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 sequence abundance correlational analyses. ECM fungal communities were sampled from root tips and mesh in-growth bags in three monodominant *A. rubra* plots and identified using Illumina-based amplification of the ITS1 gene region. We found a total of 183 ECM fungal taxa present across the plots; 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.

Missing checkerboards: an absence of competitive signal in *Alnus*-associated ectomycorrhizal fungal communities

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PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.531v1 | CC-BY 4.0 Open Access | rec: 10 Oct 2014, publ: 10 Oct 2014

Introduction

A common ecological way to assess the role of interspecific competition and/or facilitation in determining community structure is experimental manipulation involving the removal of neighboring individuals. This approach has been widely used in studies examining the biotic determinants of plant and animal communities (Connell 1983; Schoener 1983), but the ability to carry out similar manipulations in field-based studies of diverse soil microbial communities is non-feasible due to the inability to selectively manipulate species-level neighborhood composition. One widely proposed alternative is to look at species distribution patterns, with Diamond's (1975) study of bird distributions in the New Guinea archipelago being one of most well recognized examples. In that study, the presence of certain bird species on a given island was associated with the absence of other species (and vice versa on other islands), resulting in a series of 'forbidden species combinations' or 'checkerboard distributions', which were posited to be the result of competitive exclusion (Diamond 1975). This technique provided an important step forward in assessing the role of species interactions in field-based studies at the community level, but it has been widely noted that analyses of species co-occurrence patterns should also include comparisons with patterns generated from communities assembled randomly to maximize inference (Connor & Simberloff 1979; Gotelli & Graves 1996 and references therein).

Since the 1970s, species co-occurrence analyses have been used to assess the possibility of species interactions in a wide range of organisms, including both macro- and microorganisms (Gotelli & McCabe 2002; Horner-Devine et al., 2006). Plant-associated fungal communities, which have diverse ecological roles in ecosystems (Smith & Read 2008; Rodriguez et al., 2009), have shown a full range of co-occurrence patterns, including those consistent with both positive

and antagonistic interactions (Koide et al., 2005; Pan & May 2009; Gorzelak et al., 2012; Ovaskainen *et al.* 2010; Pickles et al., 2012; Toju et al., 2014). For ectomycorrhizal (ECM) fungi, the dominant microbial eukaryotes in many temperate and some tropical forest soils (Smith and Read 2008), these analyses have consistently found evidence of less species co-occurrence than expected by chance (Koide et al., 2005; Pickles et al., 2010; Pickles et al., 2012). This suggests that competitive interactions may play a significant role in structuring the communities of this fungal guild (Kennedy 2010). The initial studies of species co-occurrence patterns in ECM fungal communities looked only in forests dominated by conifer hosts, but a recent study in *Fagus sylvatica* forests in Europe also found evidence of significantly lower than expected co-occurrence patterns (Wubet et al., 2012). This latter result indicates that the predominance of antagonistic interactions in determining ECM fungal community structure may be a common, host-lineage independent phenomenon. Importantly, however, other ecological and evolutionary factors aside from species interactions can also be responsible for non-random species co-occurrence patterns (Gotelli & McCabe 2002; Ovaskainen *et al.* 2010), so caution must be applied in inferring underlying mechanisms.

In this study, we focused on assessing the community co-occurrence distributions of ECM fungi associated with the host genus *Alnus*. Unlike other ECM host genera with large geographical distributions, the ECM fungal communities associated with *Alnus* trees have been consistently found to be both species poor and highly host specific (Tedersoo et al., 2009; Kennedy & Hill 2010; Kennedy et al., 2011; Bogar & Kennedy 2013; Põlme et al., 2013; Roy et al., 2013). The mechanisms driving this atypical structure have long been thought to be related to the co-presence of nitrogen-fixing *Frankia* bacteria, which can have strong biotic and abiotic effects on *Alnus*-associated ECM fungal communities (Walker et al., 2014). In particular, the

high rates of nitrification present in *Alnus* forest soils (due to the high inputs and decomposition of nitrogen-rich leaf litter) results in significantly higher nitrate and acidity levels than those present in most other ECM-dominated forest soils (Miller et al., 1992; Martin et al., 2003; Walker et al., 2014). Elevated levels of both of these abiotic factors have been shown to inhibit the growth of many ECM fungi (Hung and Trappe 1983; Lilleskov et al., 2002) and, using an experimental pure culture approach, Huggins et al., (in press) recently demonstrated that *Alnus*-associated ECM fungi have a greater ability to tolerate high nitrate and acidity conditions compared to non-*Alnus*-associated ECM fungi.

Given the ability of *Alnus*-associated ECM fungi to grow in conditions that are generally considered abiotically stressful, we hypothesized that ECM fungal species co-occurrence patterns in *Alnus* forests may differ from those present in forests dominated by other ECM hosts. Specifically, we speculated that competitive interactions would be less prevalent in this study system, based on the fact that many studies of vascular plants have shown that the nature of species interactions often changes from antagonistic to positive with increasing levels of abiotic stress (Bertness & Callaway 1994, Gómez-Aparicio et al., 2004, but see Michalet et al., 2006). To examine this hypothesis, we examined the co-occurrence patterns of the ECM fungal communities present in three mono-dominant plots of *Alnus rubra* in the western United States. ECM fungal communities were sampled on root tips and in soil. For the latter, we used sand-filled mesh in-growth bags, which allow for efficient, well-replicated community sampling of fungal hyphae growing in soil (Wallander et al., 2001, Branco et al., 2013). To identify the ECM fungi present in the study, we used high throughput Illumina sequencing, which is being increasingly used to profile ECM fungal community composition (McGuire et al., 2013, Smith & Peay 2014).

