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# Comparative phylogeography, genetic differentiation, and contrasting reproductive modes in three fungal symbionts of a multipartite bark beetle symbiosis

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1 **TITLE**
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3 modes in three fungal symbionts of a multipartite bark beetle symbiosis.
4
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25
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28
29 **ABSTRACT**
30 Multipartite symbioses are complex symbiotic relationships involving multiple interacting
31 partners. These types of partnerships provide excellent opportunities in which to apply a
32 comparative approach to identify common historical patterns of population differentiation
33 and species-specific life history traits. Using three symbiotic blue stain fungal species
34 (Ophiostomatacea) associated with outbreaking populations of the mountain pine beetle
35 (*Dendroctonus ponderosae* Hopkins) in western Canada, we applied phylogenetic,
36 population genetic, and demographic approaches to clarify phylogeographic patterns
37 among the three fungal species. Broadly, the three species showed significant
38 population differentiation, forming northern and southern populations, despite dramatic
39 differences in haplotype diversity. Finer scale structuring and population demographic
40 patterns were less consistent, showing some interspecific incongruence. By contrasting

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41 these species simultaneously, we were able to identify differences in recombination rate
42 and ecological traits that can explain the observed patterns of incongruence among the
43 fungal species. By applying a comparative approach to partners of a multipartite
44 symbiosis we were able to distinguish congruent population structuring and species-
45 specific differences that help us to understand the complexity and evolution of this
46 symbiotic system.

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47 **INTRODUCTION**

48 Zook (1998) defined symbiotic relationships as "the acquisition and maintenance of one
49 or more organisms by another that may result in novel structures and [or] metabolism".
50 Historically, symbioses have often been viewed in a pairwise manner, involving a host
51 and a single microsymbiotic partner, but recent research, aided by new molecular and
52 analytical tools (Ruby 2008), has shown that relationships are often not this simple
53 (Stanton 2003). Many symbiotic systems are complex and involve a diversity of
54 microsymbionts interacting within a single host (Klepzig *et al.* 2009). Multipartite
55 symbioses are well known in mammalian digestive systems (e.g. Ley *et al.* 2008), and in
56 recent years, have been described in insect systems, such as bark beetles (Cardoza *et*
57 *al.* 2008; Klepzig & Six 2004), fungus farming ants (Caldera *et al.* 2009; Currie *et al.*
58 2003), termites (Husseneder 2010), and aphids (Oliver *et al.* 2010). Multipartite
59 symbioses represent dynamic communities, where spatial, temporal, and genetic
60 variation in the community may affect host fitness. To gain a realistic understanding of
61 the interactions that occur between hosts and their symbiotic fauna, we need to examine
62 co-occurring symbionts simultaneously in natural systems (Barrett *et al.* 2008a; Ruby
63 2008). Comparative phylogeography and population genetic approaches can be used to
64 identify concordant patterns of genetic variation among co-distributed organisms, and
65 may help to identify common historical factors structuring this variation (Avise 2000;
66 Bermingham & Moritz 1998). A comparative phylogeographic approach has been
67 previously used in a range of organisms (e.g. Bernatchez & Wilson 1998; Bromilow &
68 Sperling 2010; Michaux *et al.* 2005; Qu *et al.* 2010; Rocha *et al.* 2008; Szovenyi *et al.*
69 2006), including macro- and microsymbiont systems (Ballard 2004; Jones *et al.* 2006;
70 Maia Da Silva *et al.* 2007; Thompson *et al.* 2005), but has rarely been applied to multiple
71 microsymbionts in a single host (Mikheyev *et al.* 2008; Noda *et al.* 2007). We will apply a
72 comparative approach to a multipartite bark beetle-fungal symbiosis, allowing us not only

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73 to infer common historical patterns among the symbionts, but also to identify species-
74 specific traits that may explain ecological and functional differences among the
75 symbionts (Barrett *et al.* 2008a; Bleiker & Six 2009a).
76
77 A well-known host for multiple symbionts is the mountain pine beetle (MPB,
78 *Dendroctonus ponderosae* Hopkins), which has a diverse, well studied symbiont fauna
79 (Adams *et al.* 2008; Bleiker & Six 2009a; Klepzig & Six 2004; Lee *et al.* 2006a; Six &
80 Klepzig 2004, M. Evenden and H. Proctor, pers. comm.). MPB is a major pest of pines in
81 western North America, and is currently experiencing one of the largest outbreaks in
82 recorded history (Raffa *et al.* 2008). The current outbreak has seen unprecedented
83 expansions of MPB populations into Alberta (Alberta Sustainable Resource
84 Development 2009; Ono 2004; Powell 1961), so identifying factors that could impact
85 beetle fitness, such as fungal genetic variation, is an important aspect to understanding
86 MPB outbreaks.
87
88 Some of the most well known MPB fungal symbionts are blue-stain fungi in the family
89 Ophiostomatacea, specifically *Grosmannia clavigera* (Robinson-Jeffrey and Davidson)
90 Zipfel, de Beer and Wingfield, *Leptographium longiclavatum* Lee, Kim and Breuil, and
91 *Ophiostoma montium* (Rumbold) von Arx. Phylogenetically, *G. clavigera* and *L.*
92 *longiclavatum* are closely related, belonging to the same teleomorph genus *Grosmannia*,
93 while *O. montium* is nested within a more distantly related teleomorph genus
94 *Ophiostoma* (Alamouti *et al.* 2009; Zipfel *et al.* 2006). *G. clavigera* is considered the
95 primary fungal symbiont, with a long evolutionary history with MPB, while *O. montium* is
96 thought to be a recent invader (Six & Paine 1999). The relationship of the recently
97 described *L. longiclavatum* is not known (Lee *et al.* 2005), but following Six & Paine
98 (1999) it could also be considered a recent invader. Each species is obligately

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99 dependent on MPB for transport to ephemeral food sources (Six & Klepzig 2004;
100 Whitney & Farris 1970). The fungi, in turn, provide nutrition, aid in overcoming host plant
101 defenses, alteration of microclimatic conditions, and protection against antagonistic fungi
102 (Bentz & Six 2006; Raffa & Berryman 1983; Reid *et al.* 1967; Six & Klepzig 2004; Six &
103 Paine 1998). The fungi do not contribute equally to these different functional benefits
104 (Six & Bentz 2007; Six & Paine 1998), so shifts in fungal species abundance throughout
105 the host range could dramatically impact MPB outbreaks (Hofstetter *et al.* 2006).
106
107 Shifts in intraspecific strain abundance may also be important for MPB fitness, although
108 geographically extensive surveys of fungal intraspecific variation are currently lacking.
109 Even without broad-scale genetic characterization, intra-strain variation of functional
110 traits has been documented. For example, different *O. montium* stains have been shown
111 to have a range of impacts on MBP fitness, being antagonistic (Six & Paine 1998),
112 weakly mutualistic (Bleiker & Six 2007; Six & Klepzig 2004), and even important
113 mutualists in the MPB system (Bleiker & Six 2007). Virulence and nutrition also vary both
114 among and within fungal species, affecting the fitness of the beetle host. *G. clavigera*
115 has generally been found to be more virulent than either *O. monitum* and *L.*
116 *longiclavatum* (Lee *et al.* 2006b; Plattner *et al.* 2008; Reid *et al.* 1967; Rice *et al.* 2007;
117 Solheim & Krokene 1998). Nutritionally, little is known about *L. longiclavatum* and
118 beetles that feed on *G. clavigera* are larger than those that feed on *O. montium*,
119 although intra-strain variation confounds generalization (Bleiker & Six 2007).
120
121 These fungi are also known to differ in a number of important ecological characteristics.
122 First, *G. clavigera* and *L. longiclavatum* are transported almost exclusively in mycangia,
123 while *O. montium* has been found in mycangia and on the exoskeleton of the beetle host
124 (Bleiker *et al.* 2009; Six 2003). Second, these three species vary in their environmental

