Missing checkerboards? An absence of competitive signal in *Alnus*-associated ectomycorrhizal fungal communities

A number of recent studies suggest that interspecific competition plays a key role in determining the structure of ectomycorrhizal (ECM) fungal communities. Despite this growing consensus, there has been limited study of ECM fungal community dynamics in abiotically stressful environments, which are often dominated by positive rather than antagonistic interactions. In this study, we examined the ECM fungal communities associated with the host genus *Alnus*, which live in soils high in both nitrate and acidity. The nature of ECM fungal species interactions (i.e. antagonistic, neutral, or positive) was assessed using taxon co-occurrence and DNA sequence abundance correlational analyses. ECM fungal communities were sampled from root tips or mesh in-growth bags in three monodominant *A. rubra* plots at a site in Oregon, USA and identified using Illumina-based amplification of the ITS1 gene region. We found a total of 175 ECM fungal taxa; 16 of which were closely related to known *Alnus*-associated ECM fungi. Contrary to previous studies of ECM fungal communities, taxon co-occurrence analyses on both the total and *Alnus*-associated ECM datasets indicated that the ECM fungal communities in this system were not structured by interspecific competition. Instead, the co-occurrence patterns were consistent with either random assembly or significant positive interactions. Pair-wise correlational analyses were also more consistent with neutral or positive interactions. Taken together, our results suggest that interspecific competition does not appear to determine the structure of all ECM fungal communities and that abiotic conditions may be important in determining the specific type of interaction occurring among ECM fungi.

1   Missing checkerboards? An absence of competitive signal in *Alnus*-associated ectomycorrhizal

2   fungal communities

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7   Corresponding Author: Peter Kennedy, 1445 Gortner Ave., St. Paul, MN, USA. Email:

8   kennedyp@umn.edu, Phone: 612-624-8519, Fax: 612-625-1738.**Introduction**

9   A common way to assess the role of interspecific competition or facilitation in

10   determining community structure is experimental manipulation involving the removal of

11   neighboring individuals. This approach has been widely used in ecological studies examining

12   biotic determinants of plant and animal communities (Connell 1983; Schoener 1983), but

13   carrying out similar manipulations in field-based studies of soil microbial communities is less

14   feasible due to the inability to selectively manipulate species-level neighborhood composition. A

15   widely proposed alternative is to look at species distribution patterns, with Diamond's (1975)

16   study of bird distributions in the New Guinea archipelago being one of most well-recognized

17   examples. In that study, the presence of certain bird species on a given island was associated with

18   the absence of other species (and vice versa on other islands), resulting in a series of 'forbidden

19   species combinations' or 'checkerboard distributions', which were posited to be the result of

20   competitive exclusion (Diamond 1975). This technique provided an important step forward in

21   assessing the role of species interactions in field-based studies at the community level, but it has

22   been frequently noted that analyses of species co-occurrence patterns need to include

23   comparisons with patterns generated from communities assembled randomly to maximize

24   inference (Connor & Simberloff 1979; Gotelli & Graves 1996).

25   Since the 1970s, species co-occurrence analyses have been used to assess the possibility

26   of species interactions in a wide range of organisms, including both macro- and microorganisms

27   (Gotelli & McCabe 2002; Horner-Devine et al., 2006). Plant-associated fungal communities,

28   which have diverse ecological roles in ecosystems (Smith & Read 2008; Rodriguez et al., 2009),

29   have shown a full range of co-occurrence patterns, including those consistent with both positive

30   and antagonistic interactions (Koide et al., 2005; Pan & May 2009; Gorzelak et al., 2012;

31   Ovaskainen et al., 2010; Pickles et al., 2012; Toju et al., 2013). For ectomycorrhizal (ECM)

32 fungi, the dominant microbial eukaryotes in many temperate and some tropical forest soils (Smith

33 and Read 2008), these analyses have consistently found evidence of less species co-occurrence

34 than expected by chance (Koide et al., 2005; Pickles et al., 2010; Pickles et al., 2012). This

35 suggests that competitive interactions may play a significant role in structuring the communities

36 of this fungal guild (Kennedy 2010). The initial studies of species co-occurrence patterns in ECM

37 fungal communities looked only in forests dominated by conifer hosts, but a recent study in

38 *Fagus sylvatica* forests in Europe also found evidence of significantly lower than expected co-

39 occurrence patterns (Wubet et al., 2012). This latter result indicates that the predominance of

40 antagonistic interactions in determining ECM fungal community structure may be a common,

41 host-lineage independent phenomenon. Importantly, however, other ecological and evolutionary

42 factors aside from species interactions can also be responsible for non-random species co-

43 occurrence patterns (Gotelli & McCabe 2002; Ovaskainen *et al.* 2010), so caution must be

44 applied in inferring underlying mechanisms.

45   In this study, we focused on assessing the community co-occurrence distributions of ECM

46 fungi associated with the host genus *Alnus*. Unlike other ECM host genera with large

47 geographical distributions, the ECM fungal communities associated with *Alnus* trees have been

48 consistently found to be both species poor and highly host specific (Tedersoo et al., 2009;

49 Kennedy & Hill 2010; Kennedy et al., 2011; Bogar & Kennedy 2013; Põlme et al., 2013; Roy et

50 al., 2013). The mechanisms driving this atypical structure have long been thought to be related to

51 the co-presence of nitrogen-fixing *Frankia* bacteria, which can have strong biotic and abiotic

52 effects on *Alnus*-associated ECM fungal communities (Walker et al., 2014). In particular, the high

53 rates of nitrification present in *Alnus* forest soils (due to the high inputs and decomposition of

54 nitrogen-rich leaf litter) results in significantly higher nitrate and acidity levels than those present

55 in most other ECM-dominated forest soils (Daniere et al. 1986, Miller et al., 1992; Martin et al.,

56 2003; Walker et al., 2014). Elevated levels of both of these abiotic factors have been shown to

57   inhibit the growth of many ECM fungi (Hung and Trappe 1983; Lilleskov et al., 2002) and, using

58   an experimental pure culture approach, Huggins et al., (in press) recently demonstrated that

59   *Alnus*-associated ECM fungi have a greater ability to tolerate high nitrate and acidity conditions

60   compared to non-*Alnus*-associated ECM fungi.

61   Given the ability of *Alnus*-associated ECM fungi to grow in conditions that are generally

62   considered abiotically stressful, we hypothesized that ECM fungal species co-occurrence patterns

63   in *Alnus* forests may differ from those present in forests dominated by other ECM hosts.

64   Specifically, we speculated that competitive interactions would be less prevalent in this study

65   system, based on the fact that many studies of vascular plants have shown that the nature of

66   species interactions often changes from antagonistic to positive with increasing levels of abiotic

67   stress (Bertness & Callaway 1994, Gómez-Aparicio et al., 2004, but see Michalet et al., 2006). To

68   examine this hypothesis, we examined the co-occurrence patterns of the ECM fungal

69   communities present in three mono-dominant plots of *Alnus rubra* in the western United States.