Materials & Methods

Study Location

The study site was located on the eastern side of the Coast Range mountains in northwestern Oregon, U.S.A. (latitude: N 45.820 W 123.05376, elevation: 462 m). Temperatures at the site are moderate (mean annual temperature = 8.7°C, min = -1.2°C, max = 23.8°C), with significant precipitation between October and May followed by drier summer months (total = 1742 mm). The specific study location is part of a long-term research project examining the effects of different forest management practices on *A. rubra* growth (see the Hardwood Silvicultural Cooperative (HSC) website for details, http://www.cof.orst.edu/coops/hsc). The HSC site used, Scappoose (HSC 3209), was established in 1995. Prior to the implementation of the HSC work, the site was a second-growth coniferous forest, which was clear-cut and replanted with a series of monodominant *A. rubra* plots. *A. rubra* seedlings were planted from nursery stock (Brooks Tree Farm, Brooks, OR) during the beginning of their second year of growth. Seedling ECM status at the time of planting was not assessed (*Frankia* nodules were noted to be absent), but nursery fumigation practices indicate colonization was unlikely (Brooks Nursery, pers. com.).

Our experiment was conducted in three 1600 m^2 plots at HSC 3209. The plots, which were located approximately 100 m apart, differed in initial *A. rubra* stem density (Plot 2 = 628, Plot 4 = 1557, and Plot 8 = 3559 stems/ha), but had no other forest management practices applied. The understories in all three plots were well colonized by arbsucular mycorrhizal plants (dominated by *Mahonia nervosa* and *Claytonia perfoliata*), with no other ECM hosts besides *A. rubra* present. Soils were classified as well-drained Tolamy loams (USDA Soil Survey,

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Columbia County, OR). Within each plot, we located a 9 x 9 m subplot and overlaid a 100 point grid, with each point being separated by 1 m. At each point in Plot 4, which was sampled for ECM root tips, a 5 cm diameter x 10 cm deep soil core was taken on May 31, 2013. In Plots 2 and 8, which were sampled for ECM communities present in soil, a 5 x 5 cm mesh bag was buried at each point 5 cm below the soil surface. The bags were made of anti-static polyester fabric with 300 micron diameter pores. This pore size allowed fungal hyphae to grow into the bags, but prevented penetration of plant roots. We filled the bags with twice autoclaved #3 grade Monterey aquarium sand (Cemex, Marina, CA, USA). Aluminum tags on fluorescent string were added to facilitate bag recovery. The mesh bags at Plot 2 were buried on February 1, 2013 and February 22 at Plot 8. They were left undisturbed in the soil until May 31, when all were harvested. After removal from the soil, we placed the mesh bags into individual plastic bags and then onto ice for transport back to the laboratory. Soil cores and bags were stored at 4°C for <96 hours before further processing.

Molecular Analyses

We processed the root tip samples by gently washing all roots away from the soil and removing all ECM colonized root tips from each core under a 10X dissecting scope (~10-50 root tips/core). All roots from each core were extracted using individual MoBio PowerSoil kits (Hercules, CA, USA), following manufacturer's instructions for maximum DNA yields. For the mesh bags, we followed the protocol outlined in Branco et al., (2013). Briefly, each bag (including a negative control that was taken to the field, but not buried) was emptied into a sterile 50 ml centrifuge tube. We added 10 ml of sterile deionized water and vortexed each tube for two minutes, followed by a five minute settling period (hyphae have been previously observed to float to the water surface). We then transferred the top two ml top of water to a new 2 ml

centrifuge tube and contents were pelleted via centrifugation. On the same day, we extracted total genomic DNA form the pellets using the Sigma REDExtract-N-Amp kit (Sigma-Aldrich, St, Louis, MO, USA) following manufacturer's instructions. Root tips and extracts were stored for one week at -20°C prior to PCR amplification.

For the root tip samples, we combined equal quantity aliquots from all 97 DNA extractions (three cores contained no roots) into a single template for PCR. In contrast, for each mesh bag sample as well as extraction controls, we conducted individual PCR reactions. We processed these two types of samples differently because we were most interested in the spatial co-occurrence patterns in the soil ECM fungal communities and therefore only used the root tip samples to create a local sequence reference set of known *Alnus*-associated ECM taxa against which the mesh bag data could be compared. For all PCR reactions, we used the barcoded ITS1F and ITS2 primer set of Smith & Peay (2014), with each sample run in triplicate and pooled to minimize heterogeneity. Successful PCR products were determined by gel electrophoresis and magnetically cleaned using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA) according to manufacturer's instructions. Final product concentrations were quantified using a Qubit dsDNA HS Fluorometer (Life Technologies, Carlsbad, CA, USA). Root tip and bag samples were run at different sequencing facilities under the same general conditions. The single root tip PCR product was run at the University of Minnesota Genomics Center using 250 bp paired-end sequencing on the MiSeq Illumina platform. For the bags, we pooled the 192 successfully amplified bag samples at equimolar concentration and ran them on the same platform at the Stanford Functional Genomics Facility using 250 bp paired-end sequencing on the MiSeq Illumina platform. A spike of 20% and 30% PhiX was added to the runs to achieve sufficient sample heterogeneity, respectively. Raw sequence data and associated metadata from

both the root tip and bag samples were deposited at MG-RAST (http://metagenomics.anl.gov/) under project #1080.

