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Mountain pine beetle mutualist *Leptographium longiclavatum* presence in the southern Rocky Mountains during a record warm period

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We studied blue-stain fungi (Ophiostomataceae: Ophiostomatales) of mountain pine beetle in declining epidemic populations affecting three pine species in Colorado. Using morphological and molecular characterizations, we determined the presence of the mutualist *L. longiclavatum* in the southern Rocky Mountains of Colorado, where it was as common as the warm temperature adapted *O. montium* within the insect's specialized maxillary mycangium. The species was more prevalent than its "sibling species" *G. clavigera* which is the the common mycangial mutualist documented in USA populations. Findings were made during a two-year period including the warmest year on record in the state (i. e., 2012). Other studies have indicated that *L. longiclavatum* is more frequent in insect populations occurring in the northern Canadian Rockies diminishing in southern areas of that mountain range, suggesting latitude influences the frequency of this fungal mutualists, due to its better cool temperature tolerances. Our findings suggest Colorado isolates may have a greater tolerance of warmer temperatures than those from the north. These findings also increase our knowledge about the species distribution and the *in situ* conditions permissive of its occurrence in areas south of the Canadian Rockies.

Keywords: blue-stain, mycangia, mutualist, symbiont, phytopathogen.

Populations of mountain pine beetle (*Dendroctonus ponderosae*), hereafter MPB, have been in an irruptive stage across many western North American pine forests since the 1990s. Since then, outbreaks have spread to unusually high latitudes in Canada including new areas in Alberta (Cullingham et al. 2011). Populations have also reached unusually high elevations in the Rocky Mountains. In northern Colorado, attacks by the MPB at elevations higher than 3000 m are not common and have been discussed only in one internal report (DeLeon 1939, unpubl.) and a single publication (Mitton & Ferrenberg 2012). These expansions may be attributed to a warming climate.

Bark beetles can carry a wide range of phoretic associates (Mercado et al. 2014, Hofstetter et al. 2015) including important mutualists that may also become affected by changes in climate. Of these, the best understood are the ophiostomatoid blue-stain fungi. The name "blue-stain fungi" comes from the characteristic grayish blue-green hues seen on infected wood due to refraction of incident natural light by the melanin in their hyphal bodies (Zink & Fengel 1988, 1990). The relationship between MPB and its three main fungal associates *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 represents a near obligate mutualistic symbiosis in which these fungi obtain transport mainly by the MPB. Similarly, the beetle's larvae require nutrients from the beneficial blue-stain fungi to fully develop into adults (Six & Paine 1998, Myrholm & Langor 2015).

Across the USA, the occurrence of the MPB symbionts *G. clavigera* and *O. montium* has been documented for several decades (Rumbold 1941, Robinson-Jeffrey & Davidson 1968). However, *L. longiclavatum* has only recently been documented from continental USA, first in California (Lee et al. 2005, Massoumi Alamouti et al. 2011) and later from most of the states where the MPB occurs (Ojeda Alayon et al. 2017). The species has been frequently reported from MPB populations in Canada (Lee et al. 2005, Rice et al. 2008, Roe et al. 2011, among others). A caveat of previous knowledge from the USA is

that investigations utilizing techniques capable of detecting this MPB fungal symbiont in the US have been used less frequently than in Canada (Ojeda Alayon et al. 2017). Our objective was to determine the main blue-stain fungal species of MPB populations affecting areas of three common host pines in northern Colorado, using morphological and molecular characterizations.

Materials and methods

Field sampling

To infer potential effects of temperature in blue-stain fungi diversity we instrumented trees with temperature sensors (Onset, S-TMB-M002) in 2011 and within 5 days of insect attacks to measure phloem temperatures during their development. These probes were drilled into the phloem at the north and south sides. In 2012 and 2013, adult flying MPB were collected at different sites in the Roosevelt National Forest in northern Colorado from a population receding from the climax of its epidemic in 2011 (Figs. 1–2). Insect collections were made during mid to late July which corresponded to the beetle's main flight and attack period during these years. Bark beetles were not directly handled nor did these interact with other insects as these were induced to drop into individual sterile micro centrifuge tubes with lid (1.5 ml, Fisher Scientific). Insects were transported in coolers and refrigerated at the laboratory at –18 °C until processed.

Statistical analyses on fungal presence

We compared prevalence (a proportion) which inherently accounts for differences in the number of MPBs obtained across years. We used a generalized linear mixed model (GLMM) with a logit link to assess patterns in fungal presence and included MPB ID as a random effect to account for repeated observations (Hosmer et al. 2013). Differences between factors (year and species) were discerned using Tukey multiple comparisons. All statistical analyses were performed in R using the *lme4* and *multcomp* packages (Hothorn et al. 2008, R Core Team 2017).

Fungal culturing

In 2011, the first author became familiar with the blue-stain fungi transported by the mountain pine beetle in Colorado. At first, the protocol used included selective antibiotic-amended media [1 % Streptomycin (Sigma) and 2 % Cycloheximide (Acros Organics)] that allowed the near-exclusive growth of resistant blue-stain species. After becoming familiarized, amended media was not used for the rest of the study to obtain information about other MPB fungal associates for future studies.

Direct cultures of dissected mycangia and elytral surfaces were grown in un-amended 2 % MEA petri dishes (100 mm). A direct culture method of specific transporting structures of the MPB was chosen following previous studies (Whitney & Farris 1970, Six & Paine 1998). Within two to seven days after initial culture, agar plugs (2 mm) of morphologically different fungi were transferred to water agar. Two to four days later, hyphal tips were transferred to un-amended 2 % MEA plates to grow pure fungal strains. Colonies were grown at room temperature (22 °C) without protection from daytime fluorescent light exposure for a period of 28 days. Replicates of pure fungal cultures were used for DNA extraction and to study fungal growth characters in wood discs. Conidia were produced profusely in sterile wood discs from the MPB three pine hosts; therefore, these were used to examine the type of conidia and conidiophore as well.

