Structural diversity across arbuscular mycorrhizal, ectomycorrhizal, and endophytic plant–fungus networks

Hirokazu Toju[1,2*], Hirotoshi Sato[3], Satoshi Yamamoto[4], Akifumi S. Tanabe[5]

[*]Correspondence: toju.hirokazu.4c@kyoto-u.ac.jp

[1]Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113, Japan

[2]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

[3]Graduate School of Human and Environmental Studies, Kyoto University, Sakyo, Kyoto 606-8501, Japan

[4]Graduate School of Science, Kyoto University, Kitashirakawa-oiwake-cho, Kyoto 606-8502, Japan

[5]Faculty of Science and Technology, Ryukoku University, 1-5 Yokotani, Seta Oe-cho, Otsu, Shiga 520-2194, Japan

This article includes 7 Additional files (Data S1-7).

Abstract

Background: Below-ground linkage between plant and fungal communities is one of the major drivers of terrestrial ecosystem dynamics. However, we still have limited knowledge of how such plant–fungus associations vary in their community-scale properties depending on fungal functional groups and geographic locations.

Methods: Based on high-throughput sequencing of root-associated fungi in forest ecosystems, we performed a comparative analysis of arbuscular mycorrhizal, ectomycorrhizal, and saprotrophic/endophytic associations across a latitudinal gradient from cool-temperate to subtropical regions.

Results: In most of the plant–fungus networks analyzed, host–symbiont associations were significantly specialized but lacked "nested" architecture, which has been commonly reported in plant–pollinator and plant–seed disperser networks. Meanwhile, the structure of arbuscular mycorrhizal networks was differentiated from that of ectomycorrhizal and saprotrophic/endophytic networks, characterized by high connectance. Our data also suggested that geographic factors affected the organization of plant–fungus network structure. For example, the southernmost subtropical site analyzed in this study displayed lower network-level specificity of host–symbiont associations and higher (but still low) nestedness than northern localities.

Conclusions: Our comparative analyses suggest that arbuscular mycorrhizal, ectomycorrhizal, and saprotrophic/endophytic plant–fungus associations often lack nested network architecture, while those associations can vary, to some extent, in their community-scale properties along a latitudinal gradient. Overall, this study provides a basis for future studies that will examine how different types of plant–fungus associations collectively structure terrestrial ecosystems.

Keywords: biodiversity; community ecology; competitive exclusion; host specificity or preference; latitudinal gradients; microbiomes; plant–fungus interactions; plant–soil feedback; species coexistence; mycorrhizal and endophytic symbiosis

Background

Fungi in the below-ground biosphere are key drivers of terrestrial ecosystem processes [1-4]. Mycorrhizal fungi are considered to support land plants not only by provisioning soil nitrogen and phosphorous [5, 6] but also by increasing plants' resistance to biotic/abiotic stress [7, 8]. Pathogenic fungi in the soil affect the survival/mortality of young plants in a major way, possibly determining spatial distributions of plant species within forest/grassland ecosystems [9, 10]. Moreover, recent mycological studies have begun to examine the poorly explored diversity of endophytic fungi, which can enhance the nutritional conditions and pathogen resistance of mycorrhizal and non-mycorrhizal plant species [11-14]. Thus, terrestrial biomes consist of multiple layers of below-ground plant–fungus interactions [15]. Nonetheless, we still have limited knowledge of the structure of such complex webs of interactions, leaving major processes in below-ground ecosystems poorly explored.