Bioinformatic Analyses

We used the software packages QIIME (Caporaso et al., 2010) and MOTHUR (Schloss et al., 2009) to process the sample sequences. Raw sequences were demultiplexed, quality filtered using Phred = 20, trimmed to 178 base pairs, and ends were paired, followed by filtering out of sequences that had any ambiguous bases or a homopolymer run of 9 bp. Following the guidelines discussed in Nguyen et al., (in press), we employed a multi-step operational taxonomic unit (OTU) picking strategy by first clustering with reference USEARCH (including de novo chimera checking) at 97% sequence similarity, followed by UCLUST at 97% sequence similarity. We used a 97% similarity threshold because it the most commonly employed in community-level ECM fungal studies, although some lineages, including *Alnicola*, may have greater sequence similarity among species (Tedersoo et al. 2009). Alnicola have been previously noted a pilot analysis of sequence similarity among *Alnus*-associated *Tomentella* species, we observed that the 97% threshold resulted in the same number of OTUs for ITS1 and the full ITS region (i.e. ITS1, 5.8S, and ITS2). The UNITE database (Kõljalg et al., 2013) was used in both chimera checking and OTU clustering, with singleton OTUs were discarded to minimize the effects of artifactual sequences (Tedersoo et al. 2010b). We assigned taxonomic data to each OTU with NCBI BLAST+ v2.2.29 (Altschul et al., 1990), using a custom fungal ITS database containing the curated UNITE SH database (v6) (http://unite.ut.ee/repository.php, Kõljalg et al., 2013) and more than 600 vouchered fungal specimens, including 46 representative sequences from *Alnus* forests at other HSC locations in Oregon (Kennedy & Hill 2010) and Mexico (Kennedy et al. 2011). Since sequences that had low subject length:query length matches were typically non-

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fungal, we further filtered out sequences with matches ≤ 90% to BLAST (i.e. at least 90% of the bases in the input sequence matches to another sequence in the database at some identity level).

Using the remaining sequence dataset, we rarified all samples to 12946 sequences, which was lowest number of sequences obtained across the 192 samples. Since there has recently been question raised about the validity of rarifaction in next generation sequencing analyses (McMurdie and Holmes 2014), we also analyzed the data without rarifaction. We obtained very similar results (Table S1), so present the data based on rarefied samples only. ECM OTUs within each sample were parsed out using a python script that searches for genera names from a list of 189 known ECM genera and their synonyms (Branco et al., 2013, appended from Tedersoo et al., 2010a). The resulting sample x OTU matrix contained 201 ECM taxa represented by at least one sequence per sample (min = 1, median = 34, mean = 1334, max = 209,187). We found that 18 of the 201 OTUs present were highly similar (>97% similar) to ECM fungi present in the dipterocarp rainforests of Malaysia, which were concurrently being studied in the Peay lab using the same next-generation sequencing approach (Fig. 1). Because these OTUs represented accidental contamination probably during library construction, they were eliminated from the final analyses. Although an additional 84 OTUs had greater than >97% similarity to taxa found in the Borneo study, because their closest BLAST match was not from Borneo, we conservatively considered these taxa as having cosmopolitan distributions and included them in the final analyses. The final OTU × sample matrix, including taxonomic matches and representative of sequences for each OTU, can be found in Table S2.

Statistical Analyses

Taxon co-occurrence patterns of the ECM fungal communities present in bag samples were assessed using the program EcoSim (Gotelli & Entsminger 2009), with presence-absence

matrices for Plots 2 and 8 being analyzed separately. (The root data from Plot 4 could not be analyzed for sample-level co-occurrence due to the pooled sequencing approach for those samples). We utilized the C-score algorithm (Stone & Roberts 1990), which compares the number of checkerboard units (i.e. 1,0 x 0,1) between all pairs of species in the observed matrix ($C_{observed}$) to that based in random permutations of the same matrix ($C_{expected}$, i.e. the null models). Since randomized permutations of a matrix can be achieved in multiple ways (see Gotelli & Entsminger 2009 for details), we analyzed our datasets using both the 'fixed-fixed' and 'fixed-equiprobable' options (which are recommended by the program guide and used in the previous ECM fungal co-occurrence analyses). In both options, the row (i.e. taxon) totals were fixed, so that the total abundances of each taxon in the observed and null matrices were identical. In the 'fixed-equiprobable' option, however, the column (i.e. sample) totals in the null matrices were no longer equivalent to those in the observed matrix. Instead, all samples in the null matrices had an equal probability of being colonized by any of the taxa in the observed matrix, which effectively eliminates differences in taxon richness among samples.