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125 tolerances; *G. clavigera* grows faster in cooler temperatures and oxygen deficient
126 tissues than *O. montium*, which is better adapted to warmer temperatures and tissues
127 with greater oxygen availability (Rice *et al.* 2008; Six & Bentz 2007; Solheim & Krokene
128 1998), although cold tolerant *O. montium* strains have been identified (Rice & Langor
129 2009). *L. longiclavatum* has similar environmental tolerances to *G. clavigera*, but has a
130 slower growth rate albeit with higher rates of sporulation at cooler temps (Lee *et al.*
131 2005, 2006b; Rice *et al.* 2008). Third, *G. clavigera* appears to be the primary colonizer,
132 isolated ahead of *O. montium* and *L. longiclavatum* in tree tissue (Bleiker & Six 2009b;
133 Kim *et al.* 2005), but both *G. clavigera* and *O. montium* are able to colonize previously
134 occupied substrates and have been shown to coexist and exhibit fine scale resource
135 partitioning (Bleiker & Six 2009a, b). As highlighted above, intra- and interspecific
136 variability in both functional and ecological traits clouds our understanding of the roles
137 that these fungi play within the MPB system and requires closer examination.
138
139 Ultimately, MPB fitness is significantly improved in the presence of fungal symbionts
140 (Bleiker & Six 2007; Six & Paine 1998), and given the phenotypinc variability within and
141 among fungal species, it is important to characterize the genetic variation within each
142 fungal associate and to relate patterns of genetic diversity to this biological variation.
143 Earlier population genetic studies of *G. clavigera* (Lee *et al.* 2007) and MPB (Bartell *et*
144 *al.* 2008) show the presence of two distinct populations, a British Columbian and a
145 Rocky Mountain population (roughly corresponding to the northern and southern MPB
146 populations of Bartell *et al.* (2008)). Using a comparative approach, we have examined
147 the genetic diversity and geographic structuring of three MPB blue stain fungal
148 symbionts, *O. montium*, *G. clavigera*, and *L. longiclavatum*. Given the obligate nature of
149 the symbiosis between MPB and its fungi, we predicted that the geographic structuring
150 of the fungi would mirror that of the previous studies, with each species containing a

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151 northern and southern population. Moreover, we expected to observe congruent patterns
152 of genetic diversity and population demographics among the fungi given the putatively
153 similar biological constraints faced by the three symbionts. Characterization of
154 intraspecific population substructure is also an essential first step prior to identifying
155 patterns of adaptive variation in molecular markers or biological traits that could impact
156 MPB fitness (Pritchard *et al.* 2000).
157

158 **METHODS**

159 *Fungal isolation and multilocus sequence typing*

160 Detailed descriptions of field collections and culturing of fungal isolates are available in
161 Roe *et al.* (2010). MPB adults, larvae and gallery wood were sampled from 42 stands of
162 lodgepole pine (*Pinus contorta* Douglas var. *latifolia* Engelmann) and three stands of
163 lodgepole x jack pine hybrids (*P. contorta* x *P. banksiana* Lamb.). Stands were grouped
164 in 12 landscapes located in British Columbia and Alberta from January 2007 – May 2008
165 (Fig. 1). Landscapes represent different ecoregions within the sampling area. Fungi were
166 cultured on malt extract agar and scored as one of three morphotypes: *G. clavigera*, *L.*
167 *longiclavatum*, or *O. montium* (Roe *et al.* 2010). Representative strains were deposited
168 in the University of Alberta Microfungus Collection and Herbarium (Appendix S1).
169 Following morphotyping, strains were randomly selected within stands using a random
170 number generator for single spore isolation (SSI) and multilocus sequence typing
171 (MLST). For SSI, strains morphotyped as *G. clavigera* or *L. longiclavatum* were grown
172 on malt extract agar, while *O. montium* morphotypes were grown on malt extract agar
173 amended with lodgepole pine shavings to encourage sporulation. Following Roe et al.
174 (2010), fungal isolates underwent DNA extraction, PCR amplification, and sequencing
175 for four or five gene regions: actin, elongation factor 1 alpha (EF1a), beta tubulin (Btub),
176 an anonymous locus (UFM), and ITS2 (partial 5.8S + internal transcribed spacer region

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177 2 + partial 28S). Sequence data for all five loci were previously published for *G. clavigera*
178 and *L. longiclavatum* (Roe *et al.* 2010, GenBank GU370130-GU370344). *O. montium*
179 sequence data for four loci (actin, EF1a, Btub and ITS2) were submitted to GenBank
180 (HQ413347 – HQ413650, Appendix S1). The UFM locus did not amplify for *O. montium*.
181

182 *Phylogenetic relationships*. Previously aligned sequences were obtained for *G. clavigera*
183 and *L. longiclavatum* from TreeBASE (http://www.treebase.org). Sequence data from *O.*
184 *montium* were initially aligned using Sequencher 4.8 (Gene Codes, Ann Arbor, MI)
185 followed by manual adjustments to the alignment. Unique *O. montium* haplotypes were
186 determined for each locus and parsimony networks were estimated using TCS 1.21
187 (Clement *et al.* 2000). Previously published sequence data of an *O. montium* strain were
188 included for Btub and ITS2 (GenBank AY194948, AY194964). Parsimony networks were
189 available for *G. clavigera* and *L. longiclavatum* from Roe et al. (2010). Following single
190 locus analysis, sequences were concatenated into multilocus data sets for each species.
191 Maximum likelihood (ML) trees were estimated for *G. clavigera* and *L. longiclavatum*
192 (Roe *et al.* 2010), and *O. montium* (present study) using RAxML v. 7.0.4 (Stamatakis
193 2006) implemented on the CIPRES portal v. 1.0 (Cyberinfrastructure for Phylogenetic
194 Research – http://phylo.org/portal/Home.do, accessed January 28, 2010). Analyses
195 were performed on the multilocus data sets using distinct models for each locus, with
196 individual partition branch length optimization. Clade support was estimated using
197 RAxML rapid bootstrapping with 1000 replicates, obtained simultaneously with the ML
198 tree search (Stamatakis *et al.* 2008). Previously published sequence data of a closely
199 related ophiostomatoid fungal species, *O. ips* (Rumbold) Nannfeldt, was used as an
200 outgroup in the *O. montium* analysis (GenBank AY194938, AY194951). Final alignment
201 and multilocus tree files were deposited in TreeBASE (www.treebase.org).
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203 *Genetic diversity and population differentiation.* Standard genetic diversity indices for
204 both the northern and southern populations, as well as the combined data sets, were
205 calculated for each species. The following were calculated using the concatenated
206 multilocus data sets in DNAsp v. 5.10.00 (Rozas *et al.* 2003): polymorphic sites (S),
207 number of haplotypes (h), haplotype diversity (Hd) (Nei 1987), and nucleotide diversity
208 ($\pi$) (Nei 1987). Genetic variation was examined for signatures of population
209 differentiation and substructure corresponding to previous fungal and MPB studies
210 (Bartell *et al.* 2008; Lee *et al.* 2007). Arlequin v.3.11 (Excoffier *et al.* 2005) was used to
211 perform a hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992).
212 $\Phi_{ST}$ is analogous to Fst and incorporates sequence divergence among haplotypes in
213 addition to shifts in haplotype frequency to infer genetic differentiation among
214 populations (Weir & Cockerham 1984), and the statistical significance of $\Phi_{ST}$ was
215 estimated using permutation tests (1000 replicates).
216
217 *Comparative population structuring*. To identify congruent patterns of population
218 structuring among the three fungal species, we compared fungal genetic distance
219 matrices using CADM (Congruence Among Distance Matrices) (Legendre & Lapointe
220 2004). Using DNAsp v. 5.10.00 we obtained pairwise landscape $\Phi_{ST}$ genetic distance
221 matrices for each species, a distance measure that incorporates both sequence
222 divergence and shifts in MLST haplotype frequency. Pairwise geographic distance
223 between each landscape was also included to identify potential spatial autocorrelation.
224 CADM is a generalization of a Mantel test that allows the simultaneous comparison of
225 multiple distance matrices to identify both global and *a posteriori* pairwise congruence
226 among matrices (Legendre & Lapointe 2004). CADM was implemented in the R
227 framework (R Development Core Team 2010) using the ape package v. 2.5-2 (Paradis

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228 *et al.* 2004). Initially, a global test was performed with the `CADM.global` function to test
229 for overall congruence among the four distance matrices. If the null hypothesis of
230 incongruence was rejected, then *a posteriori* pairwise CADM and one-tailed Mantel tests
231 were performed to identify which combinations of matrices were congruent. One-tailed
232 Mantel tests were based upon ranks (Spearman correlation coefficient *r*). *A posteriori*
233 pairwise CADM and complementary one-tailed Mantel tests were executed with the
234 function `CADM.post`. For both the global and *a posteriori* analyses, we used 9999
235 permutations to assess the significance of matrix congruence. For the *a posteriori* tests,
236 a Holm correction (Holm 1979) was also used to correct *P*-values for multiple testing.
237
238 *Population demographics*. Population demographic patterns were examined within the
239 two populations of each fungal species. In Arlequin v. 3.11, we calculated a mismatch
240 distribution and compared this distribution to that of an expanding population which is
241 expected to be unimodal (Rogers & Harpending 1992). Fit of the observed to an
242 unimodal distribution was estimated with sum of squares deviations (SSD) and
243 Harpendings raggedness statistic (r) (Harpending 1994). We also calculated Tajima's D
244 and Fu's Fs, two tests of neutrality which are sensitive to signatures of population
245 expansion (Fu 1997; Rogers & Harpending 1992; Tajima 1989). Significance was
246 determined by comparing the observed values to a randomly generated distribution
247 (1000 permutations) assuming selective neutrality and population stationarity (Excoffier
248 *et al.* 2005). In an expanding population, an excess of low frequency variants is
249 expected for all loci, leading to negative Tajima's D and Fu's Fs values (Ramos-Onsins
250 & Rozas 2002; Tajima 1989).
251
252 *Evidence of recombination*. Three methods were used to detect evidence of
253 recombination. First, concatenated sequence data sets were used to construct split