Morphological determinations

Observed cultural characters used to distinguish *L. longiclavatum* from *G. clavigera* were those described by Lee et al. (2005). Characters used to determine *O. montium* were those defined by Rumbold (1941). Microscopic characterization using the Stereomaster (Fisher Scientific) and Eclipse TS100 (Nikon) microscopes included distinguishing the asexual morphs *Hyalorhinocladiella* from *Leptographium*, as well as the types of conidia produced by the fungi.

Molecular determinations

DNA sequences of the nuclear ribosomal ITS2-LSU (partial 5.8 + ITS 2 + partial 28S) and the protein-coding gene β-tubulin (partial gene) were used in the present study. DNA extraction and amplification were performed at the Center for Forest Mycology Research (CFMR) while the sequencing was performed at the University of Wisconsin Biotechnology Center (UWBC) in Madison, WI. DNA was extracted with a CTAB protocol following Palmer et al. (2008) and Lorch et al. (2013), but with certain modifications. A small amount of mycelium from cultures was scraped using a flattened inoculation needle and placed in 400 µl 2 % CTAB lysis buffer in 1.5 microtubes. Tubes were frozen overnight at –20 °C and then the tissue samples were ground using a plastic blue pestle. After this, the tubes were incubated in a 65 °C water bath for two hours. The samples were

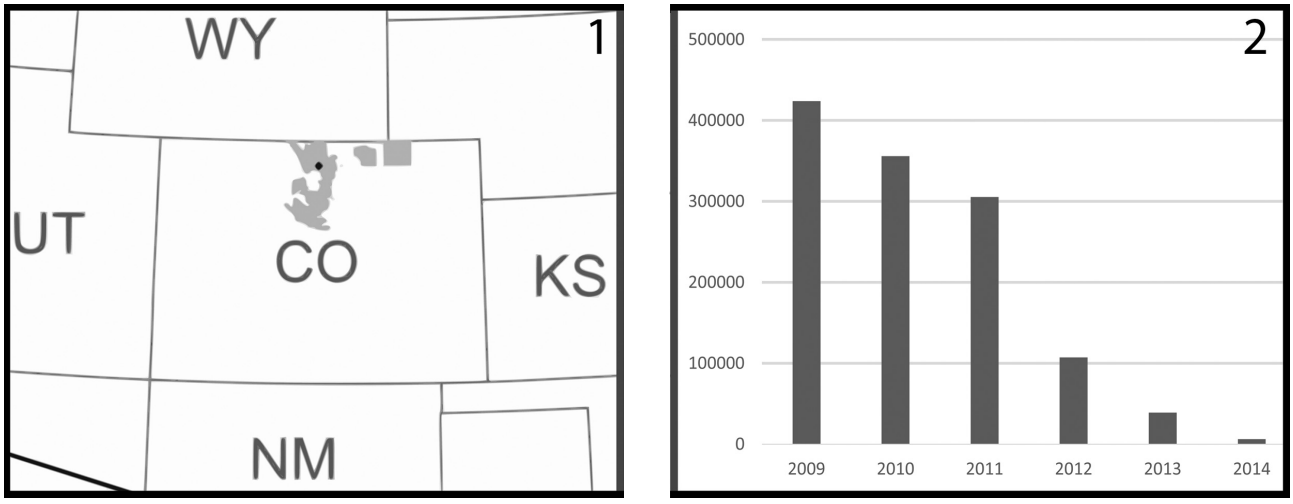


Fig. 1. Study site (star) in the Roosevelt National Forest (gray polygon). The forest is part of the southern Rocky Mountains. – **Fig. 2.** Affected forest hectares in Larimer County, Colorado from 2009 to 2013 by Mountain pine beetle relates directly to the insect' population level which was receding during the study. Most of this insect's activity in this county was located within the Roosevelt National Forest. Data adapted from USDA/Forest Health and Technologies.

then centrifuged at 13000 rcf for 6 min at room temperature (RT) and 300 µl of supernatant were transferred to a new tube. Next, 350 µl of cold 2-propanol was added to each tube and placed at –80 °C for 15 min. Samples were centrifuged at 10000 rcf for 20 min at 4 °C and then supernatant was discarded and 375 µl 75 % ethanol was added and tubes were centrifuged at 13000 rcf for 6 min at RT. Supernatant was discarded and pellets were air-dried at RT for at least 5 min, then resuspended in 50 µl water. DNA was cleaned with the GeneClean III kit as indicated by Palmer et al. (2008).

The ITS2-LSU region was amplified with primers ITS3 (White et al. 1990) and LR3 (Vilgalys & Hester 1990), while the β-tubulin was amplified with primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995). PCR was performed using 5x Green GoTaq reaction buffer and polymerase (Promega, Madison, WI) as indicated by Palmer et al. (2008). Thermo-cycler conditions for the ITS2-LSU region were: initial denaturation at 94 °C (2 min), followed by 30 cycles of denaturation at 94 °C (40 s), primer annealing at 53 °C (40 s) and elongation at 72 °C (130 s); and a final extension step of 72 °C (5 min). For the β-tubulin, the PCR conditions followed Gorton et al. (2004). PCR products were sequenced in both directions with the BigDye sequencing Kit and an ABI 3730xl capillary sequencer at UWBC.

