 + PC1 + PC2		0.043***
	Partial RDA				Tree species	0.048***

[a]Variables in the model are described in Table S3

P−value : ** ≤0.01, *** ≤0.001

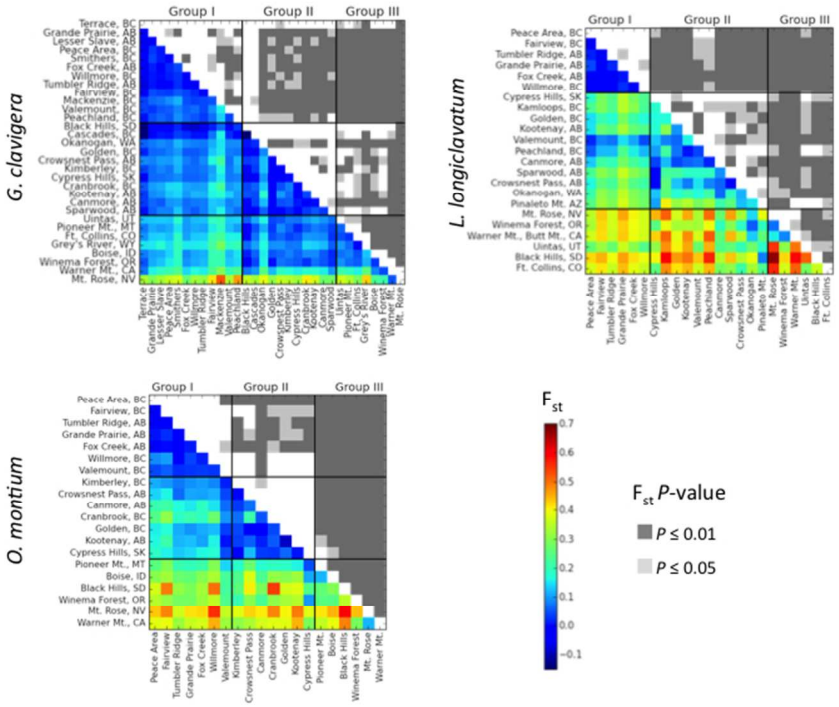
Table 3. *Grosmannia clavigera, Leptographium longiclavatum* and *Ophiostoma montium* SNP loci showing evidence of association with environmental variables as indicated by RDA. BAYENV log_{10} Bayes Factors are indicated for these loci.

SNPs	Putative function	GO – Biological Process / Molecular activity	RDA	BAYENV (log_{10} Bayes Factor)[a]
G. clavigera				
GLEAN_647	Enoyl CoA hydratase	Metabolic process / Isomerase activity	RDA1	1.60* (BIO3)
GLEAN_3226	Hydroxymethylglutaryl-lyase	Cellular ketone body metabolic process / Hydroxymethylglutaryl-CoA lyase activity	RDA3	-
GLEAN_5868	CFEM domain protein	- / Hydrolase activity	RDA1	-
GLEAN_6725_a	Legume lectin domain protein	- / -	RDA1	2.84** (PC2)
GLEAN_7505	Chromatin remodeling complex subunit	Chromatin silencing / Ion binding	RDA2	2.26** (PC3)
GLEAN_7505_a	Chromatin remodeling complex subunit	Chromatin silencing / Ion binding	RDA2	1.74* (PC2)
GLEAN_7572	Beta-glucosidase (glycoside hydrolase fam. 3)	Polysaccharide catabolic process / Hydrolase activity	RDA3	-
L. longiclavatum				
GLEAN_229	Serine-type peptidase	- / Peptidase activity, acting on L-amino acid peptides	RDA1	1.50 (PC2)
GLEAN_3884_a	ATP dependent rna helicase	rRNA processing, ribonucleoprotein complex assembly / Nucleotide binding, nucleic acid binding, hydrolase activity, acting on acid anhydrides	RDA2	1.85 (PC1)
GLEAN_3884	ATP dependent rna helicase	rRNA processing, ribonucleoprotein complex assembly / Nucleotide binding, nucleic acid binding, hydrolase activity, acting on acid anhydrides	RDA3	-
GLEAN_4629	UDP-n-acetylglucosaminyl transferase	- / Transferase activity	RDA3	-
GLEAN_4680	40s ribosomal protein s3ae	Translation, ribosome biogenesis	RDA2	-
GLEAN_5244	Class V chitinase (glycoside hydrolase fam. 18)	- / Hydrolase activity	RDA3	-
GLEAN_5244_b	Class V chitinase (glycoside hydrolase fam. 18)	- / Hydrolase activity	RDA2	-