70   ECM fungal communities were sampled on root tips and in soil. For the latter, we used sand-

71   filled mesh in-growth bags, which allow for efficient, well-replicated community sampling of

72   fungal hyphae growing in soil (Wallander et al., 2001, Branco et al., 2013). To identify the ECM

73   fungi present in the study, we used high throughput Illumina sequencing, which has been

74   increasingly used to profile ECM fungal community composition (McGuire et al., 2013, Smith &

75   Peay 2014).

76   **Materials & Methods**

77   *Study Location*

78   The study site was located on the eastern side of the Coast Range mountains in

79   northwestern Oregon, U.S.A. (latitude: N 45.820 W 123.05376, elevation: 462 m). Temperatures

80   at the site are moderate (mean annual temperature = 8.7°C, min = -1.2°C, max = 23.8°C), with

81   significant precipitation between October and May followed by drier summer months (total =

82   1742 mm). The specific study location is part of a long-term research project examining the

83   effects of different forest management practices on *A. rubra* growth (see the Hardwood

84   Silvicultural Cooperative (HSC) website for details, http://www.cof.orst.edu/coops/hsc). The

85   HSC site used, Scappoose (HSC 3209), was established in 1995. Prior to the implementation of

86   the HSC work, the site was a second-growth coniferous forest, which was clear-cut and replanted

87   with a series of monodominant *A. rubra* plots. *A. rubra* seedlings were planted from nursery

88   stock (Brooks Tree Farm, Brooks, OR) during the beginning of their second year of growth.

89   Seedling ECM status at the time of planting was not assessed (*Frankia* nodules were noted to be

90   absent), but nursery fumigation practices indicate colonization was unlikely (Brooks Nursery,

91   pers. com.).

92   Our experiment was conducted in three 1600 m^2 plots at HSC 3209. The plots, which

93   were located approximately 100 m apart, differed in initial *A. rubra* stem density (Plot 2 = 628,

94   Plot 4 = 1557, and Plot 8 = 3559 stems/ha), but had no other forest management practices

95   applied.   Despite the differences in stem density, *A. rubra* fine root density did not differ

96   significantly among the three plots (Fig. S1). The understories in all three plots were colonized by

97   arbsucular mycorrhizal plants (dominated by *Mahonia nervosa* and *Claytonia perfoliata*), with no

98   other ECM hosts besides *A. rubra* present. Soils were classified as well-drained Tolamy loams

99   (USDA Soil Survey, Columbia County, OR). Within each plot, we located a 9 x 9 m subplot and

100   overlaid a 100 point grid, with each point being separated by 1 m. We chose this subplot size to

101   avoid any dead stems in the canopy immediately above the sampling area, while at the same time

102   maximizing the number of samples taken per subplot. At each point in Plot 4, which was sampled

103   for ECM root tips, a 5 cm diameter x 10 cm deep soil core was taken on May 31, 2013. In Plots

104   2 and 8, which were sampled for ECM communities present in soil, a 5 x 5 cm mesh bag was

105   buried at each point 5 cm below the soil surface. The bags were made of anti-static polyester

106   fabric with 300 micron diameter pores. This pore size allowed fungal hyphae to grow into the

107   bags, but prevented penetration of plant roots. We filled the bags with twice autoclaved #3 grade

108   Monterey aquarium sand (Cemex, Marina, CA, USA). Aluminum tags on fluorescent string were

109   added to facilitate bag recovery. The mesh bags at Plot 2 were buried on February 1, 2013 and

110   February 22 at Plot 8. They were left undisturbed in the soil until May 31, when all were

111   harvested. After removal from the soil, we placed the mesh bags into individual plastic bags and

112   then onto ice for transport back to the laboratory. Soil cores and bags were stored at 4°C for <96

113   hours before further processing.

114   *Molecular Analyses*

115   We processed the root tip samples by gently washing all roots away from the soil and

116   removing all ECM colonized root tips from each core under a 10X dissecting scope (~10-50 root

117   tips/core). All roots from each core were extracted using individual MoBio PowerSoil kits

118   (Hercules, CA, USA), following manufacturer's instructions for maximum DNA yields. For the

119   mesh bags, we followed the protocol outlined in Branco et al., (2013), which provided a cheaper

120   and quicker protocol compared to direct DNA extraction from the sand within the mesh bags.

121   Briefly, each bag (including a negative control that was taken to the field, but not buried) was

122   emptied into a sterile 50 ml centrifuge tube. We added 10 ml of sterile deionized water and

123   vortexed each tube for two minutes, followed by a five minute settling period (hyphae have been

124   previously observed to float to the water surface). We then transferred the top two ml top of

125   water to a new 2 ml centrifuge tube and contents were pelleted via centrifugation. On the same

126   day, we extracted total genomic DNA form the pellets using the Sigma REDExtract-N-Amp kit

127   (Sigma-Aldrich, St, Louis, MO, USA) following manufacturer's instructions. Root tips and

128   extracts were stored for one week at -20°C prior to PCR amplification.

129   For the root tip samples, we combined equal quantity aliquots from all 97 DNA

130   extractions (three cores contained no roots) into a single template for PCR. In contrast, for each

131   mesh bag sample as well as extraction controls, we conducted individual PCR reactions. We

132   processed these two types of samples differently because we were primarily interested in the

133   spatial co-occurrence patterns in the soil ECM fungal communities and therefore only used the

134   root tip samples to create a local sequence reference set of known *Alnus*-associated ECM taxa

135   against which the mesh bag data could be compared. For all PCR reactions, we used the barcoded

136   ITS1F and ITS2 primer set of Smith & Peay (2014), with each sample run in triplicate and pooled

137   to minimize heterogeneity. Successful PCR products were determined by gel electrophoresis and

138   magnetically cleaned using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA)

139   according to manufacturer's instructions. Final product concentrations were quantified using a

140   Qubit dsDNA HS Fluorometer (Life Technologies, Carlsbad, CA, USA). Root tip and bag

141   samples were run at different sequencing facilities under the same general conditions. For the

142   root tips, the single PCR product was run at the University of Minnesota Genomics Center using

143   250 bp paired-end sequencing on the MiSeq Illumina platform. For the bags, we pooled the 192

144   successfully amplified bag samples at equimolar concentration and ran them on the same

145   platform at the Stanford Functional Genomics Facility using 250 bp paired-end sequencing on the

146   MiSeq Illumina platform. A spike of 20% and 30% PhiX was added to the runs to achieve

147   sufficient sample heterogeneity, respectively. Raw sequence data and associated metadata from

148   both the root tip and bag samples were deposited at MG-RAST (http://metagenomics.anl.gov/)

149   under project #1080.