Of the ECM taxa present in the final root tip and bag datasets, over 90% (167/183) belonged to species never previously encountered with *Alnus* (Table S2, *Alnus*Match = No). Unlike other ECM host systems with large geographic ranges, the ECM fungal community associated with *Alnus* hosts is remarkably well characterized at local (Tedersoo et al., 2009, Kennedy et al., 2010, Walker et al., 2014), regional (Kennedy et al., 2011, Roy et al., 2013, and global scales (Polme et al., 2013). As such, it is highly likely the majority of the novel OTUs encountered were not part of the active ECM community in our plots, but rather present simply either as spores or additional lab contaminants. To account for this issue, we divided our checkerboard analyses into five different input matrices for the bag dataset (Plots 2 and 8). The

first matrix included all 183 ECM fungal taxa (referred to as "All"). The second matrix included the 16 taxa that had >97% similarity matches to ECM samples from *Alnus* forests (referred to as *Alnus*). The third matrix included only the 8 taxa that were encountered on ECM root tips in Plot 4 (referred to as *Alnus*RootOnly). To assess the robustness of the results generated using the larger *Alnus* matrix, the fourth matrix excluded the three most frequent and abundant species (*Tomentella*3, *Alnicola*1, *Tomentella*2) (referred to as *Alnus*MinusTop3). Finally, the fifth matrix included just the 10 taxa in the genus *Tomentella* (from the larger *Alnus* matrix) to look for evidence of species interactions among this subset of closely related taxa (referred to as *AlnusTomentella*Only). For all of the aforementioned C-score analyses, taxa present in less than 5 bag samples were removed, as low frequency taxa are generally considered non-informative (Koide et al., 2005). The observed input matrices were compared to 5000 null matrices. Significant differences between the observed matrix C-score and that of the null matrices were determined along with standardized effect sizes (SES). Observed C-scores significantly higher than those generated from the null matrices are consistent with a community being structured by competitive interactions, whereas $C_{observed}$ significantly lower than the $C_{expected}$ is consistent with positive interactions.

To further assess the degree of association between known *Alnus* ECM fungal taxa, we also used an abundance-based approach (as opposed to the co-occurrence analyses, which are based on binary presence/absence data). Specifically, we calculated the pair-wise Spearman rank correlation coefficients among all pairs of the 16 *Alnus*-associated taxa using the *cor* function in R (R Development Core Team 2013). Coefficients >0.30 were tested for significance with the *cor.test* function. To account for multiple tests (n=13), we used a Bonferroni-corrected *P* value of 0.003. With the same data set, we also tested for the presence of spatial autocorrelation using

253 the *mgram* function in the ECODIST package in R. We first converted the sequence abundance
254 datasets in both Plots 2 and 8 into dissimilarity matrices using the Bray-Curtis Index and then
255 compared those to a Euclidean distance matrix of sampling points for each plot. For the Mantel
256 correlogram tests, we used the *n.class=0* option, which uses Sturge's equation to determine the
257 appropriate number of distance classes.

258

259 **Results**

260 We found 183 total ECM fungal taxa across all three plots (Table S2); 16 of which
261 matched closely to known *Alnus*-associated ECM fungi. In the mesh bags, *Alnus*-associated
262 ECM fungal taxa represented six of the ten most abundant OTUs present, including the dominant
263 ECM fungal taxon, *Tomentella*3, which was present in all the bag samples in both plots and had
264 sequence abundances nearly ten-fold higher than any other taxon (Fig. 2a,b). Two other *Alnus*-
265 associated taxa, *Alnicola*1 and *Tomentella*2, were also present in all samples, whereas the
266 remaining *Alnus*-associated ECM fungal taxa had frequencies varying from 2-96% (Plot 2 mean
267 = 25%, Plot 8 mean = 31%) and lower sequence abundances. Eight of the 16 *Alnus*-associated
268 ECM fungal taxa were present on both roots and in the bags, with abundances that were very
269 similar (Fig. 1a). Of the eight ECM fungal taxa found on root tips, all were previously
270 encountered on *A. rubra* root tips at other sites in Oregon, while the eight taxa found exclusively
271 in bags had not been previously documented (Kennedy & Hill 2010).

272 ECM fungal taxon co-occurrence patterns were largely consistent between plots, but
273 different between null models. Of the ten tests (i.e. 5 matrix types x 2 plots) using the 'fixed-
274 fixed' permutation option, nine indicated that the observed ECM fungal community did not

differ significantly from random assembly (Table 1). In one case, Plot 2 All, the observed ECM fungal community had significantly more co-occurrence than expected by chance. In contrast, in the ten tests using the 'fixed-equiprobable' permutation option, three indicated that the observed ECM fungal community did not differ significantly from random assembly, while seven found that the observed ECM fungal community had significantly more co-occurrence than expected by chance. Results remained the same for *Alnus* ECM fungal communities whether the top three taxa were removed or not. The *Alnus* and *Alnus*RootOnly analyses did differ under the 'fixed-equiprobable' option, with the former showing greater than expected co-occurrence and the latter having a pattern no different than one based on random assembly. Additionally, in the *AlnusTomentella*Only analysis, the ECM fungal community showed greater than expected co-occurrence in Plot 2 but not in Plot 8. In all of these cases, significant antagonistic patterns were not observed.

Spearman rank analyses revealed that pair-wise sequence abundances of some of the 16 *Alnus* ECM fungal taxa were significantly positively correlated (Table 2). The specific significant combinations varied between plots, with only taxon pair (*Alnicola*1 & *Tomentella*9) showing significant positive correlations in both plots. Although a number of pair-wise correlations had negative values (suggesting negative rather than positive interactions), none of them were significant, even when considered at a *P* value of 0.05. In addition, the Mantel correlogram analyses found no clear evidence of spatial autocorrelation in the *Alnus*-associated ECM fungal communities. In Plot 2, there was no significant autocorrelation at any distance, while in Plot 8 there was a single significant positive correlation between samples located 1-2 m apart (Fig. S1, S2).