Phylogenetic analyses

DNA sequences used for molecular identification and phylogenetic comparison were compared with other sequences available in GenBank (Benson et al. 2013) via BLASTn search. Ten new ITS2-LSU and β-tubulin sequences were generated, and 27 sequences pertaining to the genera *Leptographium* and *Grosmannia* were retrieved from GenBank. The selection of sequences from GenBank was performed considering the BLASTn search results and the species grouped within the *Grosmannia* clade (Zipfel et al. 2006, Roe et al. 2010) and the *Grosmannia clavigera* complex (Six et al. 2011) in previous studies. The information for these isolates is provided in Table 1. Newly generated sequences were edited with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan) and were deposited in GenBank (KY940824-KY940843). DNA sequences were aligned with MAFFT 7 (Katoh & Standley 2013). The Q-INS-I algorithm was used to align both the ITS2-LSU and β-tubulin sequences. The alignments were manually adjusted with MacClade 4.08 (Maddison & Maddison 2002). The final alignment was deposited in TreeBASE (S20898). Phylogenetic analyses of the combined ITS2-LSU + β-tubulin dataset were performed using maximum likelihood (ML) and Bayesian (BY) methods. Maximum likelihood analysis was performed using RAxML black box (Stamatakis et al. 2008) under the GTR model with GAMMA distributed rate heterogeneity and 100 rapid bootstrap replicates. Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al. 2012) on XSEDE through the CIPRES Science Gateway (Miller et al. 2010) for 3000000 generations in two runs and four chains with trees sampled every 1000 generations.

The burn-in period was set to 0.25. Phylogenetic trees were visualized and edited via FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and rooted to midpoint.

Voucher material

Studied specimens (Tab. 1) were isolated from *D. ponderosae* active in the Roosevelt National Forest in Colorado, USA. by the first author in the summers of 2012 and 2013. All isolates are maintained in the Rocky Mountain Research Station (RMRS) in Fort Collins, CO and duplicates have also been deposited at the Center for Forest Mycology Research Culture Collection (CFMR) in Madison, WI.

Results

Field study

In 2012, at our mid-elevation sites and prior to MPB emergence, we registered seven periods in which temperatures exceeded 30.0 °C, and theoretically were able to limit the growth of *G. clavigera* and *L. longiclavatum* (Fig. 3). These periods were short, none exceeding a couple of hours. These temperatures were measured on the south (warmer) side within 13 days prior our estimated MPB emergence date of July 2.

Overall, we found all three species of fungi were more abundant in 2012 than in 2013 samples (Fig. 4). In both years, *L. longiclavatum* and *O. montium* were the most common fungi. Although their frequency diminished nearly by half from 2012 to 2013, there were no significant difference in their population prevalence in the two years (p-value = 0.71). *Grosmannia clavigera* was significantly scarcer than *L. longiclavatum* (p-value = 0.0002) and also than *O. montium* (p-value = 0.0017) during both years (Fig. 5). Although 2013 was a climatically moderate year in contrast to the record warm 2012, there was no reduction on the previously considered warm-adapted *O. montium* (Rice et al. 2008) or an increment of the considered cold-tolerant *L. longiclavatum* and *G. clavigera* (Rice et al. 2008, Addison et al. 2013).

Morphological determinations

Morphological characterization by the leading author led to the identification of three species, *G. clavigera*, *L. longiclavatum*, and *O. montium*, three well known mutualistic blue-staining ophiostomatoids of the MPB. Determinations were based on the study of both cultural and microscopic characteristics as detailed below.

Two types of colonies were common among the growing cultures, those with an "entire" or a "sinuous" outer margin. Organisms with the entire outer margin (Fig. 6) were separated by color (i.e. sepia vs. olive-brown). Strains of the sepia colored organisms in agar media and wood discs produced *Hyalorhinocladiella* asexual morphs (Fig. 8) and short and oval conidia, diagnostic characters of *O. montium* (Rumbold 1941). The olive-brown colonies produced *Leptographium* asexual morphs (Fig. 9) in media and wood discs. Microscopically, conidiophores produced long septate-clavate conidia, characters used for the diagnosis of *G. clavigera* (Lee et al. 2005).

Ophiostomatoids with a sinuous outer margin of their colonies also differed by their dark-olive color (Fig. 7). As in *G. clavigera*, wood-disc subcultures of this third ophiostomatoid produced *Leptographium* type conidiophores with long and clavate-septate conidia. However, this strain was distinguished from *G. clavigera* by a greater conidial production near the center of the colony, a diagnostic character of *L. longiclavatum* (Lee et al. 2005).

Molecular determinations

The combined ITS2-LSU + β-tubulin dataset consisted of 37 ingroup sequences with a total of 1013 characters (616 characters for ITS2-LSU and 397 characters for β-tubulin), including 7 species of *Leptographium* and 4 of *Grosmannia*. The tree topologies obtained from the ML and BY analyses were congruent therefore only the BY tree topology is presented here (Fig. 10). Three main clades (strongly supported) were obtained in the present

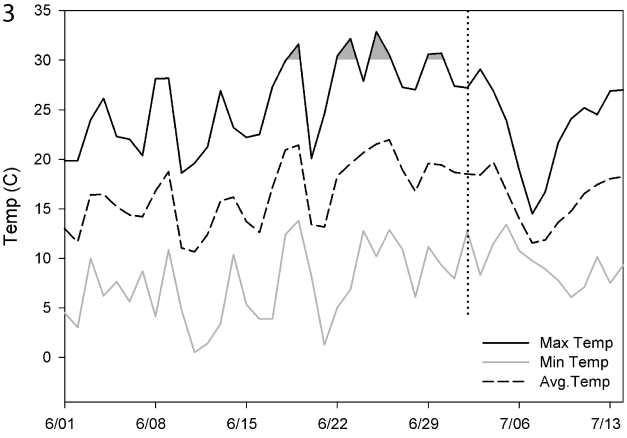


Fig. 3. Recorded phloem temperature (°C) around the time of mountain pine beetle emergence in 2012. Vertical dotted line indicates the estimated date of beetle emergence determined by examining captures from flight intercept traps in 2012 at 2800m. Gray-shaded areas represent the theoretical growth advantage of *O. montium* over the other species.