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254 networks in SplitsTree v. 4.10 using the neighbor-net algorithm (Bryant & Moulton 2004).
255 This algorithm uses uncorrected pairwise distances to estimate relationships among
256 MLST haplotypes, using reticulations to represent incompatibilities within the data set
257 (Huson & Bryant 2006). A reticulated network provides an implicit representation of
258 evolutionary patterns, and may indicate the presence of homoplasy or recombination
259 (Huson & Bryant 2006). Second, we used the pairwise homoplasy index ($\Phi_w$) (Bruen *et*
260 *al.* 2006), implemented in SplitsTree. The test is robust to demographic history and
261 mutation rate and relies on the premise that physically close sites will be less likely to be
262 disassociated by recombination than distant sites. Using a 100 bp window, compatibility
263 among sites was calculated and significance determined with a permutation test
264 assuming no recombination. Third, an index of association ($I_A$), a test for clonality or lack
265 of recombination, was calculated using Multilocus v.1.3b (Agapow & Burt 2001) on a
266 clone-corrected data set. The observed MLST haplotype distribution was compared to
267 an expected haplotype distribution generated from 1000 randomly reshuffled haplotype
268 combinations. The test assumes an infinite amount of recombination so significant
269 departure from the simulated data set suggests the presence of clonality. Clone
270 corrected data sets were obtained by removing identical MLST haplotypes at the level of
271 the stand to reduce the chances of sampling the same fungal strain multiple times.
272

273 **RESULTS**

274 *Phylogeographic analyses*. A total of 143 *O. montium*, 155 *G. clavigera*, and 169 *L.*
275 *longiclavatum* isolates from 45 stands in 12 landscapes were sequenced (Fig. 1). Five
276 loci (actin, EF1a, Btub, UFM, and ITS2) were amplified for *G. clavigera* and *L.*
277 *longiclavatum*, and four loci (missing UFM) were amplified for *O. montium*. Haplotype
278 networks inferred from each locus are presented in Appendix S2, including pruned
279 networks for *G. clavigera* and *L. longiclavatum* which were described previously (Roe *et*

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280 *al.* 2010). Sequences were concatenated, creating a multilocus data set for each
281 species, from which unique MLST haplotypes were selected for ML analysis. A summary
282 of the phylogenetic data and ML model parameters for *O. montium* are presented in
283 Table 1, with similar data available for the other two species in Roe *et al.* (2010, Table
284 2). ML phylograms for *O. montium* (Fig. 2), *G. clavigera* and *L. longiclavatum* (Fig. 3) are
285 shown, in which *O. montium* was paraphyletic, while both *G. clavigera* and *L.*
286 *longiclavatum* formed monophyletic clades. ML phylograms of all three species have
287 short internal branches and longer terminal branches, with some very long branches
288 indicating highly divergent *O. montium* MLST haplotypes (Fig. 2, e.g. M37). Given the
289 congruence between MPB and *G. clavigera* population structure (Bartell *et al.* 2008; Lee
290 *et al.* 2007), we looked for similar congruence our fungal data sets. *O. montium* was the
291 most diverse of the three species. A total of 66 MLST haplotypes were found, with over
292 half found only in a single strain (Fig. 2). Few MLST haplotypes were shared between
293 northern and southern populations, with more shared between landscapes within the
294 populations than between populations. Based on the ML relationships, phylogenetic
295 structuring exists among *O. montium* haplotypes, partially corresponding to geographic
296 location, although these relationships were poorly supported (Fig. 2). Single locus data
297 showed a similar pattern (Appendix S2A), with only half of the haplotypes shared
298 between northern and southern populations, although correspondence between
299 phylogenetic relationship and geographic location was less evident. In contrast, *G.*
300 *clavigera* and *L. longiclavatum* (Fig. 3) had far fewer MLST haplotypes and showed little
301 correspondence between phylogenetic structuring and geographic location. In *G.*
302 *clavigera* 12 MLST haplotypes were found, seven of which were very common and
303 shared between northern and southern populations, while all but one of the remaining
304 haplotypes were only in the southern population. *L. longiclavatum* had six MLST
305 haplotypes, two of which were very common and were shared between northern and

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306 southern populations, with the remaining haplotypes found only in the southern
307 population.
308
309 *Genetic diversity and population differentiation.* Indices of genetic diversity for each
310 species, as well as for northern and southern populations within each species, are
311 provided in Table 2. *O. montium* had the highest overall Hd and $\pi$, followed by *G.*
312 *clavigera*, and then *L. longiclavatum*. All species had higher Hd in the southern
313 population. *O. montium* had similar levels of $\pi$ in both populations, while *G. clavigera*
314 and *L. longiclavatum* both had slightly higher $\pi$ in the southern population. Using
315 AMOVA we estimated population differentiation and structure for each fungal species.
316 For all three species, variation within populations accounted for the majority of the total
317 variation (Table 3). The percentage of variation among the northern and southern
318 populations ranged from 4.47% (*O. montium*) to 21.67% (*L. longiclavatum*), and all $\Phi_{ST}$
319 values were significant (Table 3).
320
321 *Comparative population structuring*. With CADM we tested for congruence among three
322 fungal $\Phi_{ST}$ distance matrices and a landscape-level geographic distance matrix (Table
323 4). The global CADM test rejected the null model of incongruence among the matrices
324 and all *a posteriori* CADM results indicate that each matrix was congruent with at least
325 one other matrix. One-tailed Mantel tests showed that the $\Phi_{ST}$ distance matrices of *G.*
326 *clavigera* and *L. longiclavatum* were congruent ($P < 0.01$), suggesting similar landscape-
327 level population structuring. *L. longiclavatum* genetic distance matrix was also congruent
328 with geographic distance ($P < 0.05$), indicating significant spatial autocorrelation. *O.*
329 *montium* was incongruent with both other fungal matrices, but was congruent with
330 geographic distance ($P < 0.01$), indicating significant spatial autocorrelation.

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332 *Population demographics*. Comparing several tests for population expansion, conflicting
333 population demographic patterns occurred within and between species. Following Grant
334 & Bowen (1998), *O. montium* was characterized by high Hd (>0.5) and low $\pi$ (<0.5),
335 consistent with a past population bottleneck followed by rapid population expansion.
336 Mismatch distributions for both *O. montium* populations were multi-modal, containing
337 multiple distinct peaks (Fig. 4). The demography of the northern population was
338 significantly different from the null model of sudden expansion, while the southern
339 population was consistent with the model of a sudden expansion. Both populations had
340 a non-significant Harpendings raggedness index. In *O. montium* the northern population
341 had a small cluster of highly divergent pairwise differences (Fig. 4), which may
342 correspond to mismatches between divergent haplotypes (e.g. M37 or M65; Fig. 2).
343 Using the tests of neutrality, Tajima's D was significant for both northern and southern *O.*
344 *montium* populations, suggesting population expansion, while only Fu's Fs was
345 significant for the southern population. Like *O. montium*, *G. clavigera* was characterized
346 by high Hd (>0.5) and low $\pi$ (<0.5), suggesting a past population bottleneck followed by
347 rapid population expansion. Mismatch distributions of both *G. clavigera* populations were
348 unimodal, were not significantly different from the null model of sudden expansion, and
349 had a non-significant Harpendings raggedness index (Fig. 4). In contrast, neither Fu's Fs
350 or Tajima's D were significant for populations of *G. clavigera*, refuting a population
351 expansion scenario. Unlike the previous species, *L. longiclavatum* had relatively low Hd
352 (≤0.5) and low $\pi$ (<0.5), making it difficult to differentiate between a bottleneck and
353 recent population expansion, or a population bottleneck with few founders. Mismatch
354 distributions in *L. longiclavatum* were difficult to define due to the low Hd, particularly in
355 the northern population (only two MLST haplotypes were present). The distribution in the