Table 3. *Continued*

O. montium				
Glean_4a	Beta-galactosidase B	Glycerolipid metabolic process / beta-galactosidase activity	RDA2	-
Glean_20	Sulfate permease	Sulfate transmembrane transport / Secondary active sulfate transmembrane transporter activity	RDA1	1.40* (BIO3)
Glean_20a	Sulfate permease	Sulfate transmembrane transport / Secondary active sulfate transmembrane transporter activity	RDA1	-
Glean_47a	Benzoate 4-monooxygenase	Redox process / Monooxygenase activity, iron ion binding, heme binding,	RDA2	1.54* (BIO15)
Glean_65	Chitin synthase	Chitin biosynthetic process, cell wall organization / Chitin synthase activity	RDA1	1.23* (PC1)
Glean_81	Heat shock protein	- / ATP binding	RDA1	-
Glean_89	AMP dependent ligase	- / -	RDA2	1.59** (PC2)
Glean_97a	MFS transporter	Mycelium development, transmembrane transport	RDA3	-
Glean_101	Succinate semialdehyde dehydrogenase	Redox process, tyrosine metabolic process / Succinate-semialdehyde dehydrogenase [NAD(P)+] activity	RDA3	1.45* (BIO3)

[a]Only SNPs with a Log10 value ≥1.5 and/or part of the top 5% (indicated with an asterisk) are indicated; environmental variables correlated with the SNP are indicated between brackets.



Pairwise genetic differentiation (Nei's unbiased Fst-values) among genetic groups and populations of Grosmannia clavigera, Leptographium longiclavatum and Ophiostoma montium; below diagonal are Nei's unbiased Fst-values and above diagonal Fst P-values estimated from 1 000 permutations of haplotypes between populations.

282x204mm (72 x 72 DPI)