In enhancing our understanding of community- or ecosystem-level processes of below-ground plant–fungus interactions, analyses on community-scale properties of such host–symbiont associations provide essential insights. For example, if a pathogenic fungal community consists mainly of species with narrow host ranges, it as a whole is expected to restrict the emergence of dominant plant species through "negative plant–soil feedback", contributing to the maintenance of plant species diversity within an ecosystem [16-18]. In contrast, with a high proportion of mycorrhizal fungi with narrow host ranges, their specific host species, such as Pinaceae plants hosting Suillaceae ectomycorrhizal fungi [19], will dominate the plant community through positive plant–soil feedback [18, 20]. Meanwhile, endophytic and arbuscular mycorrhizal fungi with broad host ranges [21-23] may diminish such negative and positive feedback by interlinking otherwise compartmentalized ecological dynamics (but see [24]). Therefore, concomitant analyses of community-scale properties of those multiple plant–fungus associations are of particular importance in understanding how plant–soil feedbacks organize terrestrial ecological processes.

Since the application of network science to ecology and mycology, researchers have evaluated the architecture of networks that represent linkage between plant and fungal

communities [25]. Those studies have shown that arbuscular mycorrhizal [26-28], ectomycorrhizal [29], and ericaceous [30] plant–fungus networks exhibit moderate or low levels of host–symbiont specificity, while they are structured to avoid overlap of host plant ranges within fungal communities. In addition, many of those plant–fungus networks [15, 29, 31] are known to lack "nested" architecture (i.e., structure of networks wherein specialist species interact with subsets of partners of generalist species [32]), which has been commonly reported in above-ground networks of plant–pollinator and plant–seed-disperser interactions [32-34] (but see [35]). However, in those previous studies, data of different types of plant–fungus networks have been collected from different geographic localities with different sampling strategy, precluding the chance of simultaneously evaluating the effects of interaction type and geographic factors. Although comparative studies of published data provide invaluable insights [25], compiled data often vary in the molecular markers used and they may differ in appropriate null model assumptions in statistically examining network topological properties.

In this study, we compared community-scale properties of arbuscular-mycorrhizal, ectomycorrhizal, and endophytic associations across eight forest sites spanning from cool-temperate to subtropical regions in Japan. Based on high-throughput sequencing of root-associated fungi, we obtained network data depicting how multiple plant species are associated with respective functional groups of fungi in each of the eight forests. We then examined how structure varied depending on categories of plant–fungus associations and geographic locations. Overall, this study provides a first step for integrating insights into community-scale properties of multiple types of below-ground plant–fungus associations and their ecosystem-level consequences.

Methods

Terminology

In analyzing metadata of community-scale properties of plant–fungus associations, we need to use consistent terminology that can be applied to a wide range of host–symbiont

102 associations. While plant–fungus network properties have been compared within a single
103 functional group of fungi (e.g., arbuscular mycorrhizal or ectomycorrhizal fungi) in most
104 studies, we herein target not only arbuscular mycorrhizal and ectomycorrhizal fungi but also
105 pathogenic and saprotrophic/endophytic fungi. Given that those functional groups of fungi
106 vary considerably in their microscopic structure within plant tissue [8], developing a general
107 criterion for mutualistic/antagonistic interactions with host plants is impossible. Thus, we
108 targeted all the fungi detected by high-throughput sequencing and the data described below
109 could contain not only mutualistic/antagonistic fungi but also commensalistic fungi merely
110 adhering to plant roots [36]. In this sense, our data represented symbiotic relationships in the
111 broad sense, i.e., intimate physical connections between organisms [15, 37].

112

113 **Sampling**

114 We collected root samples at eight forest sites (four cool-temperate, one warm-temperate, and
115 three subtropical forests) across the entire range of the Japanese Archipelago (45.042–24.407
116 ºN; Fig. 1A; Additional file 1: Data S1). In each forest, 2-cm segment of terminal roots were
117 collected from 3-cm below the soil surface at 1-m horizontal intervals. In each forest, 383
118 terminal root samples were collected. Those roots were collected indiscriminately regarding
119 root morphology or apparent mycorrhizal type so that the samples as a whole represented the
120 relative frequency of plant–fungal associations in the horizon in each forest [38]. Therefore,
121 while the sample sets consisted mainly of woody plants, they also included herbaceous plants
122 (Additional file 2: Data S2). Each root sample was preserved in 70% ethanol and stored at -25
123 ºC until DNA extraction.