150   *Bioinformatic Analyses*

151   We used the software packages QIIME (Caporaso et al., 2010) and MOTHUR (Schloss et

152   al., 2009) to process the sample sequences. Raw sequences were demultiplexed, quality filtered

153   using Phred = 20, trimmed to 178 base pairs, and ends were paired, followed by filtering out of

154   sequences that had any ambiguous bases or a homopolymer run of 9 bp. Following the guidelines

155   discussed in Nguyen et al., (in press), we employed a multi-step operational taxonomic unit

156   (OTU) picking strategy by first clustering with reference USEARCH (including de novo chimera

157   checking) at 97% sequence similarity, followed by UCLUST at 97% sequence similarity. We

158   used a 97% similarity threshold because it the most commonly employed in community-level

159   ECM fungal studies, although some lineages, including *Alnicola*, may have greater sequence

160   similarity among species (Tedersoo et al. 2009, Rochet et al. 2011). To assess the validity of the

161   97% threshold for sequences based on only ITS1 versus the full ITS region (i.e. ITS1, 5.8S, and

162   ITS2), we examined seven known *Alnus*-associated *Tomentella* taxa (i.e. those present in

163   Kennedy et al., 2011) and found that that threshold resulted in the same number of OTUs in both

164   cases (data not shown). The UNITE database (Kõljalg et al., 2013) was used in both chimera

165   checking and OTU clustering, with singleton OTUs discarded to minimize the effects of

166   artifactual sequences (Tedersoo et al. 2010b). We assigned taxonomic data to each OTU with

167   NCBI BLAST+ v2.2.29 (Altschul et al., 1990), using a custom fungal ITS database containing

168   the curated UNITE SH database (v6) (http://unite.ut.ee/repository.php, Kõljalg et al., 2013) and

169   more than 600 vouchered fungal specimens, including 46 representative sequences from *Alnus*

170   forests at other HSC locations in Oregon (Kennedy & Hill 2010) and Mexico (Kennedy et al.

171   2011). Since sequences that had low subject length:query length matches were typically non-

172   fungal, we further filtered out sequences with matches ≤ 90% to BLAST (i.e. at least 90% of the

173   bases in the input sequence matches to another sequence in the database at some identity level).

174   Using the remaining sequence dataset, we rarefied all samples to 12946 sequences, which

175   was the lowest number of sequences obtained across the 192 samples. Since there has recently

176   been a question raised about the validity of rarefaction in next generation sequencing analyses

177   (McMurdie and Holmes 2014), we also analyzed the data without rarefaction. We obtained very

178 similar results (Table S1), so present the data based on rarefied samples only. ECM OTUs within

179 each sample were parsed out using a python script that searches for genera names from a list of

180 189 known ECM genera and their synonyms (Branco et al., 2013, appended from Tedersoo et al.,

181 2010a). While this script provides a strong general filter for sorting the data by fungal lifestyle,

182 some taxa belonging to clades that are polyphyletic for the ECM habit (e.g. *Lyophyllum*,

183 *Sebacinales*) as well as taxa with low matches to Genbank (e.g. Uncultured Fungus) can be of

184 questionable trophic status. For each of these groups, we carefully checked both the sequence

185 matches and placement of our OTUs within phylogenetic trees of the clades to determine whether

186 these taxa were properly classified at ECM. The resulting sample x OTU matrix contained 190

187 ECM taxa represented by at least one sequence per sample (min = 1, median = 34, mean = 1334,

188 max = 209,187). We found that 15 of the 190 OTUs present were highly similar (>97% similar)

189 to ECM fungi present in the dipterocarp rainforests of Malaysia, which were concurrently being

190 studied in the Peay lab using the same next-generation sequencing approach (Fig. 1). Because

191 these OTUs represented accidental contamination probably during library construction, they were

192 eliminated from the final analyses. Although an additional 80 OTUs had greater than >97%

193 similarity to taxa found in the Borneo study, because their closest BLAST match was not from

194 Borneo, we conservatively considered these taxa as having cosmopolitan distributions and

195 included them in the final analyses. The final OTU × sample matrix, including taxonomic

196 matches and representative of sequences for each OTU, can be found in Table S2.

197 *Statistical Analyses*

198   Taxon co-occurrence patterns of the ECM fungal communities present in bag samples

199 were assessed using the program EcoSim (Gotelli & Entsminger 2009), with presence-absence

200 matrices for Plots 2 and 8 being analyzed separately. (The root data from Plot 4 could not be

201 analyzed for sample-level co-occurrence due to the pooled sequencing approach for those

202   samples). We utilized the C-score algorithm (Stone & Roberts 1990), which compares the

203   number of checkerboard units (i.e. 1,0 x 0,1) between all pairs of species in the observed matrix

204   ($C_{observed}$) to that based in random permutations of the same matrix ($C_{expected}$, i.e. the null models).

205   Since randomized permutations of a matrix can be achieved in multiple ways (see Gotelli &

206   Entsminger 2009 for details), we analyzed our datasets using both the 'fixed-fixed' and 'fixed-

207   equiprobable' options (which are recommended by the program guide and used in the previous

208   ECM fungal co-occurrence analyses). In both options, the row (i.e. taxon) totals were fixed, so

209   that the total abundances of each taxon in the observed and null matrices were identical. In the

210   'fixed-equiprobable' option, however, the column (i.e. sample) totals in the null matrices were no

211   longer equivalent to those in the observed matrix. Instead, all samples in the null matrices had an

212   equal probability of being colonized by any of the taxa in the observed matrix, which effectively

213   eliminates differences in taxon richness among samples.

214   Of the ECM taxa present in the final root tip and bag datasets, over 90% (167/183)

215   belonged to species never previously encountered with *Alnus* (Table S2, *Alnus*Match = No).

216   Unlike other ECM host systems with large geographic ranges, the ECM fungal community

217   associated with *Alnus* hosts is remarkably well characterized at local (Tedersoo et al., 2009,

218   Kennedy et al., 2010, Walker et al., 2014), regional (Kennedy et al., 2011, Roy et al., 2013, and

219   global scales (Polme et al., 2013). As such, it is highly likely the majority of the novel OTUs

220   encountered were not part of the active ECM community in our plots, but rather present simply

221   either as spores or additional lab contaminants. To account for this issue, we divided our

222   checkerboard analyses into five different input matrices for the bag dataset (Plots 2 and 8). The

223   first matrix included all 183 ECM fungal taxa (referred to as "All"). The second matrix included

224   the 16 taxa that had >97% similarity matches to ECM samples from *Alnus* forests (referred to as

225   *Alnus*). The third matrix included only the 8 taxa that were encountered on ECM root tips in Plot

226   4 (referred to as *Alnus*RootOnly). To assess the robustness of the results generated using the

227   larger *Alnus* matrix, the fourth matrix excluded the three most frequent and abundant species

228   (*Tomentella*3, *Alnicola*1, *Tomentella*2) (referred to as *Alnus*MinusTop3). Finally, the fifth matrix

229   included just the 10 taxa in the genus *Tomentella* (from the larger *Alnus* matrix) to look for

230   evidence of species interactions among this subset of closely related taxa (referred to as

231   *AlnusTomentella*Only). For all of the aforementioned C-score analyses, taxa present in less than 5

232   bag samples were removed, as low frequency taxa are generally considered non-informative

233   (Koide et al., 2005). The observed input matrices were compared to 5000 null matrices.