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356 northern population was significantly different from the null model of sudden expansion,
357 although this may be due to the low Hd characterizing this population. Both populations
358 had non-significant Harpending's raggedness index. Like *G. clavigera*, neither test of
359 neutrality (Fu's Fs or Tajima's D) was significant for populations of *L. longiclavatum*.
360
361 *Evidence for recombination*. Using the concatenated data set, networks were produced
362 for each species using the neighbor-net algorithm. Each species showed a reticulate
363 topology, although the level of reticulation varied between species (Fig. 5). *O. montium*
364 had the most reticulation among haplotypes, followed by *G. clavigera*. Reticulation
365 among haplotypes within these two species occurred between internal nodes, as well as
366 between terminal branches, a pattern indicative of recombination (Rosendahl *et al.*
367 2009). Reticulation among *L. longiclavatum* haplotypes, on the other hand, was
368 restricted to internal nodes as expected from homoplasy. Using $\Phi_w$, another metric for
369 presence of recombination, only *O. montium* was found to have significant evidence for
370 recombination. We should note that $\Phi_w$ has been demonstrated to be too conservative
371 when sequence diversity is low and populations are growing (Bruen *et al.* 2006), so it is
372 possible that this test failed to detect recombination in *G. clavigera* and *L. longiclavatum*
373 (Type II error). To further assess the presence (or lack thereof) of recombination, we
374 used $I_A$ (Agapow & Burt 2001). Using clone-corrected data sets we examined $I_A$ for each
375 fungal species and population, although we were unable to calculate $I_A$ for the northern
376 *L. longiclavatum* population due to the low Hd. Based on $I_A$, we could not reject the
377 presence of recombination in *O. montium* or *L. longiclavatum*, both at the species level
378 and at the population level. In *G. clavigera*, $I_A$ was significant at the species level, as well
379 as in the southern population, suggesting that the southern population, at least, shows
380 evidence for clonality.

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382 **DISCUSSION**

383 The current MPB outbreak in western Canada has been characterized by large
384 demographic and range expansions (Raffa *et al.* 2008). Given the obligate nature of the
385 MPB-fungal symbiosis, we had an ideal opportunity to use a comparative approach to
386 infer common historical patterns in multiple co-distributed species, test for evidence of
387 recombination that may affect the genetic diversity of each species, and to relate the
388 observed genetic diversity to biological characteristics of each species. This type of
389 comparative study is of particular value in pathogenic species (Barrett *et al.* 2008a) and
390 can improve our understanding of population structure and demographic processes
391 affecting these economically important organisms. Despite similar needs (e.g.
392 transportation to ephemeral food sources), these three fungi were surprisingly different,
393 even with concordant broad-scale population structuring. Comparative, simultaneous
394 comparisons of multiple co-distributed species also serves to demonstrate the level of
395 complexity observed within multipartite symbioses and should be considered for future
396 studies on multipartite symbioses.

397

398 Broad-scale population structuring was congruent across the three fungal species, with
399 each species differentiating into southern and northern populations (Table 3), similar to
400 previously observed patterns in *G. clavigera* (Lee *et al.* 2007) and MPB (Bartell *et al.*
401 2008). We observed higher levels of Hd in all three southern fungal populations that, as
402 suggested in Lee et al. (2007), could be evidence of earlier MPB outbreaks in western
403 Canada. Over the past 100 years, three MPB outbreaks have been recorded in this
404 region. The first two recorded outbreaks (1934-43, 1977-85) occurred in south-central
405 British Columbia and expanded into southern Alberta (Ono 2004; Powell 1961). If
406 remnant populations of MPB and fungi from these previous outbreaks persisted at

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407 endemic levels in Alberta, there would have been time for the fungal lineages to
408 differentiate. When the disjunct populations in British Columbia expanded during the
409 current outbreak, signatures of an endemic southern Alberta population remained as
410 suggested by our data.
411
412 While we did see congruent broad-scale population differentiation, when we compared
413 these three species at a finer landscape-level, this congruence was less apparent. The
414 population structuring of two species (*G. clavigera* and *L. longiclavatum*) were
415 congruent, while *O. montium* was incongruent. Interestingly both *L. longiclavatum* and *O.*
416 *montium* showed evidence of spatial autocorrelation, although no evidence was found in
417 *G. clavigera.* The extremely high levels of haplotype diversity in *O. montium* could
418 explain this incongruence, while the lack of spatial autocorrelation in *G.clavigera* is
419 harder to interpret. *G. clavigera* is often considered the primary fungal symbiont,
420 providing the MPB greater fitness benefit than other fungal species (Bleiker & Six 2007;
421 Six & Paine 1998). It is possible that *G. clavigera* is experiencing different dispersal
422 patterns or rates than the other two fungal species, influencing its phylogeographic
423 patterns and creating incongruence with the other two symbionts.
424
425 Interestingly, neither broad nor finer scale population structuring was observed among
426 the phylogenetic relationships of haplotypes within the fungal species. We found little
427 phylogeographic structuring among the MLST haplotypes, and the intraspecific
428 relationships were poorly supported. We were surprised to see little evidence for the
429 previously detected cryptic diversity in *G. clavigera* (Groups 1 & 2, Lee *et al.* 2007). It is
430 possible that our conserved nuclear and ribosomal markers were unable to separate
431 these two groups, which were identified using more variable AFLP markers.
432 Alternatively, it is also possible that members of one of the two groups were not

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433 sampled. The relationships among our haplotypes were star-like, with short internal
434 nodes and long terminal branches (Figs. 2, 3, 5), characteristic of populations that have
435 recently undergone a rapid expansion (Excoffier *et al.* 2009). Given the observed
436 demographic and spatial expansion of the current MPB outbreak, it is not surprising that
437 the obligate symbiotic fungi are also experiencing similar, detectable, population
438 expansions. Moreover, these outbreaks have occurred very recently, so there may not
439 have been time for lineage sorting to result in detectable phylogenetic signal. Expanding
440 populations are expected to have an excess of rare alleles and low frequency mutations
441 with a skew towards singletons, gene trees with long terminal branches and star-like
442 topologies, negative Tajima's D and Fu's Fs, and unimodal mismatch distributions
443 (Excoffier *et al.* 2009; Harpending & Rogers 2000; Slatkin & Hudson 1991)). Many of
444 these genetic patterns were observed in the fungi (Figs 2, 3, 4, and Appendix 2), albeit
445 with some conflicting results. These conflicts may be due to the recent nature of the
446 current MPB population expansion, low levels of nucleotide diversity that decrease the
447 power to detect signatures of population expansion, or unrecognized cryptic species,
448 which could be giving misleading results for the tests of neutrality and confound the
449 interpretation of mismatch distributions.

450

451 Incongruent genetic diversity, demographic patterns, and landscape-level population
452 structuring could result from a number of biological differences between the three
453 species (Figs. 2, 3, Tables 2, 3, 4). First, differential recombination rates provide one
454 possible explanation for these interspecific differences. Differences in reproductive
455 mode (e.g. sexual versus asexual) is known to affect a range of population
456 characteristics (Barrett *et al.* 2008b), such as genetic diversity (Milgroom 1996),
457 population growth rate (Heitman 2006), persistence (Barrett *et al.* 2007), and rate of
458 evolutionary change (McDonald & Linde 2002). Sexual reproduction or, more broadly,

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459 recombination (including parasexual recombination) creates mosaic sequences and
460 provides a means of creating new genetic combinations. Fungal species show a great
461 diversity in levels of recombination, ranging from fully asexual lineages to obligate
462 outcrossers (Milgroom 1996) and detection of this process is paramount to
463 understanding fungal evolution.
464
465 Among the study species, morphological evidence for recombination (sexual
466 reproduction) is uneven. Sexual states have been observed in *O. montium* (Rumbold
467 1931, A.V. Rice, K. Bleiker, unpublished), and evidence for sexual reproduction is quite
468 common in *O. ips* and *O. pulvinisporum* Zhou & Wingfield, two closely related species
469 (Zhou *et al.* 2007; Zhou *et al.* 2004). In contrast, sexual states in *G. clavigera* have rarely
470 been reported since they were originally described by Robinson-Jeffrey & Davidson
471 (1968), despite efforts to produce sexual states in artificial pairings (Six *et al.* 2003; Six &
472 Paine 1997). The anamorphic genus *Leptographium* is considered to include the asexual
473 forms of *Grosmannia* species (Lee *et al.* 2005; Zipfel *et al.* 2006), so it would be
474 surprising to detect recombination in members of this group, such as *L. longiclavatum*.
475
476 Using a neighbor-net algorithm, $\Phi_w$, and $I_A$, we consistently detected evidence for
477 recombination in *O. montium*, while recombination in the other two species was weakly
478 supported (Table 2, Fig. 5). A species capable of recombination would be expected to
479 have higher genotypic diversity than an asexual species, and would also be expected to
480 have a high number of unique, recombinant genotypes (Barrett *et al.* 2008a; Burdon &
481 Roelfs 1985), similar to our observations for *O. montium*. Recombination, rather than
482 cryptic species diversity, could explain the highly divergent MLST haplotypes observed
483 in *O. montium*, such as ME8 in EF1a (Appendix S2). Conversely, asexual species, or
484 species where recombination is rare would have a number of common genotypes