Tab. 1. *Grosmannia* and *Leptographium* isolates used in the study with GenBank accession numbers for sequences.

Species	Isolate	Location	GenBank accession no.	
			ITS-LSU	β-tubulin
G. aurea	CMW 438.69	Canada-BC	JF798473	JF798454
	UC03DL06	Canada-AB	GU370267	GU370181
	UC16G21	Canada-AB	GU370293	GU370207
G. clavigera	CO-JM-13	USA-CO	KY940831	KY940841
	CO-JM-17	USA-CO	KY940832	KY940842
	CO-JM-41	USA-CO	KY940833	KY940843
	SL-Kw1407	Canada-BC	AY544615	AY263195
	UC27G29	Canada-BC	GU370261	GU370175
	UC17DL22	Canada-AB	GU370273	GU370187
G. huntii	CBS398.77	Canada	AY707208	AY707194
	UAMH4997	Canada	AY544617	AY349023
G. robusta	CMW 668	USA-ID	AY553397	AY534945
	CMW 2805	USA-ID	AY553396	AY534944
L. koreanum	DUCC 3020	Korea	KR012411	KR012443
	MUCL 46362	China-Shanxi	EU502799	EU502811
L. longiclavatum	CO-JM-8	USA-CO	KY940824	KY940834
	CO-JM-37	USA-CO	KY940825	KY940835
	CO-JM-42	USA-CO	KY940826	KY940836
	CO-JM-43	USA-CO	KY940827	KY940837
	CO-JM-44	USA-CO	KY940828	KY940838
	CO-JM-45	USA-CO	KY940829	KY940839
	CO-JM-46	USA-CO	KY940830	KY940840
	SL-Kp11	Canada-BC	AY816687	AY816712
	SL-Kw1436	Canada-BC	AY816686	AY288934
	SL-Pw5	Canada-BC	AY816689	AY288935
	UL02G20	Canada-BC	GU370299	GU370213
	UL13G22	Canada-BC	GU370300	GU370214
	UL02G23	Canada-AB	GU370262	GU370176
L. lundbergii	CMW 217	Sweden	DQ062065	AY707185
	UAMH 9584	Sweden	AY544603	AY263184
L. procerum	MUCL 46323	China-Shanxi	EU296775	EU296781
	MUCL 47244	China-Shanxi	EU502796	EU502807
L. terebrantis	ATCC 58098	USA-MN	JF798476	JF798460
	CBS337.70	USA-LA	JF798477	JF798459
L. wingfieldii	CMW 2095	France	AY553400	AY534948
	CMW 2096	France	AY553398	AY534946
L. yunnanense	CMW 5152	China-Vimen	AY707207	AY707193

study. The first main clade includes three subclades, the *L. longiclavatum* clade (strongly supported and consisting of *L. longiclavatum* isolates from Canada and Colorado, USA), the *G. clavigera* clade [not supported and consisting of isolates of *G. clavigera*, *G. robusta* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., and *L. wingfieldii* M. Morelet], and the *G. aurea* clade [strongly supported and consisting of isolates of *G. aurea* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. and *L. terebrantis* S.J. Barras & T.J. Perry]. The second main clade includes two subclades, the *L. koreanum* clade (supported in the BY analysis, consisting of isolates of *L. koreanum* J.J. Kim & G.H. Kim, *L. yunnanense* X.D. Zhou, K. Jacobs, M.J. Wingf. & M. Morelet and *L. lundbergii* Lagerb. & Melin and