234   Significant differences between the observed matrix C-score and that of the null matrices were

235   determined along with standardized effect sizes (SES). Observed C-scores significantly higher

236   than those generated from the null matrices are consistent with a community being structured by

237   competitive interactions, whereas $C_{observed}$ significantly lower than the $C_{expected}$ is consistent with

238   positive interactions.

239   To further assess the degree of association between known *Alnus* ECM fungal taxa, we

240   also used an abundance-based approach (as opposed to the co-occurrence analyses, which are

241   based on binary presence/absence data). Specifically, we calculated the pair-wise Spearman rank

242   correlation coefficients among all pairs of the 16 *Alnus*-associated taxa using the *cor* function in

243   R (R Development Core Team 2013). Coefficients >0.30 were tested for significance with the

244   *cor.test* function. To account for multiple tests (n=13), we used a Bonferroni-corrected *P* value of

245   0.003. With the same data set, we also tested for the presence of spatial autocorrelation using the

246   *mgram* function in the ECODIST package in R. We first converted the sequence abundance

247   datasets in both Plots 2 and 8 into dissimilarity matrices using the Bray-Curtis Index and then

248   compared those to a Euclidean distance matrix of sampling points for each plot. For the Mantel

249   correlogram tests, we used the *n.class=0* option, which uses Sturge's equation to determine the

250   appropriate number of distance classes.

251   **Results**

252   We found 183 total ECM fungal taxa in the study (Table S2); 16 of which matched closely

253   to known *Alnus*-associated ECM fungi. In the mesh bags, *Alnus*-associated ECM fungal taxa

254   represented six of the ten most abundant OTUs present, including the dominant ECM fungal

255   taxon, *Tomentella*3, which was present in all the bag samples in both plots and had sequence

256   abundances nearly ten-fold higher than any other taxon (Fig. 2a,b). Two other *Alnus*-associated

257   taxa, *Alnicola*1 and *Tomentella*2, were also present in all samples, whereas the remaining *Alnus*-

258   associated ECM fungal taxa had frequencies varying from 2-96% (Plot 2 mean = 25%, Plot 8

259   mean = 31%) and lower sequence abundances. Eight of the 16 *Alnus*-associated ECM fungal taxa

260   were present on both roots and in the bags, with abundances that were very similar (Fig. 1a). Of

261   the eight ECM fungal taxa found on root tips, all were previously encountered on *A. rubra* root

262   tips at other sites in Oregon, while the eight taxa found exclusively in bags had not been

263   previously documented (Kennedy & Hill 2010).

264   ECM fungal taxon co-occurrence patterns were largely consistent between plots, but

265   different between null models. Of the ten tests (i.e. 5 matrix types x 2 plots) using the 'fixed-

266   fixed' permutation option, nine indicated that the observed ECM fungal community did not differ

267   significantly from random assembly (Table 1). In one case, Plot 2 All, the observed ECM fungal

268   community had significantly more co-occurrence than expected by chance. In contrast, in the ten

269   tests using the 'fixed-equiprobable' permutation option, three indicated that the observed ECM

270   fungal community did not differ significantly from random assembly, while seven found that the

271   observed ECM fungal community had significantly more co-occurrence than expected by chance.

272   Results remained the same for *Alnus* ECM fungal communities whether the top three taxa were

273   removed or not. The *Alnus* and *Alnus*RootOnly analyses did differ under the 'fixed-equiprobable'

274   option, with the former showing greater than expected co-occurrence and the latter having a

275   pattern no different than one based on random assembly. Additionally, in the

276   *AlnusTomentella*Only analysis, the ECM fungal community showed greater than expected co-

277   occurrence in Plot 2 but not in Plot 8. In all of these cases, significant antagonistic patterns were

278   not observed.

279   Spearman rank analyses revealed that pair-wise sequence abundances of some of the 16

280   *Alnus* ECM fungal taxa were significantly positively correlated (Table 2). The specific significant

281   combinations varied between plots, with only taxon pair (*Alnicola*1 & *Tomentella*9) showing

282   significant positive correlations in both plots. Although a number of pair-wise correlations had

283   negative values (suggesting negative rather than positive interactions), none of them were

284   significant, even when considered at a *P* value of 0.05. In addition, the Mantel correlogram

285   analyses found no clear evidence of spatial autocorrelation in the *Alnus*-associated ECM fungal

286   communities. In Plot 2, there was no significant autocorrelation at any distance, while in Plot 8

287   there was a single significant positive correlation between samples located 1-2 m apart (Fig. S2,

288   S3).

289   **Discussion**

290   We found that the ECM fungal communities in *A. rubra* forests displayed a different

291   pattern of taxon co-occurrence compared to those seen for other ECM fungi. Unlike the

292   consistent previous findings of less co-occurrence among species than expected by chance (Koide

293   et al., 2005; Pickles et al., 2012; Wadet et al., 2012), we observed no evidence of spatial patterns

294   consistent with interspecific competition in *Alnus*-associated ECM fungal communities. In

295   contrast, we consistently found co-occurrence patterns that were either no different from random

296   assembly or consistent with positive interactions. Although we did not measure soil nitrate and

297   acidity conditions in this study (see Martin et al., (2003) and Walker et al., (2014) for values from

298   comparable age *A. rubra* forests at other sites in Oregon), *Alnus* soils are consistently

299   characterized by abiotic conditions are generally considered stressful to ECM fungi. The results

300   we obtained are thus consistent with the 'stress gradient hypothesis', which posits that species

301   interactions shift from negative to positive as environmental conditions become harsher (Bertness

302   & Callaway 1994). Although we emphasize that the patterns we found in this study are based

303   solely on correlative inference, there is some experimental evidence that may support the stress

304   gradient hypothesis for ECM fungal community dynamics. Koide et al., (2005) found a shift from

305   significant negative co-occurrence patterns in their control plots to non-significant co-occurrence

306   patterns in plots where either tannins or nitrogen were added experimentally. While they did not

307   explicitly analyze these manipulations in terms of stress, both increased tannin and nitrogen

308   levels have been shown to inhibit the growth of multiple ECM taxa (Koide et al., 1998; Cox et

309   al., 2010). The direction of the response in the Koide et al., (2005) study is consistent with greater

310   abiotic stress resulting in a decrease in antagonistic ECM fungal interactions. At the same time, it

311   is plausible that resource limitation was eliminated with the addition of nitrogen, which could

312   have allowed for greater spatial co-existence among ECM fungi. Since the *Alnus* system has

313   naturally higher nitrogen availability than most ECM forests due to the co-presence of nitrogen-

314   fixing *Frankia* bacteria, it is also possible that greater resource abundance could drive the co-

315   occurrence patterns we observed. Given the fact that the pattern could be explained by either

316   increasing stress or resource availability, additional experimental tests are needed to distinguish

317   among these explanations. One promising approach would be to examine the taxon co-occurrence

318   patterns in younger and older *Alnus* forests, since soil nitrate and acidity concentrations increase

319   in these forests over time (Daniere et al. 1986, Martin et al., 2003). If the stress gradient

320   hypothesis were the most plausible explanation, then we would expect to see competitive and

321   facilitative interactions to be dominant, respectively.