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485 shared between populations and few unique strains, as we observed in *L. longiclavatum*
486 and *G. clavigera* (Table 2, Fig. 5).
487
488 Asexual reproduction, or clonality, is considered a common adaptation to mutualistic
489 relationships (Wulff 1985), allowing symbiotic partners to co-evolve with optimally
490 adapted clones without the confounding force of recombination disassociating
491 successful gene combinations. However, truly asexual lineages may be prone to the
492 accumulation of deleterious alleles and may be unable to rapidly adapt to heterogeneous
493 environments (Lushai *et al.* 2003). Organisms with recombination, on the other hand,
494 have the advantage of purifying selection and the ability to create new gene
495 combinations allowing rapid adaptation to changing conditions. Many fungi have the
496 ability to switch between recombining and clonal reproduction (Taylor *et al.* 1999),
497 providing great adaptive potential. Recombination can produce highly adapted
498 genotypes, which then can increase in frequency through clonal reproduction (Barrett *et*
499 *al.* 2008a; McDonald & Linde 2002). It will be interesting to further explore patterns and
500 rates of recombination among these species, relating the differences in mode of
501 reproduction to their functional roles in the MPB-fungal symbiosis.
502
503 In addition to recombination, differences in other biological traits relating to the functional
504 roles of each fungal species could explain the observed incongruence. First, while *G.*
505 *clavigera* and *L. longiclavatum* are transmitted exclusively in the mycangia, *O. montium*
506 can be transmitted in the mycangia, as well as on the exoskeleton of the beetle host
507 (Lee *et al.* 2005; Six 2003). This apparent disparity in transmission efficiency could result
508 in the transmission of a greater diversity of *O. montium* strains relative to the exclusively
509 mycangial associates. Coupled with higher rates of recombination, these dual modes of
510 transportation may contribute to the maintenance of higher levels of genetic diversity in

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511 *O. montium*, creating discordance between the different fungal species. Second,
512 mycangial transport requires acquisition of fungal spores by the beetle prior to
513 emergence from the natal host (Six 2003). If the beetle is preferentially selecting spores
514 of certain fungal strains, this could dramatically influence the genetic diversity,
515 population structuring and demographic patterns of the mycangial species. Non-random
516 selection of fungal strains by the beetle would act like purifying selection on the fungal
517 populations, reducing genetic diversity particularly if the selected species are asexual or
518 have low rates of recombination. Third, *G. clavigera* is considered the primary symbiont
519 of the MPB (Bleiker & Six 2007; Six & Paine 1998) with a long evolutionary history with
520 this beetle host, while *O. montium* is a recent invader of the system (Six & Paine 1999).
521 It is possible that the long evolutionary history with MPB has resulted in a loss of genetic
522 diversity in *G. clavigera*, a pattern not yet observed in *O. montium*. On the other hand,
523 high genetic diversity as a result of recombination may have permitted the invasion and
524 persistence of *O. montium* in the MPB system.
525
526 **CONCLUSION**
527 As general understanding of the multipartite MPB-fungal symbiosis expands, it is
528 apparent that we have only begun to comprehend the complexity of this system. Our
529 comparative examination of three co-occurring fungal symbionts identified similar broad-
530 scale population structuring, confirming the presence of northern and southern fungal
531 populations. However, finer scale population structuring showed surprising levels of
532 incongruence, refuting our initial hypotheses. In our results *O. montium* was
533 characterized by high haplotype diversity with evidence of high rates of recombination,
534 while haplotype diversity for *G. clavigera*, and to a greater extent *L. longiclavatum*, were
535 much lower and showed little to no evidence for recombination. Characterizing the
536 differences among fungal species, such as recombination rate and standing genetic

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537 variation, is critical to understanding fungal evolution and adaptation. This is particularly
538 true in symbiotic relationships, where the fitness and adaptability of one symbiont
539 directly affects the other. While asexuality is often viewed as beneficial in symbiotic
540 relationships, recombination creates variation that can allow rapid adaptation to
541 changing environments (Croll & Sanders 2009). This creation of novel gene
542 combinations can result in phenotypic changes, which may confer an adaptive
543 advantage to a recombinant strain (Awadalla 2003), which may in turn provide an
544 advantage to the symbiont host. Recently, Wilkinson *et al.* (2010) demonstrated that
545 high intraspecific diversity of an ectomycorrhizal fungus had a significant impact on its
546 contribution to ecosystem productivity and ecological function. This work serves to
547 highlight the importance of individuals within studies seeking to clarify the functional
548 roles of fungal symbionts. Similar types of phenotypic variability have been
549 demonstrated among MPB fungal symbionts, leading to conflicting results between
550 studies. Given the potential individual variability, it will be essential to take strain
551 genotype into account when designing studies that examine the functional roles of fungal
552 symbionts, as individual strains may broadly vary in their environmental tolerance,
553 nutritive value and virulence, all factors that could impact MPB fitness.
554
555 From our study, other exciting avenues for future work have emerged. For example, how
556 does the genetic diversity of the mycangial symbionts following beetle emergence
557 compare to the community within the gallery? Does the beetle select for particular fungal
558 genotypes? Given that *O. montium* is transmitted both mycangially and phoretically, is
559 the genetic diversity of the mycangial strains different than those on the exoskeleton?
560 Does the observed genetic diversity and recombination rates within these symbionts
561 correlate with variation seen in other traits, such as ergosterol content (Bentz & Six
562 2006), virulence (Lee *et al.* 2006b; Plattner *et al.* 2008; Rice *et al.* 2007; Solheim &

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563 Krokene 1998), and environmental tolerance (Adams & Six 2007; Bleiker & Six 2009a;
564 Rice *et al.* 2008)? Do different populations of MPB (e.g. northern vs. southern) have
565 differentially adapted symbionts? Ultimately, with this new understanding of genetic
566 variation within the MPB symbiont community we now have the tools to further explore
567 MPB fungal symbiont evolution and help resolve the complexities of this system.
568

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Table 1: Parameters for individual loci and the concatenated multilocus maximum likelihood analysis of *O. montium* sequence data. A partitioned ML analysis was employed, and character information is presented for each locus partition. Ingroup character information includes the representative sequence data obtained from GenBank.

| | actin | EF1a | Btub | ITS2 | Combined[a] |
|---|---|---|---|---|---|
| # haplotypes [b] | 6 | 9 | 17 | 7 | 66 |
| # sites | 702 | 568 | 632 | 918 | 2820 |
| # constant char. [b] | 693 | 541 | 597 | 907 | 2738 |
| # variable char. (uninformative) [b] | 0 | 8 | 5 | 0 | 13 |
| # pars. inform. char. [b] | 9 | 19 | 30 | 11 | 69 |
| % informative [b] | 1.28% | 3.34% | 4.75% | 1.20% | 2.44% |
| Base freq.[c] | | | | | |
| A | 0.1920 | 0.2041 | 0.1811 | 0.2090 | - |
| C | 0.2924 | 0.3220 | 0.3235 | 0.2852 | - |
| G | 0.3220 | 0.2076 | 0.2745 | 0.3108 | - |
| T | 0.1935 | 0.2662 | 0.2208 | 0.1949 | - |
| Rate Matrix [c] | | | | | |
| A-C | 1.1732 | 2.8713 | 1.9422 | 1.6953 | - |
| A-G | 16.4090 | 1.4441 | 5.8509 | 3.9262 | - |
| A-T | 2.0000E-5 | 1.6941 | 3.7643 | 2.6116 | - |
| C-G | 6.4245 | 0.8930 | 2.0085 | 2.0000E-5 | - |
| C-T | 1.1050 | 8.0788 | 7.5822 | 23.1454 | - |
| G-T | 1.0000 | 1.0000 | 1.0000 | 1.0000 | - |
| $\Gamma$ (alpha)[c] | 0.02000 | 0.02000 | 0.02000 | 0.02000 | |

[a] GTR+$\Gamma$ ML model ; -ln = -5114.4; $\propto$ gaps/missing = 0.06755
[b] ingroup only
[c] partitioned