298 **Discussion**

299 We found that the ECM fungal communities in *A. rubra* forests displayed a different
300 pattern of taxon co-occurrence compared to those seen for other ECM fungi. Unlike the
301 consistent previous findings of less co-occurrence among species than expected by chance
302 (Koide et al., 2005; Pickles et al., 2012; Wadet et al., 2012), we observed no evidence of spatial
303 patterns consistent with interspecific competition in *Alnus*-associated ECM fungal communities.
304 In contrast, we consistently found co-occurrence patterns that were either no different from
305 random assembly or consistent with positive interactions. Although we did not measure soil
306 nitrate and acidity conditions in this study (see Martin et al., (2003) and Walker et al., (2014) for
307 values from comparable age *A. rubra* forests at other sites in Oregon), *Alnus* soils are
308 consistently characterized by abiotic conditions are generally considered stressful to ECM fungi.
309 As such, our results are largely congruent with the 'stress gradient hypothesis', which posits that
310 species interactions shift from negative to positive as environmental conditions become harsher
311 (Bertness & Callaway 1994). Further support for this hypothesis was also seen in the abundance-
312 based correlations of sequence reads, which also lacked any results suggestive of strong pair-
313 wise antagonistic interactions and instead found multiple instances consistent with positive
314 interactions.

315 Although the patterns demonstrated in our study are based solely on correlative inference,
316 there is some experimental evidence that may support the stress gradient hypothesis for ECM
317 fungal community dynamics. Koide et al., (2005) found a shift from significant negative co-
318 occurrence patterns in their control plots to non-significant co-occurrence patterns in plots where
319 either tannins or nitrogen were added experimentally. While they did not explicitly analyze these
320 manipulations in terms of stress, both increased tannin and nitrogen levels have been shown to

inhibit the growth of multiple ECM taxa (Koide et al., 1998; Cox et al., 2010). The direction of the response in the Koide et al., (2005) study is consistent with greater abiotic stress resulting in a decrease in antagonistic ECM fungal interactions. At the same time, it is plausible that resource limitation was eliminated with the addition of nitrogen, which could have allowed for greater spatial co-existence among ECM fungi. Since the *Alnus* system has naturally higher nitrogen availability than most ECM forests due to the co-presence of nitrogen-fixing *Frankia* bacteria, it is also possible that greater resource abundance could drive the co-occurrence patterns we observed. Given the fact that the pattern could potentially be explained by increasing stress or resource availability, additional experimental tests are needed to distinguish among these explanations. One promising approach would be to examine the taxon co-occurrence patterns in younger and older *Alnus* forests, since soil nitrate and acidity concentrations increase in these forests over time (Martin et al., 2003). If the stress gradient hypothesis were the most plausible explanation, then we would expect to see competitive and facilitative interactions to be dominant, respectively.

The presence of co-occurrence patterns consistent with significant negative species interactions was also missing in our analysis of more closely related ECM fungal taxa. For the ten *Alnus*-associated members of the genus *Tomentella*, co-occurrence patterns either did not differ significantly from random assembly or reflected an effect of positive interactions. Like the larger community analyses, this result also differs from previous experimental studies, where strong antagonistic interactions among closely related ECM fungal taxa have been observed (Kennedy 2010). In a similarly designed study that also assessed ECM fungi with taxon co-occurrence analyses, Pickles et al., (2012) found patterns consistent with strong interspecific competition among a suite of *Cortinarius* species in a Scottish *Pinus sylvestris* forest. Although

it has long been assumed that competition may be stronger in more closely related species due to greater overlap in resource utilization, a meta-analysis by Cahill et al., (2008) found little consistent evidence to support this supposition. Mayfield & Levine (2010) further questioned the validity of phylogenetic relatedness as a good proxy for competitive strength by showing that in certain abiotic environments competition may actually select for more closely related taxa than expected by chance (i.e. phylogenetic clustering). The *Alnus* ECM system is particularly interesting in this respect because while the fungal communities associated with *Alnus* hosts are both species poor and highly host specific, they include taxa from a number of distantly related lineages (Rochet et al., 2011). Although explanations for this higher-level phylogenetic patterning are still lacking, our current results suggest that competitive processes among both closely and more distantly related taxa are not a key factor generating the atypical structure of *Alnus* ECM fungal communities.

Some positive spatial associations have been observed in other studies of ECM fungal communities (Agerer et al., 2002; Koide et al., 2005; Pickles et al., 2012), and have been suggested to be due to complementary resource acquisition abilities of among individual taxa (Jones et al., 2010). We speculate that in *Alnus* forests positive associations among ECM fungi may also reflect amelioration of local abiotic conditions. Huggins et al., (in press) found that *Alnus*-associated ECM fungi could more effectively buffer changes in local pH environments than non-*Alnus* ECM fungi, which may be key to persistence in the high acidity soils present in *Alnus* forests. While the exact buffering mechanism is not yet known, if it involves the release of molecules into the external environment, growing directly adjacent to another ECM fungus may result in greater buffering of local pH conditions than when growing in isolation. We believe it is important to note, however, that the patterning of positive associations were patchy and not

consistent between plots, so it is hard to determine if local pH buffering is actually significant without local measurements of pH for each sample. Furthermore, sequence abundance of individual taxa has been shown not to correlate linearly with initial fungal tissue or DNA abundance in other studies using NGS techniques (Amend et al., 2010, Nguyen et al., 2014), so caution must be applied in using sequence abundance as an accurate ecological proxy.