322   The presence of co-occurrence patterns consistent with significant negative species

323   interactions was also missing in our analysis of more closely related ECM fungal taxa. For the ten

324   *Alnus*-associated members of the genus *Tomentella*, co-occurrence patterns either did not differ

325   significantly from random assembly or reflected an effect of positive interactions. Like the larger

326   community analyses, this result also differs from previous experimental studies, where strong

327   antagonistic interactions among closely related ECM fungal taxa have been observed (Kennedy

328   2010). In a similarly designed study that also assessed ECM fungi with taxon co-occurrence

329   analyses, Pickles et al., (2012) found patterns consistent with strong interspecific competition

330   among a suite of *Cortinarius* species in a Scottish *Pinus sylvestris* forest. Although it has long

331   been assumed that competition may be stronger in more closely related species due to greater

332   overlap in resource utilization, a meta-analysis by Cahill et al., (2008) found little consistent

333   evidence to support this supposition. Mayfield & Levine (2010) further questioned the validity of

334   phylogenetic relatedness as a good proxy for competitive strength by showing that in certain

335   abiotic environments competition may actually select for more closely related taxa than expected

336   by chance (i.e. phylogenetic clustering). The *Alnus* ECM system is particularly interesting in this

337   respect because while the fungal communities associated with *Alnus* hosts are both species poor

338   and highly host specific, they include taxa from a number of distantly related lineages (Rochet et

339   al., 2011). Although explanations for this higher-level phylogenetic patterning are still lacking,

340   our current results suggest that competitive processes among both closely and more distantly

341   related taxa are not a key factor generating the atypical structure of *Alnus* ECM fungal

342   communities.

343   Some positive spatial associations have been observed in other studies of ECM fungal

344   communities (Agerer et al., 2002; Koide et al., 2005; Pickles et al., 2012), and have been

345   suggested to be due to complementary resource acquisition abilities of among individual taxa

346   (Jones et al., 2010). We speculate that in *Alnus* forests positive associations among ECM fungi

347   could also reflect possible amelioration of local abiotic conditions. Huggins et al., (in press)

348   found that *Alnus*-associated ECM fungi could more effectively buffer changes in local pH

349   environments than non-*Alnus* ECM fungi, which may be key to persistence in the high acidity

350   soils present in *Alnus* forests. While the exact buffering mechanism is not yet known, if it

351   involves the release of molecules into the external environment, growing directly adjacent to

352   another ECM fungus may result in greater buffering of local pH conditions than when growing in

353   isolation. We believe it is important to note, however, that the patterning of positive associations

354   were patchy and not consistent between plots, so it is hard to determine if local pH buffering is

355   actually significant without local measurements of pH for each sample. Furthermore, sequence

356   abundance of individual taxa has been shown not to correlate linearly with initial fungal tissue or

357   DNA abundance in other studies using NGS techniques (Amend et al., 2010, Nguyen et al.,

358   2014), so caution must be applied in using sequence abundance as an accurate ecological proxy.

359   Like the co-occurrence and correlation-based patterns, we found that spatial

360   autocorrelation patterns observed in *Alnus* ECM fungal communities were also anomalous

361   relative to other studies. The specific distance of spatial autocorrelation appears to vary among

362   systems, but there is typically strong spatial autocorrelation among community samples located

363   less than 5 m apart (e.g. Lilleskov et al. 2004, Bahram et al. 2013). While the spatial extent of

364   our study was very limited (the most distant samples within plots were only ~12 m apart), the

365   absence of spatial signal was not surprising based on previous studies of *Alnus* ECM fungal

366   communities. Both Pritsch et al. (2010) and Kennedy et al. (2011) found individual *Alnus* ECM

367   fungal taxa that were almost identical genetically (at least in the ITS region) in forests located

368   thousands of kilometers apart and, in a global scale analysis, Polme et al. (2013) found many

369   *Alnus* ECM OTUs were distributed across geographically distant samples. Theoretically, the

370   absence of dispersal limitation should make the detection of non-random distribution patterns

371   more likely if biotic interactions (either negative or positive) are strong determinants of

372   community structure. The classic work of Diamond (1975) is a good example, as the bird

373   populations studied across the New Guinea archipelago were not dispersal limited, yet exhibited

374   many checkerboard distribution patterns. As such, we do not think the atypical nature of the taxon

375   co-occurrence patterns in *Alnus* ECM fungal communities that we observed were driven by the

376   also atypical spatial correlation patterns.

377   As the results observed in this study differed in multiple ways from those found

378   previously, we had some concern they were caused by an artifact of our identification or

379   sampling methodology. Unlike previous examinations of taxon co-occurrence for ECM fungi, we

380   used next-generation sequencing (NGS) to identify the communities present. NGS methods

381   provide much greater sequencing depth per sample (Smith and Peay 2014), which may have

382   allowed us to more effectively document the ECM fungal communities present in each sample

383   compared to previous studies. We found that the three most abundant *Alnus*-associated ECM

384   fungi were present in every bag sample in both plots, which has not been observed in other

385   systems. Although the presence of spatially ubiquitous taxa will result in a lower total number of

386   checkerboard units observed (as 1,0 is possible but not 0,1), it has the same effect on both the

387   observed and null matrices and therefore should not bias statistical comparisons of $C_{observed}$ versus

388    $C_{expected}$. We checked this by eliminating the three ECM fungal taxa present in every sample and

389 found functionally identical results to those when those taxa were included (Table 2). A second

390 difference between this and related studies was the sampling of ECM fungal hyphal communities

391 in mesh bags. Previous studies assessing co-occurrence patterns have largely focused on ECM

392 root tips, but Koide et al., (2005) found very similar taxon co-occurrence patterns for root-tip and

393 soil-based analyses of ECM fungal communities in the same *Pinus resinosa* forest. Based on that

394 result, and the fact that the sequence abundances of all the ECM fungi present on *A. rubra* root

395 tips and the mesh bags showed highly similar patterns (Table S2), we do not believe assessing

396 ECM hyphal communities was the source of our incongruous results either (however, in

397 hindsight, a better experimental design would have been to sample the mesh bags and the ECM

398 root tips directly around them within each plot). A third difference is the restricted taxonomic

399 richness of *Alnus* ECM fungal communities. This explanation, however, also seems unwarranted,

400 as Pickles et al., (2012) showed highly significant negative co-occurrence patterns in matrices of

401 equivalent sizes. It is also possible that variation in soil nutrient availability could drive *Alnus*

402 ECM fungal community structure and, because it was relatively homogenous in our small-sized

403 plots, the resulting taxon distribution patterns were largely random. While we reiterate that we

404 did not directly measure soil nutrient availability in this study, other studies of *Alnus* ECM fungi

405 have shown some significant correlations between community structure and soil organic matter

406 and nutrients such as K and Ca (Becerra et al., 2005, Tedersoo et al., 2009, Roy et al., 2013,

407 Polme et al., 2013; see Richard (1968) for a possible mechanism). In those studies, however, the

408 percent of variance explained by soil nutrients was generally low, so we believe it is unlikely that

409 variation in resource availability was the primary determinant of the distribution patterns

410 observed. We recognize that additional differences likely exist, but feel confident that the co-

411 occurrence results we observed are ecologically accurate and not generated by methodological or

412 sampling artifact.