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Table 2: Genetic diversity and recombination indices for populations of *O. montium*, *G. clavigera*, and *L. longiclavatum*.

| | Genetic Diversity Indices | | | | | Tests of Neutrality | | Recombination | |
|---|---|---|---|---|---|---|---|---|---|
| | n | S | h | Hd (SD) | $\pi$ (SD) | Fs | D | $I_A$ | $\Phi_w$ |
| *O. montium* | | | | | | | | | |
| North | 80 | 56 | 29 | 0.93(0.014) | 0.0019(0.00045) | -6.22 ns | -1.86** | 0.088 ns | - |
| South | 63 | 42 | 43 | 0.98(0.0060) | 0.0019(0.00021) | -25.26*** | -1.41* | -0.094 ns | - |
| Total | 143 | 76 | 66 | 0.97(0.0070) | 0.0020(0.00027) | - | -1.96* | -0.051 ns | ** |
| *G. clavigera* | | | | | | | | | |
| North | 79 | 5 | 8 | 0.74(0.040) | 0.00045(0.000040) | -0.51 ns | 0.75 ns | 0.018 ns | - |
| South | 76 | 6 | 11 | 0.82(0.023) | 0.00046(0.000040) | -3.23 ns | 0.37 ns | 0.24*** | - |
| Total | 155 | 6 | 12 | 0.81(0.021) | 0.00049(0.000030) | - | 0.73 ns | 0.12** | ns |
| *L. longiclavatum* | | | | | | | | | |
| North | 122 | 1 | 2 | 0.41(0.036) | 0.00015(0.000010) | 5.73 ns | 1.30 ns | -[a] | - |
| South | 47 | 4 | 6 | 0.57(0.068) | 0.00026(0.000320) | 0.72 ns | -0.38 ns | 0.13 ns | - |
| Total | 169 | 4 | 6 | 0.52(0.022) | 0.00021(0.000020) | - | -0.33 ns | 0.057 ns | ns |

n, number of strains; S, segregating sites; h, number of haplotypes; Hd, haplotype diversity; SD, standard deviation; Pi, nucleotide diversity; Fs, Fu's Fs; D, Tajima's D; $I_A$, Index of association; $\Phi_w$, pairwise homoplasy index.
* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns: not significant.
[a]Lack of genetic variability prevents calculation

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Table 3: Analysis of molecular variance for northern and southern populations of MPB associated fungi.

| | d.f. | SSD | Var. | % Var. | $\Phi_{ST}$ |
|---|---|---|---|---|---|
| *O. montium* | | | | | |
| Among N, S pops. | 1 | 14.092 | 0.16*** | 4.47 | 0.045*** |
| Within N-S pops. | 137 | 462.43 | 3.38 | 95.53 | |
| *G. clavigera* | | | | | |
| Among N, S pops. | 1 | 5.058 | 0.055*** | 6.52 | 0.065*** |
| Within N-S pops. | 152 | 120.76 | 0.79 | 93.48 | |
| *L. longiclavatum* | | | | | |
| Among N, S pops. | 1 | 13.46 | 0.19*** | 21.67 | 0.22*** |
| Within N-S pops. | 166 | 114.70 | 0.69 | 78.33 | |

***$P$<0.0001

d.f., degrees of freedom; SSD, sum of squares deviation; Var., components of variance; % Var., percent of variance.

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**Table 4:** Congruence among pairwise $\Phi_{ST}$ values calculated from concatenated MLST sequence data of three fungal species and geographic distance among landscapes. Tests were calculated using CADM in the ape package implemented in the R framework.

| | | | | |
|---|---|---|---|---|
| **Global congruence** | $H_0$: matrices are incongruent | | | |
| Kendall's *W*: | 0.4612 | | | |
| Friedman's $\chi^2$: | 119.8787*** | | | |
| | | | | |
| ***A posteriori* pairwise congruence** | $H_0$: matrix is incongruent with remaining matrices | | | |
| | $H_1$: matrix is congruent with at least one other matrix | | | |
| | *P*-value | adjusted *P*-value[a] | | |
| *G. clavigera* | * | * | | |
| *L. longiclavatum* | ** | * | | |
| *O. montium* | ** | * | | |
| Geographic distance | ** | ** | | |
| | | | | |
| **One-tailed Mantel tests** | $H_0$: $r = 0$; $H_1$: $r > 0$ | | | |
| | *G. clavigera* | *L. longiclavatum* | *O. montium* | Geographic distance |
| *G. clavigera* | 1.0000 | 0.4208** | 0.1901ns | 0.1496ns |
| *L. longiclavatum* | | 1.0000 | 0.1469ns | 0.3661* |
| *O. montium* | | | 1.0000 | 0.4151** |
| Geographic distance | | | | 1.0000 |

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns: not significant

[a]Holm adjustment for multiple tests

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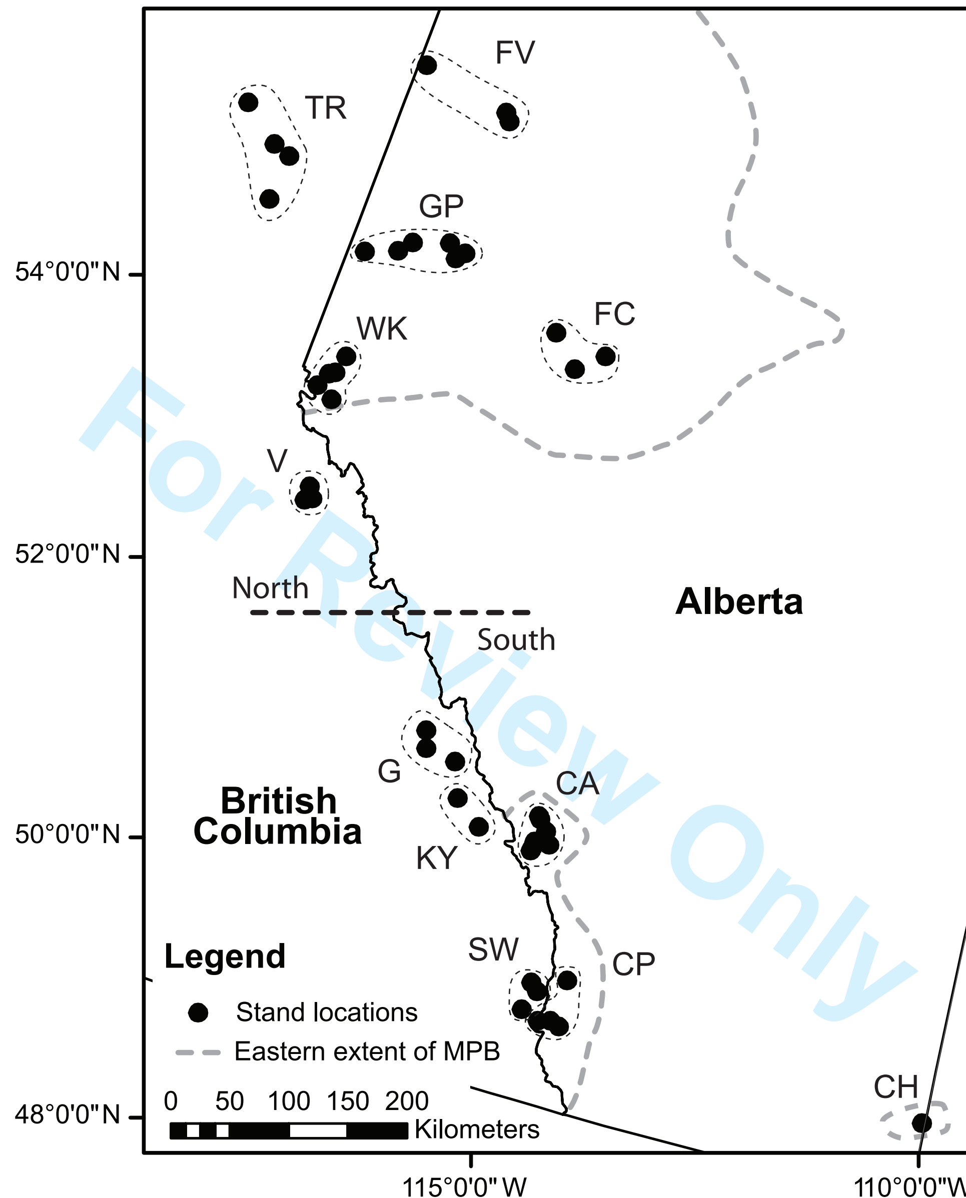


**Figure 1.** Location of collection sites for MPB fungal symbionts and the eastern boundary of MPB outbreak in Alberta. Sites grouped by landscapes. Northern landscapes: FC, Fox Creek; FV, Fairview; GP, Grande Prairie; TR, Tumbler Ridge; V, Valemont; WK, Willmore-Kakwa. Southern landscapes: CH, Cypress Hills; CA, Canmore; CP, Crowsnest Pass; G, Golden; KY, Kootenay-Yoho; SW, Sparwood. MPB outbreak boundary based on 2009 aerial survey data from Alberta Sustainable Resource Development (www.mpb.alberta.ca/Files/MPB-AerialOverview-2009.pdf, accessed 06-05-10).