As the results observed in this study differed from those found previously, we had some concern they were caused by an artifact of our identification or sampling methodology. Unlike previous examinations of taxon co-occurrence for ECM fungi, we used next-generation sequencing (NGS) to identify the communities present. NGS methods provide much greater sequencing depth per sample (Smith and Peay 2014), which may have allowed us to more effectively document the ECM fungal communities present in each sample compared to previous studies. We found that the three most abundant *Alnus*-associated ECM fungi were present in every bag sample in both plots, which has not been observed in other systems. Although the presence of spatially ubiquitous taxa will result in a lower total number of checkerboard units observed (as 1,0 is possible but not 0,1), it has the same effect on both the observed and null matrices and therefore should not bias statistical comparisons of $C_{observed}$ versus $C_{expected}$. We checked this by eliminating the three ECM fungal taxa present in every sample and found functionally identical results to those when those taxa were included (Table 2). A second difference between this and related studies was the sampling of ECM fungal hyphal communities in mesh bags. Previous studies assessing co-occurrence patterns have largely focused on ECM root tips, but Koide et al., (2005) found very similar taxon co-occurrence patterns for root-tip and soil-based analyses of ECM fungal communities in the same *Pinus resinosa* forest. Based on that result, and the fact that the sequence abundances of all the ECM fungi present on *A. rubra* root

tips and the mesh bags showed highly similar patterns, we do not believe assessing ECM hyphal communities was the source of our incongruous results either. A third difference is the restricted taxonomic richness of *Alnus* ECM fungal communities. This explanation, however, also seems non-applicable, as Pickles et al., (2012) showed highly significant negative co-occurrence patterns in matrices of equivalent sizes. Finally, it is possible that variation in soil nutrient availability could drive *Alnus* ECM fungal community structure and, because it was relatively homogenous in our small-sized plots, the resulting taxon distribution patterns were largely random. While we did not measure soil nutrient availability in this study, other studies of *Alnus* ECM fungi have shown some significant correlations between community structure and soil organic matter and nutrients such as K and Ca (Becerra et al., 2005, Tedersoo et al., 2009, Roy et al., 2013, Polme et al., 2013*)*. In those studies, however, the percent of variance explained by soil nutrients was generally low, so we believe it is unlikely that variation in resource availability was the primary determinant of the distribution patterns observed. We recognize that additional differences likely exist, but feel confident that the co-occurrence results we observed are ecologically accurate and not generated by methodological or sampling artifact.

NGS techniques clearly represent a powerful and efficient way to assess the richness and dynamics of fungal communities (Smith & Peay 2014), but we found that additional data quality control analyses beyond the standard sequence quality thresholds and chimera checking were needed to properly characterize ECM fungal community composition. Specifically, we found that a relatively high number of ECM fungal taxa present that appeared to be the result of PCR contamination. The PCR reactions of our extraction and PCR controls produced no bands indicating positive product, but the sensitivity of NGS techniques and the Illumina platform in particular makes the amplification of single DNA molecules highly probable (Tedersoo et al.,

2010b; Peay et al., 2013). Fortunately, the atypical and well-described nature of *Alnus* ECM fungal communities made it relatively easy to identify the most obvious non-*Alnus* associated taxa and remove them prior to the final analyses. For taxa that belonged to ECM fungal lineages known to associate with *Alnus* hosts but which had not been previously documented, it was more difficult to determine their status (i.e. whether they represented PCR contaminants, were present in *A. rubra* soils as spores, or actually colonizing *A. rubra* root tips). In particular, the status of Thelephoraceae1, which had the third highest sequence abundance in the full dataset, was interesting because the closest BLAST match to Thelephoraceae1 was an ECM fungal root tip sample from *Betula occidentalis* in British Columbia, Canada. Bogar & Kennedy (2013) found that ECM fungal communities present on *Alnus* and *Betula* hosts can overlap, so it is possible this taxon was overlooked in previous surveys of *Alnus* ECM fungal communities that used less sensitive methods. However, the absence of this taxon from any the root tip samples in Plot 4 suggests that it was most likely present simply as spores rather than an active member of the *Alnus*-associated ECM fungal community. Despite the unclear status of this taxon as well as many others with lower abundance, the co-occurrence patterns showed the same general results whether taxa of unknown status were included or not, suggesting the overall results were robust. In less well-characterized ECM fungal and other microbial systems, however, the potential for inclusion of spurious taxa is sufficiently high that we strongly recommend the sequencing of negative extraction and PCR controls to help try to account for any lab-based contamination (Nguyen et al., in press).

Taken together, our results suggest that while many ECM fungal communities appear to be strongly affected by competitive interactions, those present in *Alnus* forests are not. Although the reasons for this difference are not fully resolved in this study, the possibility of greater

abiotic stress changing the way in which species interact in *Alnus* forests is likely an important factor. The application of ecological theories such as the stress gradient hypothesis to better understand the factors driving ECM fungal community structure has grown rapidly in recent years (Peay et al., 2008; Koide et al., 2014) and new technologies such as next generation sequencing continue to make the study of ECM fungi increasingly tractable for ecologists. While we welcome this synergy, we stress the importance of a solid foundation in fungal biology as well as critical awareness of the limitations of molecular-based identification techniques to successfully integrate ECM fungi into the ecological mainstream.

Acknowledgements

We thank A. Bluhm and D. Hibbs for assistance with using the HSC study location, L. Bogar, V. Engebretson, J. Huggins, P. King for assistance with experiment implementation and harvest, J. Walker for assistance with DNA extractions, D. Smith for assistance with NGS processing, and members of the Peay Lab as well as C. Fernandez and XX anonymous reviewers for critical comments on a previous version of this manuscript.