124

125 **Molecular analysis**

126 Each root sample was placed in 70% ethanol with 1-mm zirconium balls and it was then
127 shaken at 15 Hz for 2 min with a TissueLyser II (Qiagen) [21]. The washed roots were
128 subsequently pulverized by shaking with 4-mm zirconium balls at 25 Hz for 3 min. DNA

129 extraction was then performed with a cetyltrimethylammonium bromide method [39].

130 The internal transcribed spacer 1 (ITS1) region of root-associated fungi was amplified
131 with the primers ITS1F_KYO1 and ITS2_KYO2, which target not only Ascomycota and
132 Basidiomycota fungi but also diverse non-Dikarya (e.g., Glomeromycota) taxa [40]. We used
133 the forward primer ITS1F-KYO1 fused with 3–6-mer Ns for improved Illumina sequencing
134 quality [41] and the forward Illumina sequencing primer (5'- TCG TCG GCA GCG TCA
135 GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [ITS1-KYO2] -3') and the reverse primer
136 ITS2-KYO2 fused with 3–6-mer Ns and the reverse sequencing primer (5'- GTC TCG TGG
137 GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [ITS2_KYO2] -3'). The
138 DNA polymerase system of KOD FX Neo (Toyobo) was used with a temperature profile of
139 94 ºC for 2 min, followed by 35 cycles at 98 ºC for 10 s, 50 ºC for 30 s, 68 ºC for 50 s, and a
140 final extension at 68 ºC for 5 min. The ramp rate was set to 1 ºC/sec to prevent the generation
141 of chimeric sequences [42]. Illumina sequencing adaptors were then added to each sample in
142 the subsequent PCR using the forward primers consisting of the P5 Illumina adaptor, 8-mer
143 tags for sample identification [43], and a partial sequence of the sequencing primer (5'- AAT
144 GAT ACG GCG ACC ACC GAG ATC TAC AC - [8-mer index] - TCG TCG GCA GCG TC
145 -3') and the reverse primers consisting of the P7 adaptor, 8-mer tags, and a partial sequence of
146 the sequencing primer (5'- CAA GCA GAA GAC GGC ATA CGA GAT - [8-mer index] -
147 GTC TCG TGG GCT CGG -3'). In the reaction, KOD FX Neo was used with a temperature
148 profile of 94 ºC for 2 min, followed by 8 cycles at 98 ºC for 10 s, 55 ºC for 30 s, 68 ºC for 50
149 s, and a final extension at 68 ºC for 5 min. The PCR amplicons of 384 samples in each forest
150 (including one PCR negative control) were pooled with equal volume after a
151 purification/equalization process with AMPureXP Kit (Beckman Coulter).

152 For the identification of plants, another set of PCR was performed targeting chloroplast
153 *rbcL* region with rbcL_F3 and rbcL_R4 primers [38]. The fusion primer design, DNA
154 polymerase system, temperature profiles, and purification processes used in the *rbcL* analysis
155 were the same as those of the fungal ITS analysis. The ITS and *rbcL* libraries were processed
156 in two Illumina MiSeq runs, in each of which samples of four forest sites were combined (run
157 center: KYOTO-HE) (2 × 250 cycles; 15% PhiX spike-in).