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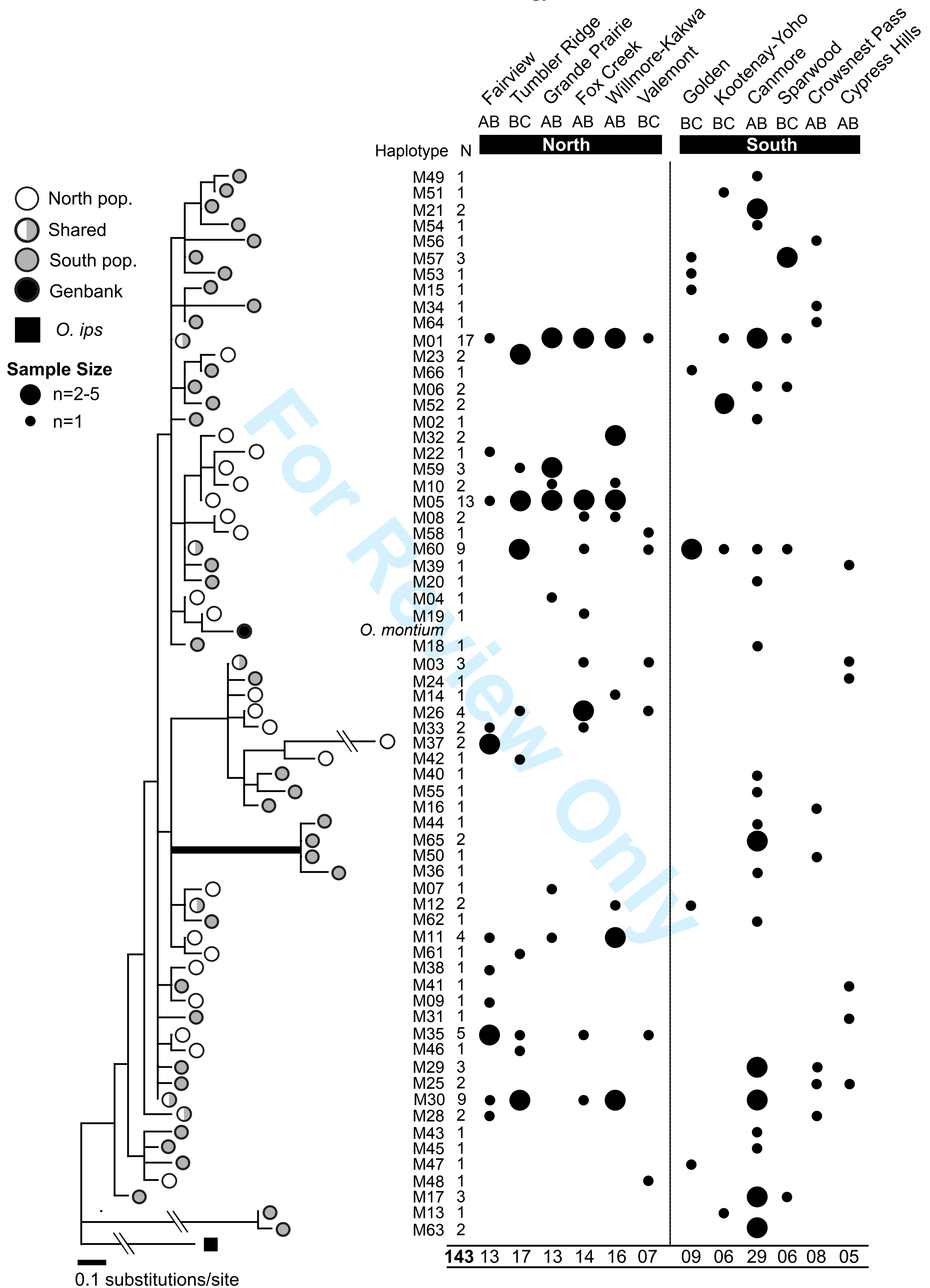


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**Figure 2**. Maximum likelihood phylogram for the *O. montium* concatenated multilocus data set of four independent loci (actin, EF1a, Btub, ITS2). Thickened branches indicate clade support greater than 80%. For each haplotype, population assignment (north, south, or shared) and landscape of origin is shown.

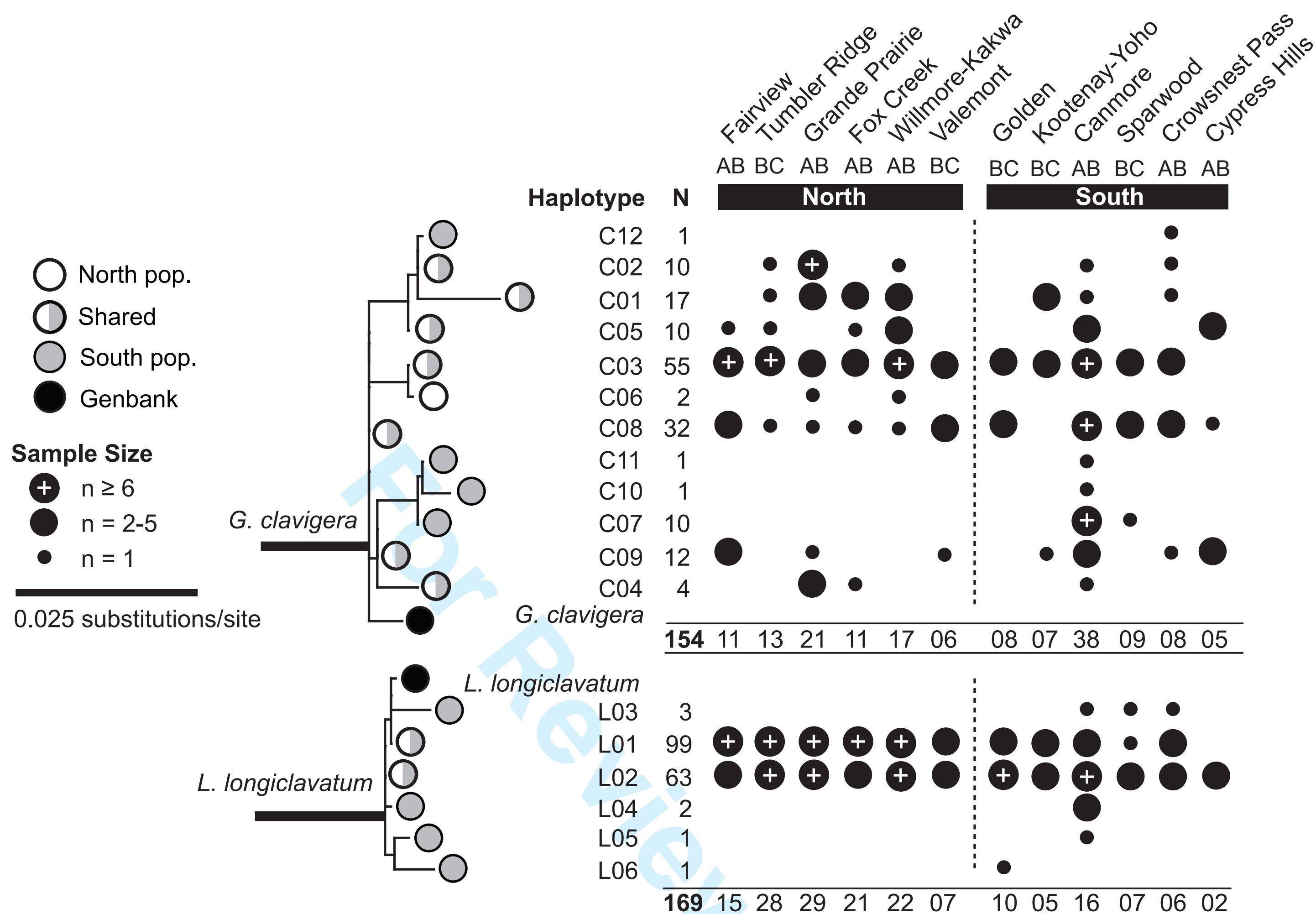


**Figure 3**. Maximum likelihood phylograms for the *G. clavigera* and *L. longiclavatum* concatenated multilocus data sets of five independent loci (actin, EF1a, Btub, UFM, ITS2). Thickened branches indicate clade support greater than 80%. For each haplotype, population assignment (north, south, or shared) and landscape of origin is shown. Phylograms were adapted from Roe *et al.* 2010.