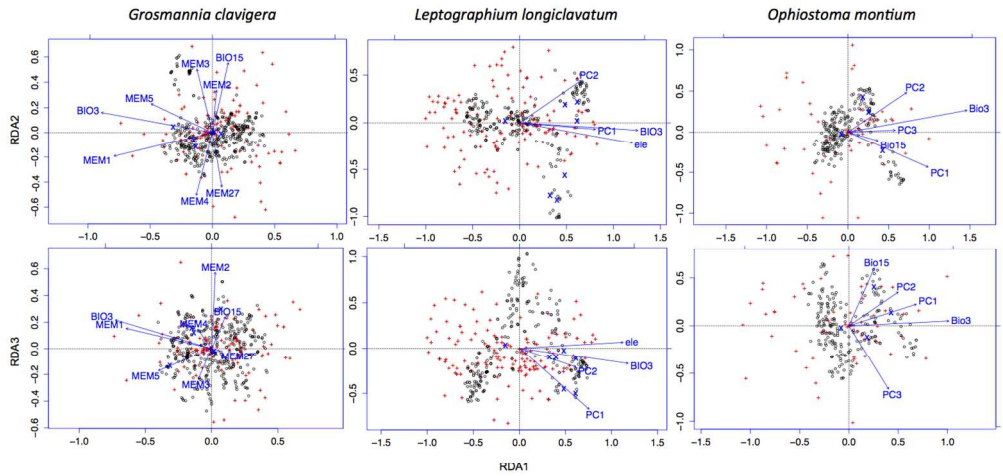
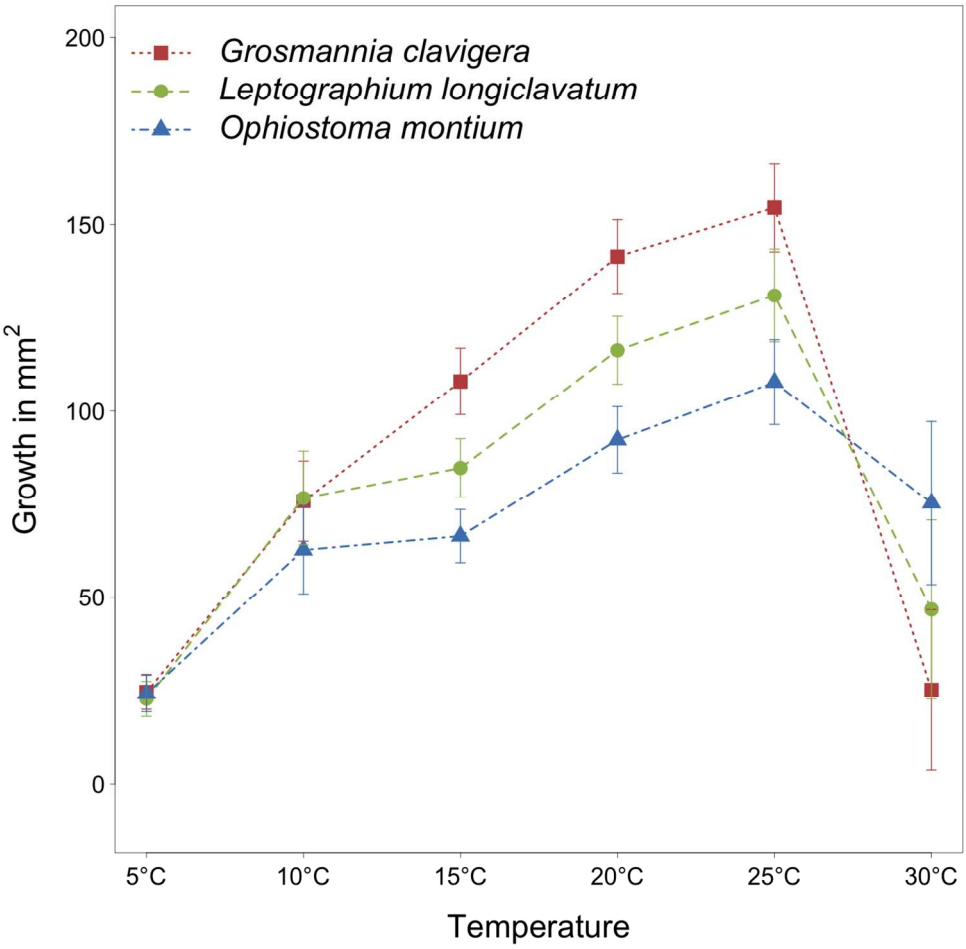


Figure 2. Biplot scores from the redundancy analysis (RDA) using SNPs as the dependant variable and environmental variables as the predictors. Variables are described in Table S3; MEMS are distance-based Moran's eigenvector maps calculated from the coordinates of the samples. The best model was selected for each fungus by forward selection based on Adj-R-square values and significance of the contribution ($P < 0.01$). The first three axes of the RDA accounted for cumulative values of 72%, 90%, and 83% of the total genetic variation in Grosmannia clavigera, Leptographium longiclavatum and Ophiostoma montium, respectively. The red crosses represent the locus scores and are the coordinates of each locus in the ordination space. The black circles represent the individual sample scores. The blue crosses are non-numerical factor scores and represent tree host species. The biplot arrows represent correlation of scores and environmental variables retained in the model.

254x190mm (150 x 150 DPI)



Growth rate for 48 isolates of the three fungal symbionts (16 G. clavigera, 18 L. longiclavatum and 14 O. montium) at six temperatures (from 5 to 30°C with steps of 5°C). The whiskers represent the 95% confidence interval of the mean.

529x529mm (72 x 72 DPI)