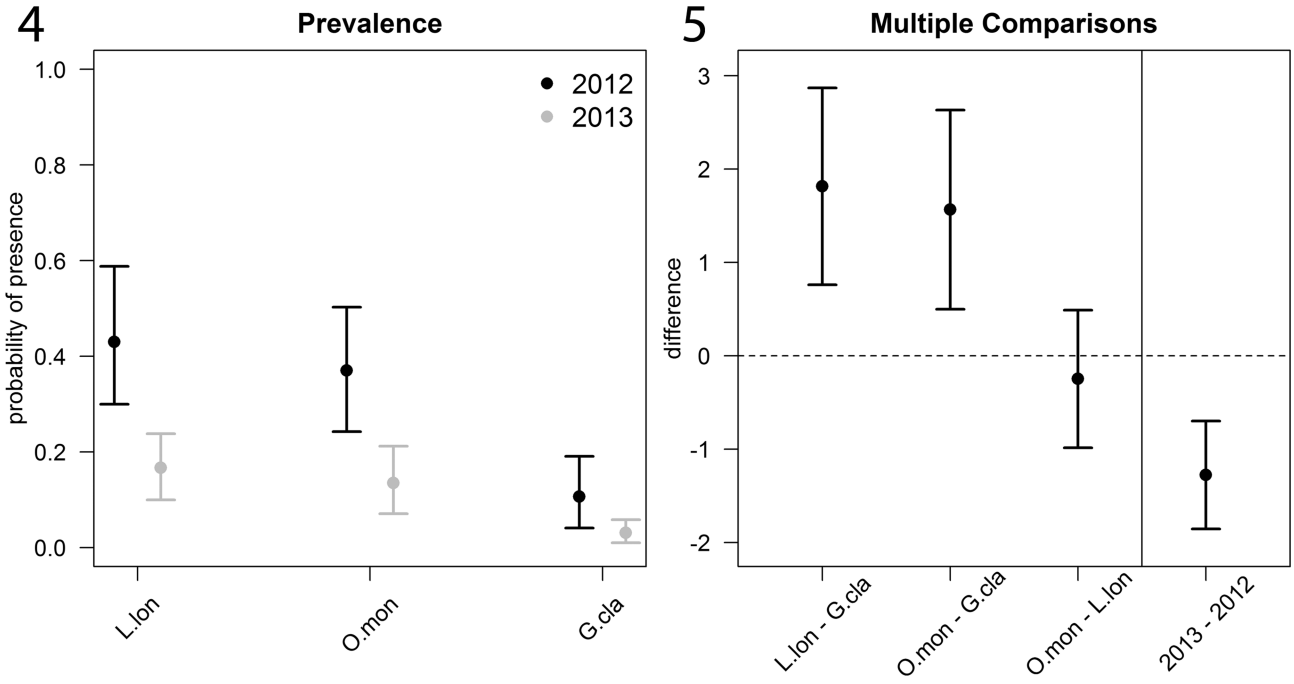


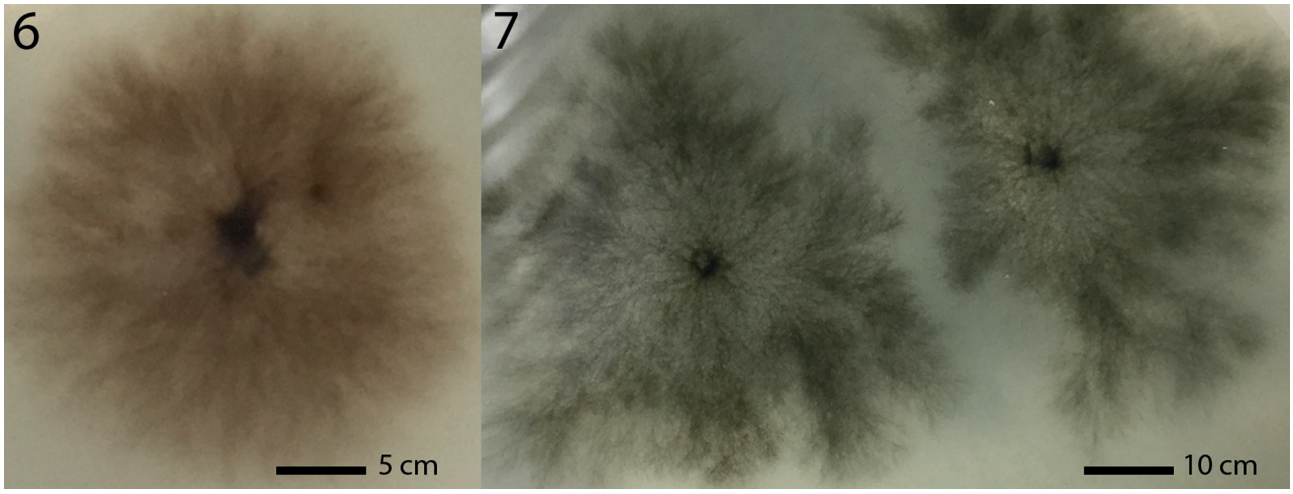
Fig. 4. Predicted probability of species prevalence between the two years. – **Fig. 5.** Results of linear multiple comparisons on logit scale with corresponding 95 % confidence intervals; confidence intervals which overlap zero (horizontal dashed line) indicate no significant difference. Abbreviations are "L.lon" for *L. longiclavatum*, "G.cla" for *G. clavigera*, and "O.mon" for *O. montium*.

the *G. huntii* clade [consisting of *G. huntii* (Rob.-Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. isolates]. The third main clade includes isolates of *L. procerum* (W.B. Kendr.) M.J. Wingf. and appears not closely related to the other two.

Discussion

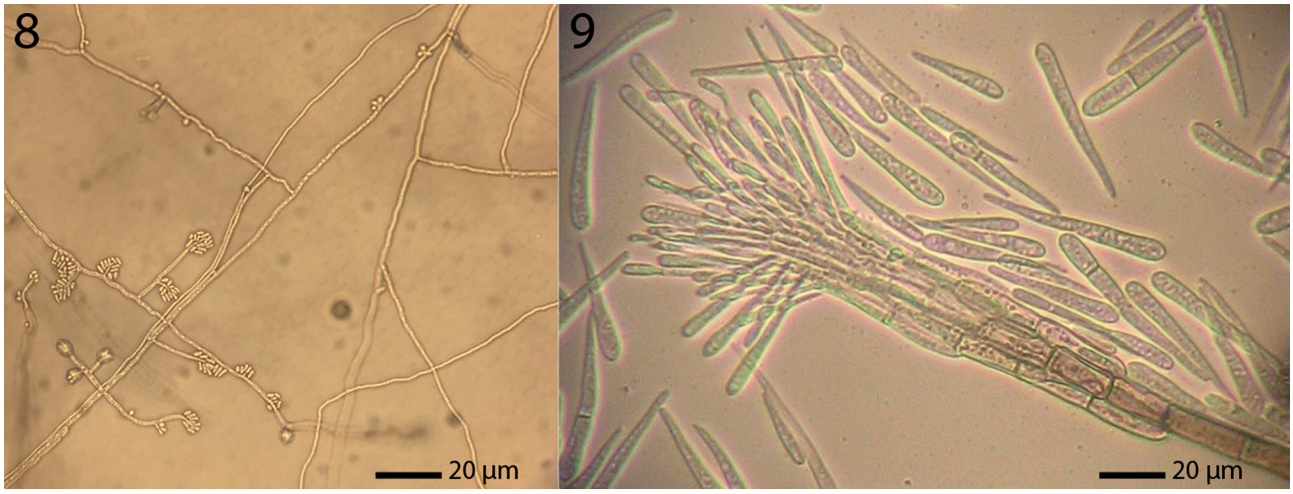
Using morphological and molecular characterizations we identified three species of blue-stain fungi from MPB collections in Colorado. These species represent *Grosmannia clavigera*, *Leptographium longiclavatum*, and *Ophiostoma montium*. Isolates of *O. montium* were not included in the phylogenetic analyses in the present study, but ITS and ITS-LSU BLASTn queries indicated that the nearest matches represented strains of *O. montium* (exceeding 97 % similarity with isolates CMW 13220, CMW 1321 and WIN 1635). Isolates of *G. clavigera* and *L. longiclavatum* from this study grouped with those from Canada (Fig. 6), confirming the identity of these species and the presence of *L. longiclavatum* in Colorado. The phylogenetic analyses also suggest the differentiation between *Grosmannia* and *Longiclavatum* species, grouped within the *Grosmannia clavigera* complex or *Grosmannia* clade in previous studies (Zipfel et al. 2006, Roe et al. 2010, Six et al. 2011, De Beer & Wingfield 2013).