413   NGS techniques clearly represent a powerful and efficient way to assess the richness and

414   dynamics of fungal communities (Smith & Peay 2014), but we found that additional data quality

415   control analyses beyond the standard sequence quality thresholds and chimera checking were

416   needed to properly characterize ECM fungal community composition. Specifically, we found that

417   a relatively high number of ECM fungal taxa present that appeared to be the result of PCR

418   contamination. The PCR reactions of our extraction and PCR controls produced no bands

419   indicating positive product, but the sensitivity of NGS techniques and the Illumina platform in

420   particular makes the amplification of single DNA molecules highly probable (Tedersoo et al.,

421   2010b; Peay et al., 2013). Fortunately, the atypical and well-described nature of *Alnus* ECM

422   fungal communities made it relatively easy to identify the most obvious non-*Alnus* associated

423   taxa and remove them prior to the final analyses. For taxa that belonged to ECM fungal lineages

424   known to associate with *Alnus* hosts but which had not been previously documented, it was more

425   difficult to determine their status (i.e. whether they represented PCR contaminants, were present

426   in *A. rubra* soils as spores, or actually colonizing *A. rubra* root tips). In particular, the status of

427   Thelephoraceae1, which had the third highest sequence abundance in the full dataset, was

428   interesting because the closest BLAST match to Thelephoraceae1 was an ECM fungal root tip

429   sample from *Betula occidentalis* in British Columbia, Canada. Bogar & Kennedy (2013) found

430   that ECM fungal communities present on *Alnus* and *Betula* hosts can overlap, so it is possible this

431   taxon was overlooked in previous surveys of *Alnus* ECM fungal communities that used less

432   sensitive methods. However, the absence of this taxon from any the root tip samples in Plot 4

433   suggests that it was most likely present simply as spores rather than an active member of the

434   *Alnus*-associated ECM fungal community. Despite the unclear status of this taxon as well as

435   many others with lower abundance, the co-occurrence patterns showed the same general results

436   whether taxa of unknown status were included or not, suggesting the overall results were robust.

437   In less well-characterized ECM fungal and other microbial systems, however, the potential for

438 inclusion of spurious taxa is sufficiently high that we strongly recommend the sequencing of

439 negative extraction and PCR controls to help try to account for any lab-based contamination

440 (Nguyen et al., in press).

441 Taken together, our results suggest that while many ECM fungal communities appear to

442 be strongly affected by competitive interactions, those present in *Alnus* forests are not. Although

443 the reasons for this difference are not fully resolved in this study, the possibility of greater abiotic

444 stress changing the way in which species interact in *Alnus* forests is likely an important factor.

445 The application of ecological theories such as the stress gradient hypothesis to better understand

446 the factors driving ECM fungal community structure has grown rapidly in recent years (Peay et

447 al., 2008; Koide et al., 2014) and new technologies such as next generation sequencing continue

448 to make the study of ECM fungi increasingly tractable for ecologists. While we welcome this

449 synergy, we stress the importance of a solid foundation in fungal biology as well as critical

450 awareness of the limitations of molecular-based identification techniques to successfully integrate

451 ECM fungi into the ecological mainstream.

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Figure 1

Rank-abundance plot of all 190 (inset) and top 20 ectomycorrhizal (ECM) fungal taxa sampled in this study.

The top 20 ECM fungal taxa are color coded by whether there are known to be associated with *Alnus* hosts (black), of unknown host origin (grey), or laboratory contaminants (white).