158

159 **Bioinformatics**

160 In total, 17,724,456 and 17,228,848 reads were obtained for the first and second MiSeq runs.
161 The raw sequencing data were converted into FASTQ files using the program bcl2fastq 1.8.4
162 provided by Illumina. The FASTQ files were then demultiplexed using the program Claident
163 v0.2.2016.07.05 [44, 45]. To avoid possible errors resulting from low-quality index sequences,
164 the sequencing reads whose 8-mer index positions included nucleotides with low (< 30)
165 quality scores were discarded in this process. As reverse sequences output by Illumina
166 sequencers have lower quality values than forward sequences, we used only forward
167 sequences after removing low-quality 3'-ends using Claident (sequencing data deposit: DDBJ
168 DRA accession: DRA006339). Noisy reads were subsequently discarded and the reads that
169 passed the filtering process were clustered using VSEARCH [46] as implemented in Claident.
170 The threshold sequencing similarities in the clustering were set to 97% for fungal ITS and
171 98% for *rbcL*, respectively. While sequence similarity values have been set to 97% in most
172 ITS analyses of Ascomycota and Basidiomycota fungi [47] (see also [48]), a recent study
173 showed that Glomeromycota fungi generally had much higher intraspecific ITS-sequence
174 variation than Dikarya fungi [49]. Therefore, we performed an additional clustering analysis
175 with a 94% cutoff similarity for defining Glomeromycota OTUs. Note that changing cut-off
176 similarities (81–97%) did not qualitatively change statistical properties of plant–fungus
177 network structure in a previous study [15]. The taxonomic assignment of the OTUs
178 (Additional files 3-4: Data S3-4) was conducted based on the combination of the
179 query-centric auto-*k*-nearest neighbor (QCauto) method [44] and the lowest common ancestor
180 (LCA) algorithm [50] as implemented in Claident. Note that taxonomic identification results
181 based on the QCauto–LCA approach were comparable to, or sometimes more accurate than,
182 those with the alternative approach combining the UCLUST algorithm [51] with the UNITE
183 database [52] [see [30] and [53] for detailed comparison of the QCautoLCA and UCLUST–
184 UNITE approaches]. The functional group of each fungal OTU was inferred using the
185 program FUNGuild 1.0 [54]. For 44.1 % (3560/8080) of fungal OTUs, functional group
186 information was inferred (Additional file 1: Data S1).

187 The obtained information of *rbcL* OTUs was used to identify each root sample, although
188 species-level taxonomic information was unavailable for some plant taxa in each forest due to
189 the relatively low variability of the chloroplast region [55]. Thus, we also used the
190 information of the ITS sequencing libraries, which included not only fungal but also host
191 plant sequencing reads: there were plant taxa that could not be identified to species even with
192 ITS information. Based on the *rbcL* and ITS information of plant sequences, possibly
193 contaminated samples were removed from the dataset.

194 For each of the eight forests, we then obtained a sample (row) × fungal OTU (column)
195 data matrix, in which a cell entry depicted the number of sequencing reads of an OTU in a
196 sample. The cell entries whose read counts represented less than 0.1% of the total read count
197 of each sample were subsequently excluded because those rare entries could derive from
198 contaminations from soil or PCR/sequencing errors [56]. The filtered matrices were then
199 rarefied to 1,000 reads per sample using the "rrarefy" function of the vegan 2.4-3 package
200 [57] of R 3.4.1 [58]. As the number of samples with 1,000 or more reads varied among the
201 eight forests examined (240–288 samples), it was equalized by randomly sampling 240
202 samples without duplication in each forest ("sample-level matrices"; Additional file 2: Data
203 S2).

204 Based on the sample-level matrices, we obtained another type of matrices, in which a cell
205 indicated the number of samples representing associations between a plant species/taxa (row)
206 and a fungal OTU (column) ("species-level matrices"; Additional file 5: Data S5). In addition
207 to the matrix indicating associations between all fungal OTUs and their host plants (ALL), a
208 series of partial network matrices representing respective fungal functional groups were
209 obtained by selecting arbuscular mycorrhizal (AM), ectomycorrhizal (ECM), potentially
210 pathogenic (PATHO), and saprotrophic/endophytic (SAPENDO) fungal OTUs in each forest
211 (Additional file 6: Data S6). Due to the limited availability of information of fungal ecology,
212 functional groups of many fungal OTUs could not be estimated and there were only 9–25
213 fungal OTUs inferred to be plant pathogens in respective forests (Additional files 1 and 5:
214 Data S1 and S5).