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Figure 1. Rank-abundance plots of all 201 (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). See Table S2 for additional details.

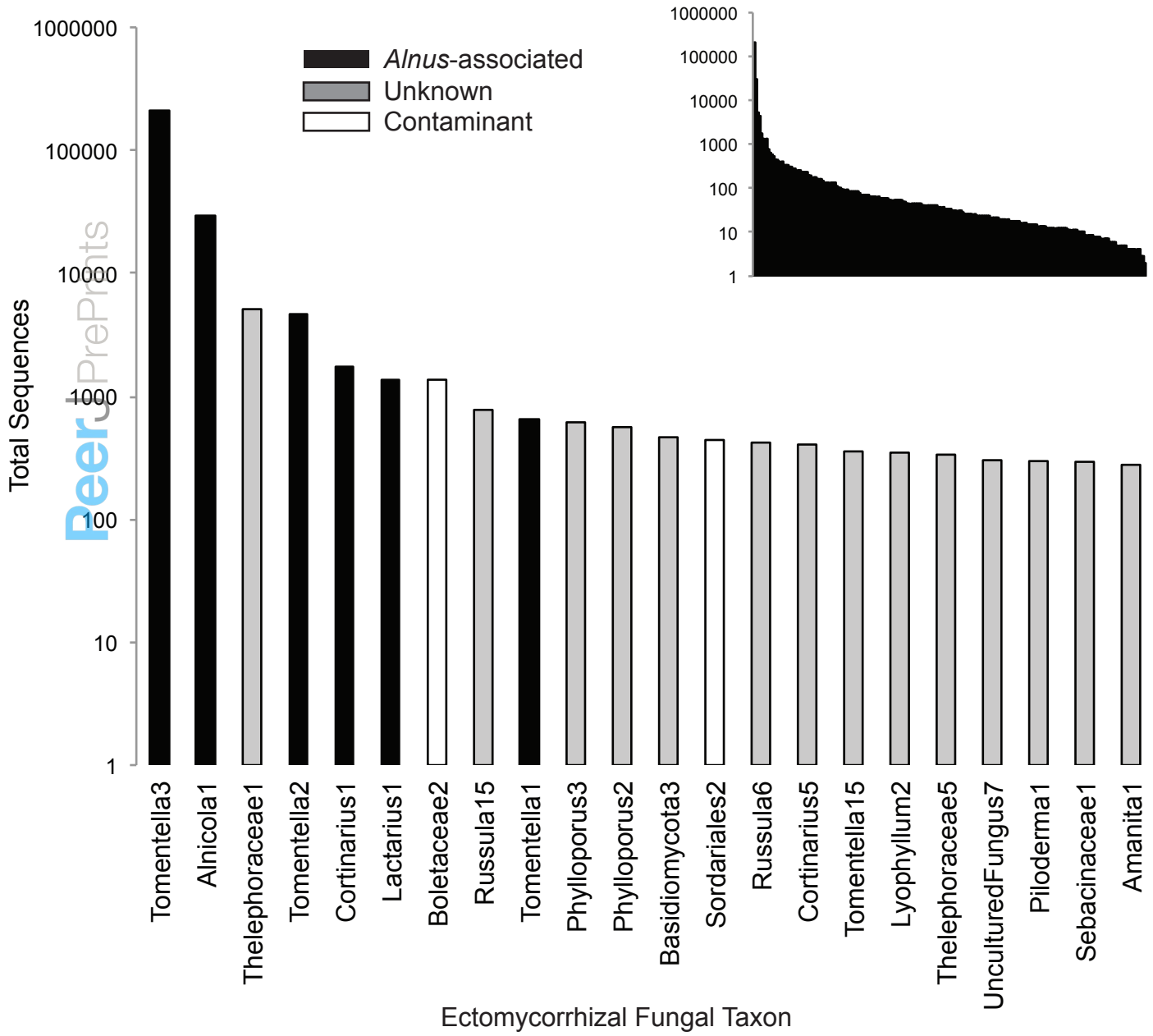

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Figure 2. Rank-abundance (A) and rank-frequency (B) plots of *Alnus*-associated ectomycorrhizal fungal taxa sampled in mesh bags and root tips.