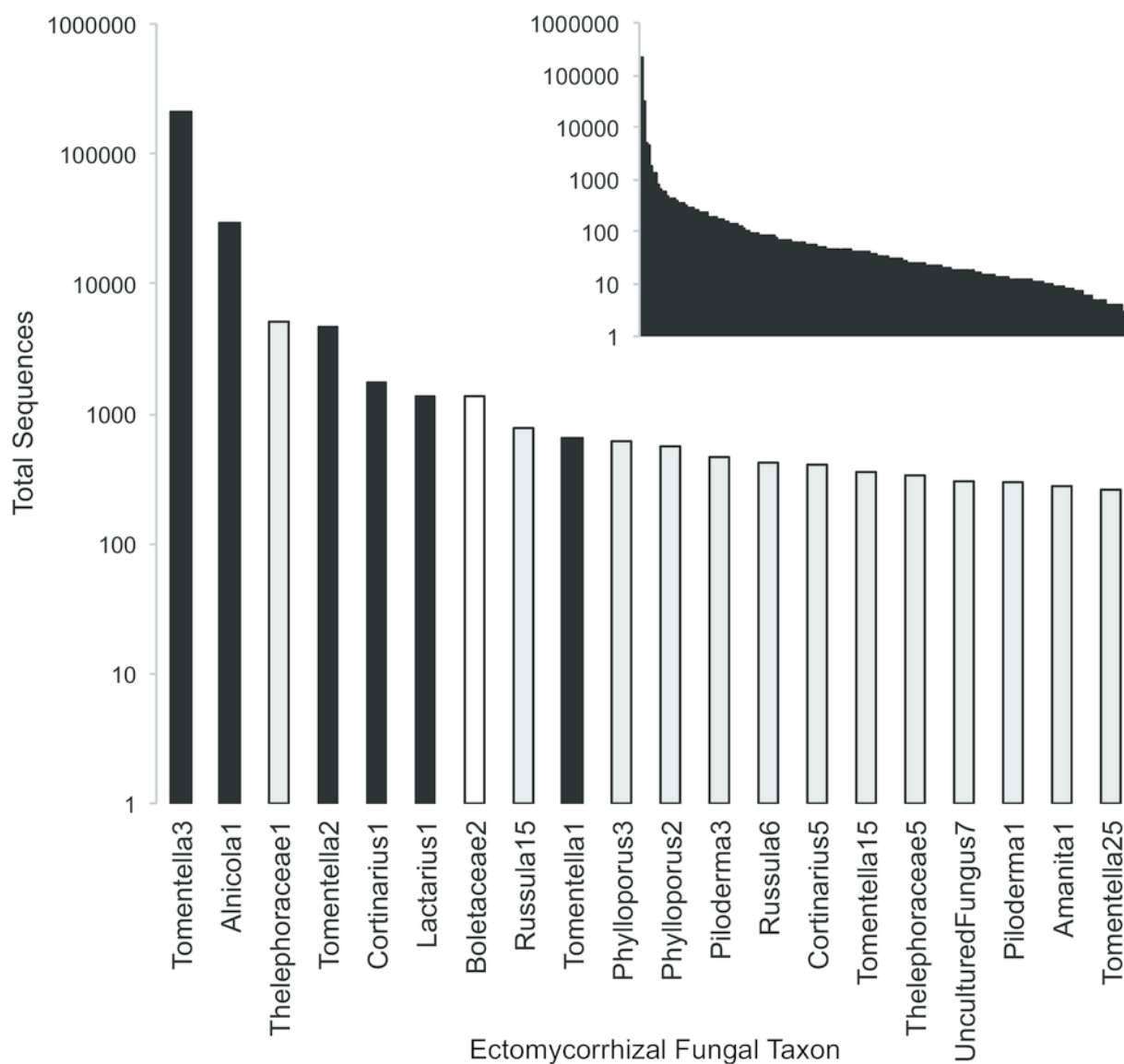
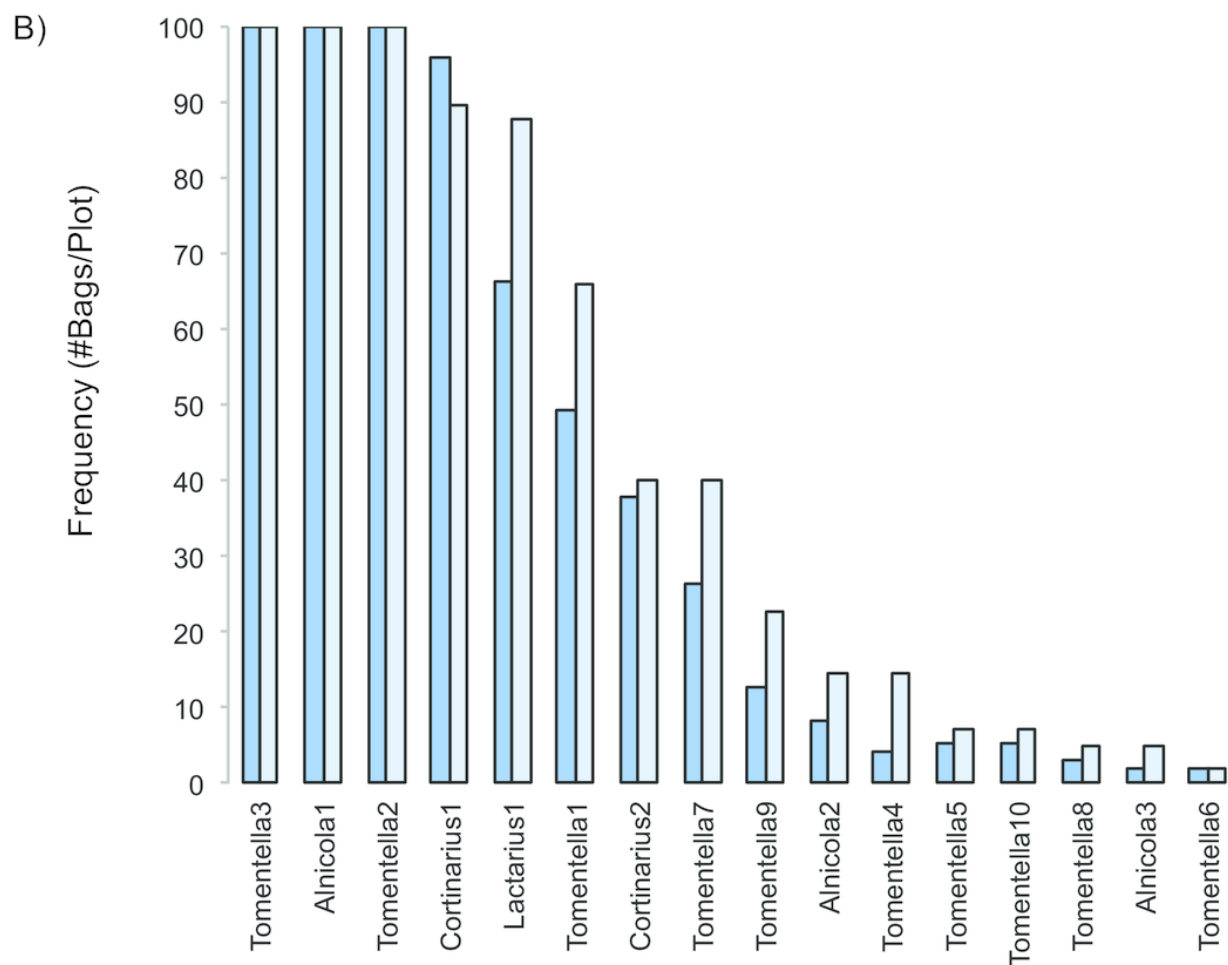
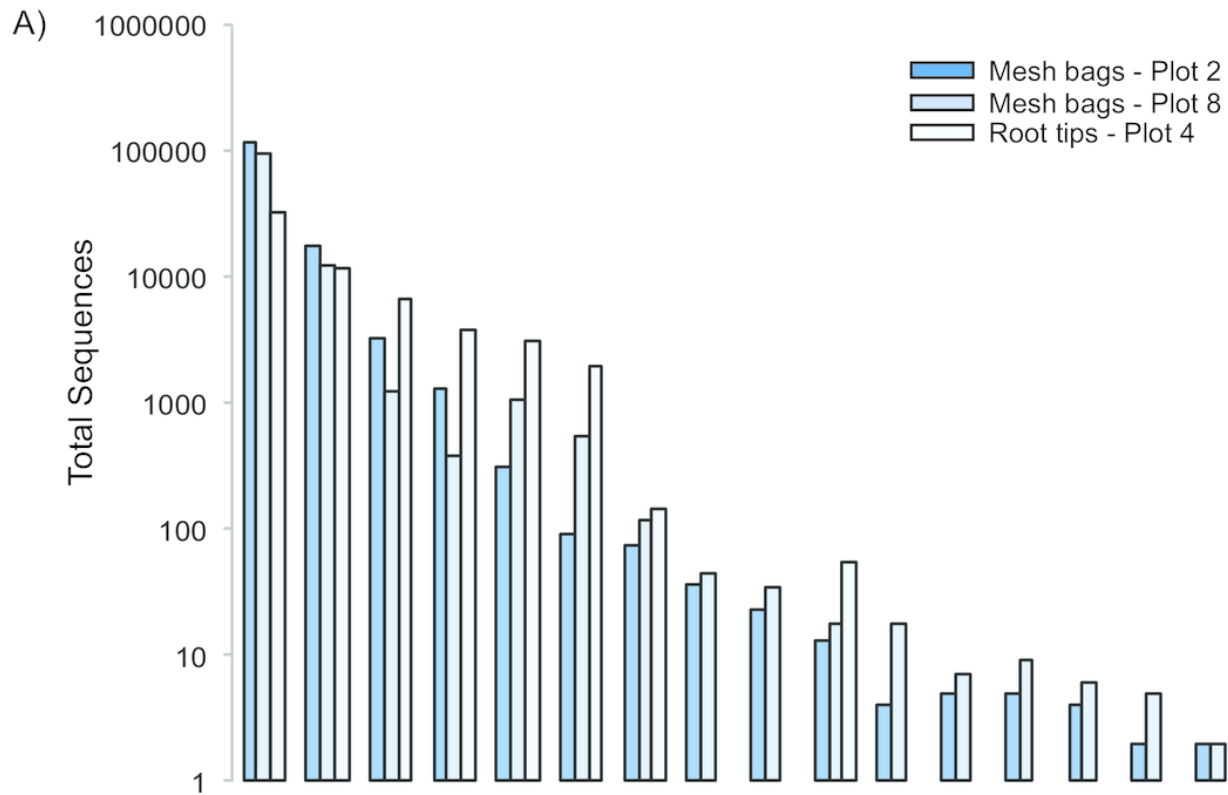



Figure 2

Rank-abundance (A) and rank-frequency (B) plots of Alnus-associated ectomycorrhizal fungal taxa sampled in mesh bags and root tips.