215

216 **Data analysis**

217 Based on the sample-level matrices, relationship between the number of samples and that of
218 observed fungal OTUs was analyzed for each forest using the "specaccum" function of the
219 vegan package. The community-scale plant–fungus associations represented by the
220 species-level matrices ("ALL" network matrices; Additional file 5: Data S5) were visualized
221 using the program GePhi 0.9.1 [59] with "ForceAtlas2" layout algorithm [60]. We then
222 analyzed the statistical properties of the ALL networks and partial networks (Additional file
223 6: Data S6) in terms of the H_2' metric of network-level interaction specificity [61], which has
224 been frequently used to measure the degree of interaction specificity in host–symbiont
225 networks [62, 63]. The plant–fungus associations were evaluated also by the weighted NODF
226 metric [64] of network nestedness [32], which measures the degree to which specialists
227 (species with narrow partner ranges) interact with partners of generalists (species with broad
228 partner ranges) in the same guild or trophic level. We further examined how host plant ranges
229 were differentiated within the fungal community of each forest based on checkerboard scores
230 [65]: a high/low score of the checkerboard index indicates host differentiation/overlap within
231 a guild or trophic level [63]. Although modularity is another important index frequently used
232 in ecological network studies [33], its computation was too time-consuming to be applied to
233 randomization analyses (see below) of our present datasets consisting of more than 1,000
234 fungal OTUs and their host plants. Note that we previously found that below-ground plant–
235 fungal associations generally showed statistically significant but low network modularity [15,
236 30, 63].

237 As estimates of network indices could vary depending on species compositions of
238 examined communities, we standardized the indices as

239 $$\text{relative index value} = [I_{\text{observed}} - \text{mean}(I_{\text{randomized}})] / \text{SD}(I_{\text{randomized}})$$

240 where I_{observed} was the index estimate of the observed data matrix, and mean($I_{\text{randomized}}$) and
241 SD($I_{\text{randomized}}$) were the mean and standardized deviation of the index values of randomized
242 matrices [63]. Randomized matrices were obtained by shuffling host-plant labels in the

243 sample-level matrices and subsequently converting the randomized sample-level matrices into
244 randomized species-level matrices. Although we used two additional methods ["r2dtable"
245 [66] and "vaznull" [67] methods] of matrix randomization in our previous studies of plant–
246 fungus networks [15, 63], they were too time-consuming to be used in the present large
247 datasets: note that the three randomization methods compared in those previous studies
248 yielded qualitatively similar results [15, 63]. The number of randomizations was set to 1,000
249 for H_2'/nestedness analyses and 100 for checkerboard-score analyses, which required
250 substantial computing time.

251 Based on the network indices, we examined how the community-scale properties of the
252 plant–fungus associations varied among local forests and fungal functional groups. For each
253 of interaction specificity (relative H_2'), nestedness (relative weighted NODF nestedness), and
254 checkerboard index (relative checkerboard values), an ANOVA model was constructed by
255 incorporating locality (forest sites), fungal functional group, number of plant species/taxa,
256 number of fungal OTUs, and network connectance (the proportion of non-zero entries in
257 community matrices) as explanatory variables. The variation in the plant–fungus network
258 properties was visualized based on a principal component analysis based on a correlation
259 matrix: the variables included were H_2' interaction specificity, NODF nestedness,
260 checkerboard index, number of plant species/taxa, number of fungal OTUs, proportion of
261 fungal OTUs to plant species/taxa, and connectance.

262

263 **Results**

264 Total fungal OTU richness was higher in warm-temperate and subtropical forests than in
265 cool-temperate forests (Figs. 1b and 2). The OTU richness of AM fungi was higher in the
266 three subtropical forests, while that of ECM fungi decreased in the southern forests (Fig. 3a).
267 The ratio of the total number of fungal OTUs to the number of plant species/taxa varied
268 among forests, although there was no systematic variation between cool-temperate and the
269 other (warm temperate and subtropical) localities (Fig. 3b). Connectance varied among forests
270 as well, while it was consistently higher in AM than in ALL, ECM, and SAPENDO

271 networks/partial networks in seven of the eight study forests (Fig. 3c). The connectance of
272 PATHO partial networks varied considerably among forests presumably due to low OTU
273 richness and the resultant uncertainty in index estimation.