Unexpectedly the usually considered "cold-adapted" *L. longiclavatum* was as common in MPB mycangia as the "warm-adapted" *O. montium* during 2012. This year was the warmest recorded year in Colorado reaching 3.6 °C above average (NOAA 2012) in late June, just before the MPB peak emergence in that year. At the moderate elevation site (2800 m), the warmest phloem temperature was recorded on June 25 (30.3 °C). Despite being close to Canadian isolates of *G. clavigera* and *L. longiclavatum* which growth is stunned or stops (Solheim & Krokene 1998, Rice et al. 2008) near this temperature, USA isolates of *G. clavigera* have been found to sporulate abundantly at 30 °C (Moore & Six 2015). Although the same has not been tested for isolates of *L. longiclavatum*, in Canada its isolates sporulate at higher temperatures than those of *G. clavigera* (Rice et al. 2008). Therefore, is likely that *L. longiclavatum* isolates from Colorado and other southern latitudes are not only adapted to warmer temperatures but also may be able to sporulate at similar temperatures than sympatric *G. clavigera* phenotypes, suggesting a life history highly synchronized to MPB in these latitudes.



Figs. 6–7. The "entire" margin *Ophiostoma montium* (**6**) colonies contrasting with the "sinuate" (branching) margin of (**7**) *Leptographium longiclavatum* cultures. Photographs J. E. Mercado.

Moreover, recent studies that independently examined fungi at one of our study sites determined that some *L. longiclavatum* isolates achieved normal growth rate at 30 °C in Colorado (Ojeda Alayon et al. 2017). These temperatures also allow the normal growth of *O. montium* (Solheim & Krokene 1998, Rice et al. 2008). In 2012, we found no thermal advantage favoring the growth of the warm-adapted *O. montium* within the month before emergence were temperatures exceeded 30 °C occasionally. The high frequency of *L. longiclavatum* in Colorado and its presence in other areas south of the Canadian Rockies suggests its association with the MPB may be as important and prevalent as that of the other two blue-stain mutualists occurring with this insect. Competitive interactions between *L. longiclavatum* and *G. clavigera* result in a higher frequency of *L. longiclavatum* in the Canadian Rockies due to its cooler temperature tolerance (Rice et al. 2008, Ojeda Alayon et al. 2017), or due to latitudinal factors (Roe et al. 2011). Nevertheless, our record of phloem temperatures indicates that conditions fostering the growth of the three blue-stain mutualists at moderate and high elevation pine forests in the southern Rockies of Colorado exist even during extremely warm years and that their prevalence may be driven by other factors. At this latitude (43 N), temperatures uniquely permissive for



Figs. 8–9. The *Hyalorhinocladiella* asexual morph (**8**) of *O. montium* is easy to distinguish from the *Leptographium* asexual morphs (**9**) of both *L. longiclavatum* and *G. clavigera*. The clavate-septate conidia of the latter are unique of the American biome in the Order Ophiostomatales. Photographs J. E. Mercado.