Alnus-associated Ectomycorrhizal Fungal Taxon

C-score taxon occurrence analyses of ECM fungal communities in Plots 2 and 8.

See methods for details about datasets and null matrix type definitions. SES = Standardized Effect Size.

Table 1. C-score taxon occurrence analyses of ECM fungal communities in Plots 2 and 8. See methods for details about datasets and null matrix type definitions. SES = Standardized Effect Size.

DataSet	Plot	Null Matrix Type	C observed	C expected	P value	SES
All	2	Fixed-Fixed	173.2	173.8	0.00	-3.35
		Fixed-Equiprobable		188.1	0.00	-21.2
All	8	Fixed-Fixed	164.4	164.7	0.73	-0.75
		Fixed-Equiprobable		172.1	0.00	-11.8
Alnus	2	Fixed-Fixed	93.5	92.5	0.21	0.76
		Fixed-Equiprobable		106.7	0.04	-1.75
Alnus	8	Fixed-Fixed	103.1	103.2	0.47	-0.07
		Fixed-Equiprobable		114.5	0.04	-1.82
*Alnus*RootOnly	2	Fixed-Fixed	77.4	76.7	0.74	0.54
		Fixed-Equiprobable		82.5	0.27	-0.59
*Alnus*RootOnly	8	Fixed-Fixed	61.2	61.6	0.45	-0.26
		Fixed-Equiprobable		78.4	0.13	-1.15
*Alnus*MinusTop3	2	Fixed-Fixed	200.3	198.4	0.27	0.59
		Fixed-Equiprobable		228.25	0.04	-1.72
*Alnus*MinusTop3	8	Fixed-Fixed	178.7	179.3	0.47	-0.20
		Fixed-Equiprobable		198.6	0.03	-1.88
*AlnusTomentella*Only	2	Fixed-Fixed	61.6	62.6	0.77	-0.55
		Fixed-Equiprobable		88.7	0.02	-1.99
*AlnusTomentella*Only	8	Fixed-Fixed	108.3	107.6	0.64	0.31
		Fixed-Equiprobable		109.1	0.47	-0.09

Spearman rank correlation coefficient matrices for ECM fungal communities in Plots 2 and 8.

Significant correlations are indicated in bold. Numbers over the columns of both matrices correspond to the number of the ECM fungal taxon identified in the first row.

Table 2. Spearman rank correlation coefficient matrices for ECM fungal communities in Plots 2 and 8. Significant correlations are indicated in bold. Numbers over the columns of both matrices correspond to the number of the ECM fungal taxon identified in the first row.

Plot2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Tomentella3	1.00															
2. Alnicola1	0.00	1.00														
3. Tomentella2	-0.02	0.00	1.00													
4. Cortinarius1	-0.05	-0.07	0.01	1.00												
5. Lactarius1	-0.07	-0.07	-0.02	0.28	1.00											
6. Tomentella1	-0.08	0.09	0.00	-0.13	-0.06	1.00										
7. Cortinarius2	-0.13	0.11	0.01	-0.05	0.00	0.26	1.00									
8. Tomentella7	0.16	-0.08	**0.65**	-0.03	0.08	-0.04	0.10	1.00								
9. Tomentella9	0.11	**0.48**	-0.06	-0.05	0.00	0.12	0.06	-0.02	1.00							
10. Alnicola2	0.07	**0.42**	0.07	-0.05	-0.04	-0.02	-0.10	0.02	0.09	1.00						
11. Tomentella4	-0.08	-0.04	**0.40**	-0.01	0.18	0.01	0.00	0.26	0.00	0.04	1.00					
12. Tomentella5	0.15	-0.07	-0.04	-0.06	-0.04	-0.03	0.00	-0.10	-0.01	-0.06	-0.05	1.00				
13. Tomentella10	0.06	0.01	-0.02	-0.03	0.00	0.08	-0.10	0.01	-0.05	-0.06	-0.05	-0.06	1.00			
14. Tomentella8	-0.07	-0.03	**0.40**	0.01	-0.03	-0.06	0.03	0.25	-0.04	0.04	**0.60**	-0.04	-0.04	1.00		
15. Alnicola3	**0.39**	-0.06	0.27	-0.04	-0.04	-0.11	0.07	**0.50**	0.03	-0.04	-0.03	0.29	-0.03	-0.02	1.00	
16. Tomentella6	**0.37**	-0.06	-0.03	-0.03	-0.02	-0.11	-0.08	0.02	0.03	-0.04	-0.03	-0.03	-0.03	-0.02	-0.02	1.00

Plot8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Tomentella3	1.00															
2. Alnicola1	0.13	1.00														
3. Tomentella2	-0.17	-0.12	1.00													
4. Cortinarius1	0.03	-0.03	0.26	1.00												
5. Lactarius1	-0.16	-0.09	0.14	0.14	1.00											
6. Tomentella1	-0.14	0.03	0.04	0.04	**0.46**	1.00										
7. Cortinarius2	0.06	**0.45**	-0.04	-0.02	-0.05	-0.06	1.00									
8. Tomentella7	-0.09	-0.06	-0.06	0.14	0.11	0.02	0.05	1.00								
9. Tomentella9	-0.12	**0.47**	0.02	-0.01	0.10	0.16	0.28	0.06	1.00							
10. Alnicola2	-0.06	0.15	0.02	0.12	-0.04	0.03	0.08	0.13	0.07	1.00						
11. Tomentella4	-0.05	-0.07	0.15	0.12	0.14	-0.02	-0.09	0.18	0.02	0.07	1.00					
12. Tomentella5	-0.05	0.05	0.07	-0.07	0.14	-0.01	-0.08	-0.08	0.08	-0.10	0.22	1.00				
13. Tomentella10	0.08	-0.04	-0.05	0.02	0.02	0.03	0.13	-0.05	-0.12	0.12	-0.04	-0.07	1.00			
14. Tomentella8	0.16	-0.07	0.01	0.06	-0.04	0.00	-0.06	0.06	-0.06	0.26	0.14	-0.06	-0.05	1.00		
15. Alnicola3	0.28	0.24	-0.10	-0.11	0.00	-0.06	0.21	-0.10	0.14	-0.08	-0.09	-0.07	0.07	-0.05	1.00	
16. Tomentella6	-0.02	-0.04	0.18	-0.04	-0.03	-0.04	0.06	-0.10	**0.33**	-0.05	-0.06	-0.04	-0.04	-0.03	-0.03	1.00