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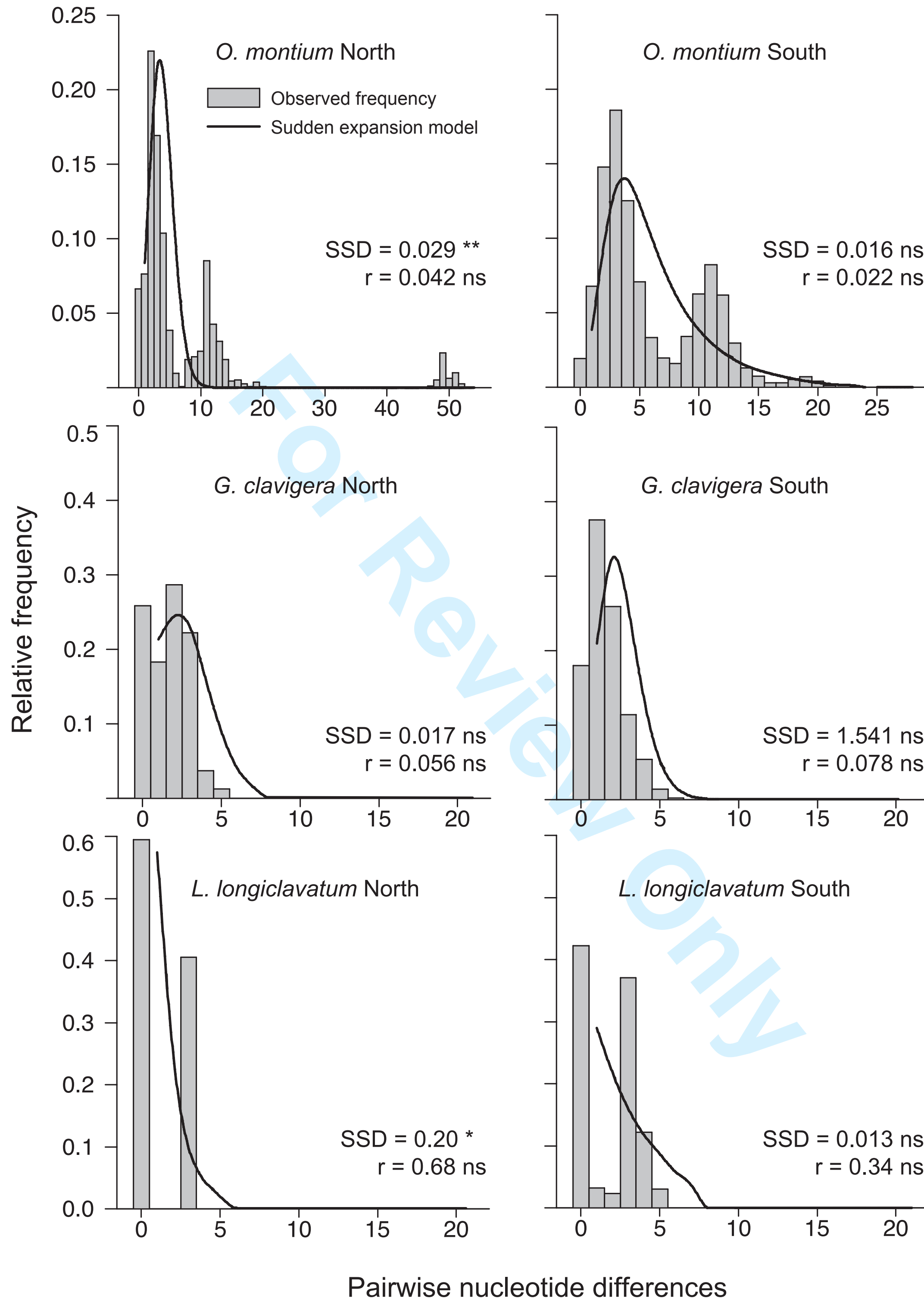


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Figure 4. Mismatch distributions for nothern and southern populations of three mountain pine beetle fungal symbionts. Bars indicate the observed mismatch difference between haplotypes and the lines represent the expected distribution under a sudden population expansion model. Deviations from the expected distribution are assessed with sums of squares deviation (SSD) and Harpendings raggedness index (r) (Harpending 1994). Measures of significance as in Table 2.

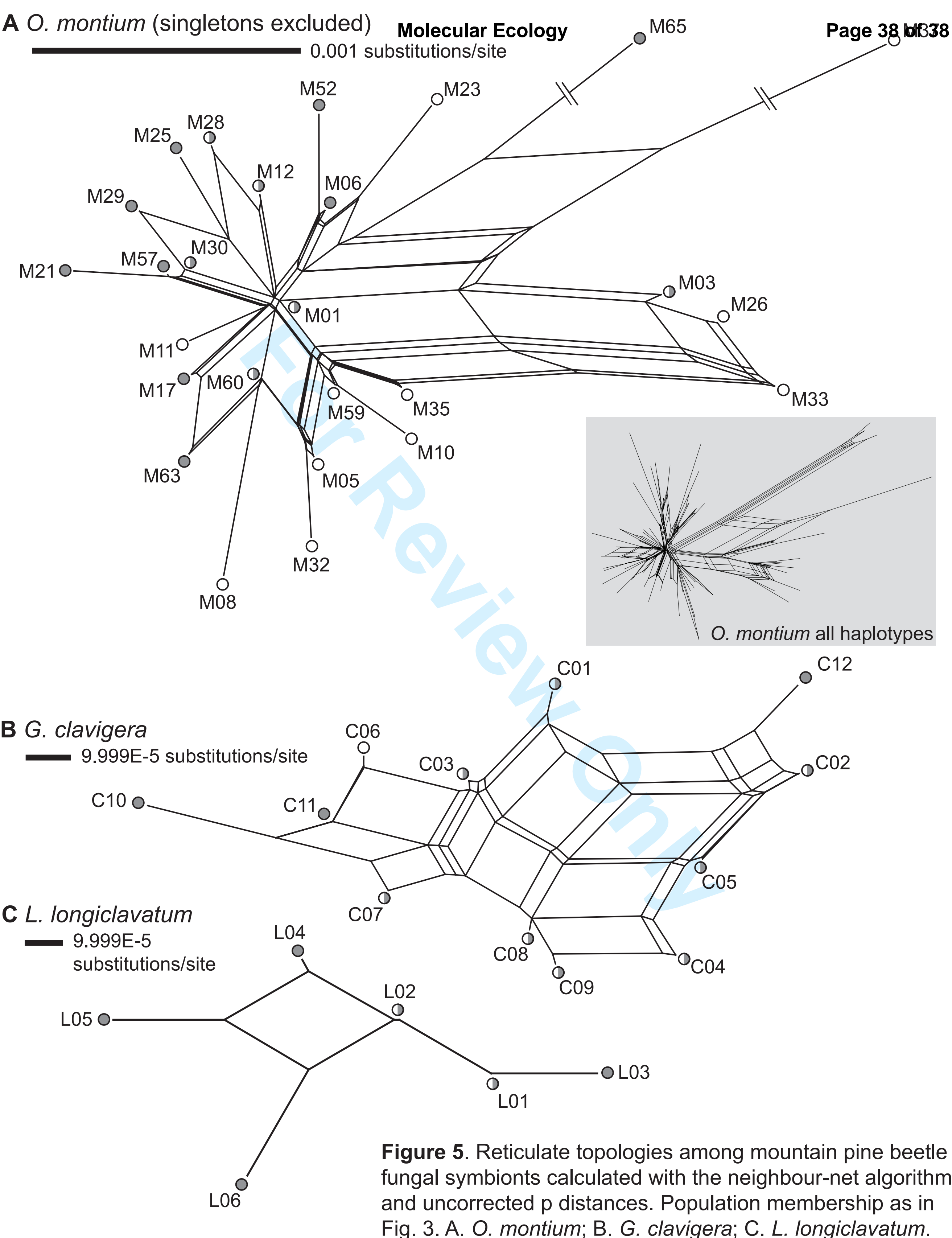


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**Figure 5**. Reticulate topologies among mountain pine beetle fungal symbionts calculated with the neighbour-net algorithm and uncorrected p distances. Population membership as in Fig. 3. A. *O. montium*; B. *G. clavigera*; C. *L. longiclavatum*.