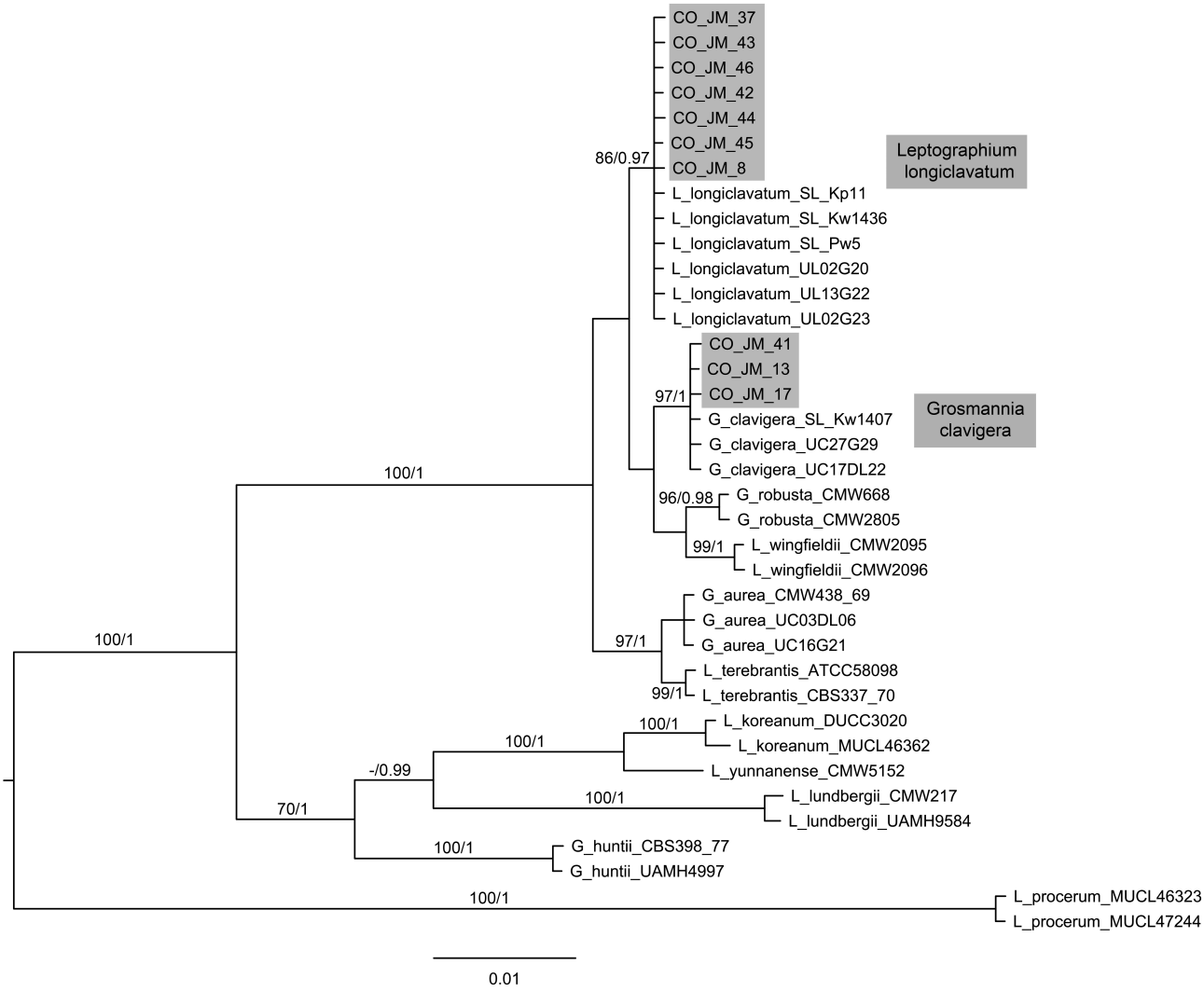


Fig. 10. Bayesian consensus tree based on combined ITS2-LSU+β-tubulin sequences. Support values along branches are from ML bootstrap (>65 %) and BY analysis (PP >0.95).

the growth of *O. montium* (i.e. >30) may be uncommon.

The matching frequencies of *L. longiclavatum* and warm temperature adapted *O. montium* during the warmer year on record suggests that currently there is no temperature barrier for the prevalence of *L. longiclavatum* in moderate to high elevation pine forests of Colorado. Furthermore, reports of the species from AZ (Ojeda Alayon et al. 2017) suggest *L. longiclavatum* could potentially tolerate even warmer temperature in western USA. Further research should explore variations across larger geographical scales to account for geographical phenotypic variability of the fungal associates of this important insect. Our findings greatly improve the knowledge of the adaptive capacity of this species, with equal repercussions for its ecologically and economically important insect carrier. It also, represents one of the first *in situ* tests of previous theoretical predictions complementing and supporting findings from *in vitro* experiments about the development of MPB fungal mutualists in this symbiosis.

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References

Addison A.L., Powell J.A., Six D.L., Moore M., Bentz B.J. (2013) The role of temperature variability in stabilizing the mountain pine beetle–fungus mutualism. *Journal of Theoretical Biology* **335**: 40–50.

Benson D.A., Cavanaugh M., Clark K., Karsch-Mizrachi I., Lipman D.J., Ostell J., Sayers E.W. (2013) GenBank. *Nucleic Acids Research* **41**: D36–D42.

Cullingham C. I., Cooke J. E., Dang S., Davis C. S., Cooke B. J., Coltman D. W. (2011) Mountain pine beetle host-range expansion threatens the boreal forest. *Molecular Ecology* **20**(10): 2157–2171.

De Beer Z.W., Winfield M.J. (2013) Emerging lineages in the Ophiostomatales. In: *The ophiostomatoid fungi: expanding frontiers* (eds. Seifert K.A., de Beer Z.W., Wingfield M.J.), CBS-KNAW Fungal Biodiversity Centre, Utrecht: 21–46.

Glass N.L., Donaldson G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**(4): 1323–1330.

Gorton C., Kim S.H., Henricot B., Webber J., Breuil C. (2004) Phylogenetic analysis of the bluestain fungus *Ophiostoma minus* based on partial ITS rDNA and beta-tubulin gene sequences. *Mycological Research* **108**(7): 759–765.

Hofstetter R.W., Dinkins-Bookwalter J., Davis T.S., Klepzig K.D. (2015) Symbiotic associations of bark beetles. In: *Bark beetles: biology and ecology of native and invasive species* (eds. Vega F., Hofstetter R. W.) Academic Press, Elsevier, San Diego, CA: 209–245.

Hosmer Jr., D.W., Lemeshow, S., Sturdivant, R.X. (2013) *Applied logistic regression*. John Wiley & Sons, New Jersey.

Hothorn T., Bretz F., Westfall, P. (2008) Simultaneous inference in general parametric models. *Biometrical Journal* **50**(3): 346–363.