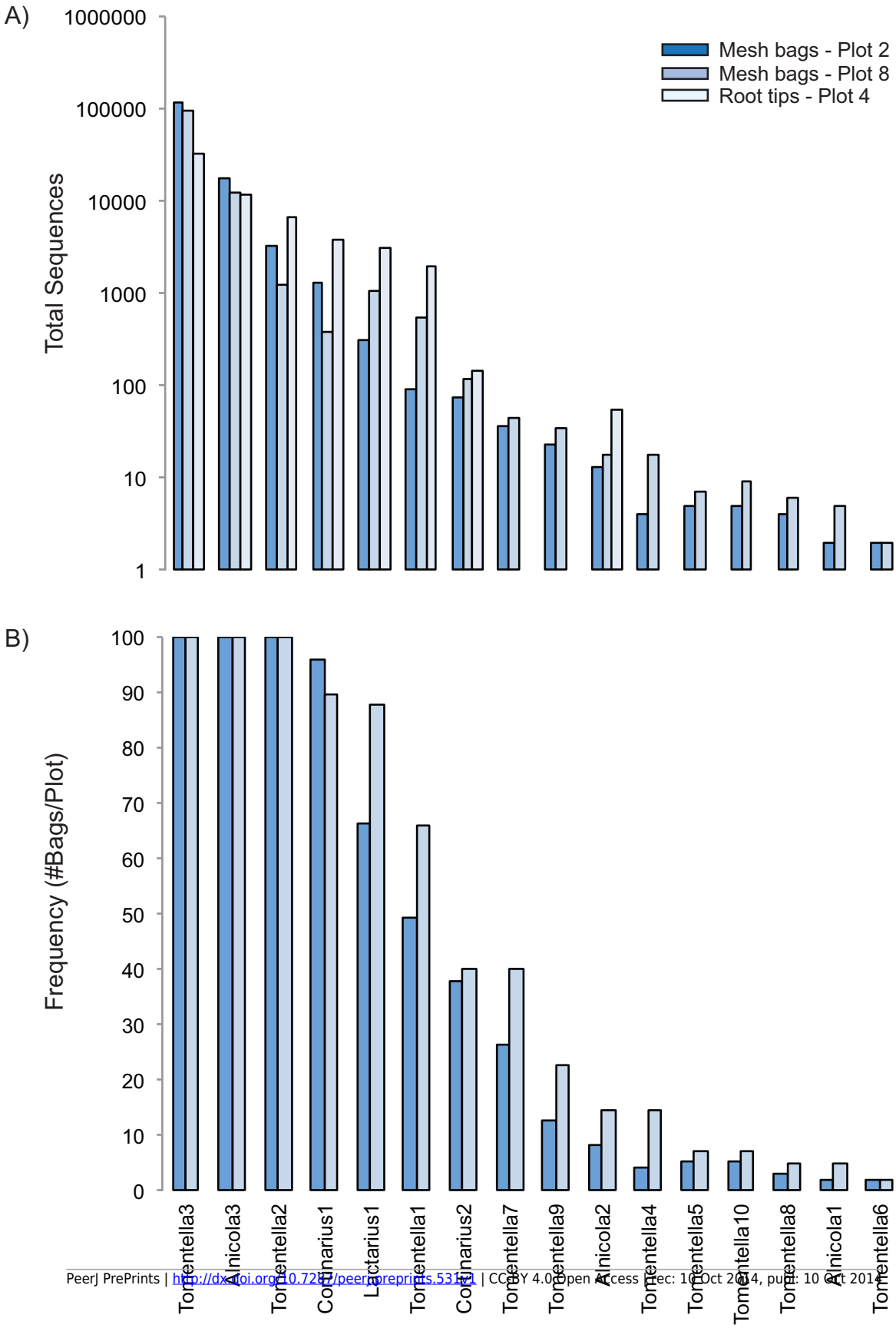

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Table 1. C-score taxon occurrence analyses of ECM fungal communities in Plots 2 and 8. See methods for details about datasets and null matrix type definitions. SES = Standardized Effect Size.

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	-0.2
		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

Table 2. Spearman rank correlation coefficient matrices for ECM fungal communities in Plots 2 and 8. Significant correlations are indicated in bold.

Plot2	Tomentella3	Alnicola1	Tomentella2	Cortinarius1	Lactarius1	Tomentella1	Cortinarius2	Tomentella7	Tomentella9	Alnicola2	Tomentella4	Tomentella5	Tomentella10	Tomentella8	Alnicola3	Tomentella6
Tomentella3	1.00															
Alnicola1	0.00	1.00														
Tomentella2	-0.02	0.00	1.00													
Cortinarius1	-0.05	-0.07	0.01	1.00												
Lactarius1	-0.07	-0.07	-0.02	0.28	1.00											
Tomentella1	-0.08	0.09	0.00	-0.13	-0.06	1.00										
Cortinarius2	-0.13	0.11	0.01	-0.05	0.00	0.26	1.00									
Tomentella7	0.16	-0.08	**0.65**	-0.03	0.08	-0.04	0.10	1.00								
Tomentella9	0.11	**0.48**	-0.06	-0.05	0.00	0.12	0.06	-0.02	1.00							
Alnicola2	0.07	**0.42**	0.07	-0.05	-0.04	-0.02	-0.10	0.02	0.09	1.00						
Tomentella4	-0.08	-0.04	**0.40**	-0.01	0.18	0.01	0.00	0.26	0.00	0.04	1.00					
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				
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			
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		
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	
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	Tomentella3	Alnicola1	Tomentella2	Cortinarius1	Lactarius1	Tomentella1	Cortinarius2	Tomentella7	Tomentella9	Alnicola2	Tomentella4	Tomentella5	Tomentella10	Tomentella8	Alnicola3	Tomentella6
Tomentella3	1.00															
Alnicola1	0.13	1.00														
Tomentella2	-0.17	-0.12	1.00													
Cortinarius1	0.03	-0.03	0.26	1.00												
Lactarius1	-0.16	-0.09	0.14	0.14	1.00											
Tomentella1	-0.14	0.03	0.04	0.04	**0.46**	1.00										
Cortinarius2	0.06	**0.45**	-0.04	-0.02	-0.05	-0.06	1.00									
Tomentella7	-0.09	-0.06	-0.06	0.14	0.11	0.02	0.05	1.00								
Tomentella9	-0.12	**0.47**	0.02	-0.01	0.10	0.16	0.28	0.06	1.00							
Alnicola2	-0.06	0.15	0.02	0.12	-0.04	0.03	0.08	0.13	0.07	1.00						
Tomentella4	-0.05	-0.07	0.15	0.12	0.14	-0.02	-0.09	0.18	0.02	0.07	1.00					
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				
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			
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		
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	
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