Katoh K., Standley D.M. (2013) MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

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

Lorch J.M., Lindner D.L., Gargas A., Muller L.K., Minnis A.M., Blehert D.S. (2013) A culture-based survey of fungi in soil from bat hibernacula in the eastern United States and its implications for detection of Geomyces destructans, the causal agent of bat white-nose syndrome. *Mycologia* **105**(2): 237–252.

Maddison D.R., Maddison W.P. (2002) *MacClade4: Analysis of phylogeny and character evolution*. Sinauer, Massachusetts.

Massoumi Alamouti, S., Wang, V., Diguistini, S., Six, D.L., Bohlmann, J., Hamelin, R.C., Feau, N., Breuil, C. (2011) Gene genealogies reveal cryptic species and host preferences for the pine fungal pathogen *Grosmannia clavigera*. *Molecular ecology* **20**(12): 2581–2602.

Mercado J.E., Hofstetter R.W., Reboletti D.M., Negrón J.F. (2014) Phoretic symbionts of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins). *Forest Science* **60**(3): 512–526.

Miller M.A., Pfeiffer W., Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop* (eds. GCE), New Orleans: 1–8.

Mitton J.B., Ferrenberg S.M. (2012) Mountain pine beetle develops an unprecedented summer generation in response to climate warming. *The American Naturalist* **179**(5): E163–E171.

Moore M.L., Six D.L. (2015) Effects of temperature on growth, sporulation, and competition of mountain pine beetle fungal symbionts. *Microbial Ecology* **70**(2): 336–347.

Myrholm C.L., Langor D.W. (2015) Assessment of the impact of symbiont Ophiostomatales (Fungi) on mountain pine beetle (Coleoptera: Curculionidae) performance on a jack pine (Pinaceae) diet using a novel in vitro rearing method. *The Canadian Entomologist* **148**(1): 68–82.

NOAA (2012) *National Climate Report - June 2012*; https://www.ncdc.noaa.gov/temp-and-precip/drought/historical-palmers/psi/201101-201210 (accessed May 16 2017).

O'Donnell K., Cigelnik E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**(1): 103–116.

Ojeda Alayon D.I., Tsui C.K., Feau N., Capron A., Dhillon B., Zhang Y., Alamouti S.M., Boone C.K., Carrol A.L., Cooke J.E.K., Roe A.D., Sperling F.A.H. (2017) Genetic and genomic evidence of niche partitioning and adaptive radiation in mountain pine beetle fungal symbionts. *Molecular Ecology* **26**(7): 2077–2091.

Palmer J.M., Lindner D.L., Volk T.J. (2008) Ectomycorrhizal characterization of an American chestnut (*Castanea dentata*)-dominated community in Western Wisconsin. *Mycorrhiza* **19**: 27–36.

R Core Team (1993) R: A language and environment for statistical computing. R Foundation for Statistical Computing; https://www.R-project.org/ (accessed July 3 2017).

Rice A.V., Thormann M.N., Langor D.W. (2008) Mountain pine beetle-associated blue-stain fungi are differentially adapted to boreal temperatures. *Forest Pathology* **38**(2): 113–123.

Robinson-Jeffrey R.C., Davidson R.W. (1968) Three new *Europhium* species with *Verticicladiella* imperfect states on blue-stained pine. *Canadian Journal of Botany* **46**(12): 1523–1527.

Roe A.D., Rice A.V., Bromilow S.E., Cooke J.E.K., Sperling F.A.H. (2010) Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. *Molecular Ecology Resources* **10**: 946–959.

Roe A.D., James P.M., Rice A.V., Cooke J.E., Sperling F.A. (2011) Spatial community structure of mountain pine beetle fungal symbionts across a latitudinal gradient. *Microbial Ecology* **62**(2): 347–360.

Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Hohna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542.

Rumbold C.T. (1941) A blue stain fungus, *Ceratostomella montium* n. sp., and some yeasts associated with two species of *Dendroctonus*. *Journal of Agricultural Research* **62**(10): 589–601.

Six D.L., Paine T.D. (1998) Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology* **27**(6): 1393–1401.

Six D.L., de Beer Z.W., Duong T.A., Carroll A.L., Wingfield M.J. (2011) Fungal associates of the lodgepole pine beetle, *Den-*

droctonus murrayanae. *Antonie van Leuwenhoek* **100**: 231–244.

Solheim H., Krokene P. (1998) Growth and virulence of *Ceratocystis rufipenni* and three blue-stain fungi isolated from the Douglas-fir beetle. *Canadian Journal of Botany* **76**(10): 1763–1769.

Stamatakis A., Hoover P., Rougemont J. (2008) A rapid bootstrap algorithm for the RAxML web-servers. *Systematic Biology* **57**(5): 758–771.

Vilgalys R., Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**(8): 4238–4246.

White T.J., Bruns T., Lee S.S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In*: PCR protocols: a guide to methods and applications* (eds. Innis M.A., Gelfand D.H., Sninsky J.J., White T.J.), Academic Press, New York: 315–322.

Whitney H.S., Farris S.H. (1970) Maxillary mycangium in the mountain pine beetle. *Science* **167**(3914): 54–55.

Zink P., Fengel D. (1988) Studies on the colouring matter of blue-stain fungi. Part 1. General characterization and the associated compounds. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* **42**(4): 217–220.

Zink P., Fengel D. (1990) Studies on the colouring matter of blue-stain fungi. Part 3. Spectroscopic studies on fungal and synthetic melanins. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* **44**(3): 163–168.

Zipfel R.D. de Beer Z.W., Jacobs K., Wingfield B.D., Wingfield M.J. (2006) Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* **55**: 75–97.

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