274 The relative H_2' metric of interaction specificity significantly varied among forests but
275 not among fungal functional groups when the effects of plant diversity, fungal OTU richness,
276 and connectance were controlled in an ANOVA model (Table 1; Fig. 3d). The relative
277 nestedness of the ALL matrices of plant–fungus associations was lower than zero in most
278 forests but not in the southern most subtropical forest (Fig. 3e; Additional file 7: Data S7).
279 Overall, plant–fungus associations in ALL networks were more specialized (Fig. 3d) and less
280 nested (Fig. 3e) than those of partial networks. In addition, fungal OTUs in ALL networks
281 displayed stronger differentiation of host ranges than those in partial networks (Fig. 3f).

282 After taking into account plant and fungal diversity in an ANOVA model, neither locality
283 nor fungal functional group explained the variation in relative nestedness (Table 1). The
284 relative checkerboard scores varied among localities (Fig. 3f), although the effects of locality
285 were non-significant in an ANOVA model (Table 1). The ANOVA model showed that the
286 variation in relative checkerboard scores was explained, to some extent, by fungal functional
287 groups.

288 In the principal component analysis of network indices, ALL, PATHO and other
289 networks/partial networks were separated by the first principal component, which represented
290 high plant diversity, fungal OTU richness, relative H_2', and relative checkerboard scores as
291 well as low relative nestedness (Fig. 4a). By incorporating the third principal component,
292 which represented high fungal OTU richness and connectance, the cluster of AM partial
293 networks and that of ECM and SAPENDO partial networks were grouped with some overlap
294 (Fig. 4b).

295

296 **Discussion**

297 Our data, which included 17–55 plant species/taxa and more than 1000 fungal OTUs in each

298 of the eight forests, provided a novel opportunity to evaluate how different types of
299 below-ground plant–fungus associations varied in their community-scale characteristics along
300 a latitudinal gradient. We then found that network structural properties differed among
301 different types of plant–fungus associations (Fig. 3), while geographic factors contributed to
302 the variation found in network structure (Table 1). Specifically, arbuscular mycorrhizal
303 networks differed in their architecture from ectomycorrhizal and saprotrophic/endophytic
304 networks, characterized by high connectance (Fig. 4). We also found that networks consisting
305 of all functional groups of fungi and their host plants had higher network-level interaction
306 specificity, more differentiated host ranges between fungi, and lower network nestedness than
307 the partial networks of arbuscular mycorrhizal, ectomycorrhizal, and saprotrophic/endophytic
308 associations (Figs. 3 and 4). As in previous studies, our data included many fungal OTUs
309 unassigned to functional groups due to the paucity of the information of fungal functions and
310 guilds in databases [54]. However, by extending findings in previous plant–fungus network
311 studies [28, 29, 63], in which sampling strategies, interaction type, or geographic factors were
312 not controlled systematically, this study offers a basis for discussing how different types of
313 below-ground plant–fungus associations collectively build plant–soil feedback in terrestrial
314 ecosystems.

315 Among the network indices examined in this study, nestedness showed an idiosyncratic
316 tendency in light of other types of interaction networks examined in community ecology
317 [32-34]. We found that below-ground plant–fungus networks often displayed "anti-nested"
318 architecture, in which scores representing nested network structure were lower than those
319 expected by chance (i.e., negative values of relative nestedness; Fig. 3e), as suggested also in
320 previous studies [15, 29, 63]. Although factors organizing anti-nested network architecture
321 remain to be investigated, competition for host plants among fungal species has been inferred
322 to decrease nestedness of plant–fungus associations [63]. In addition, a previous comparative
323 study suggested that plant–fungus network nestedness decreased with increasing annual mean
324 temperature on a global scale [25].

325 The prevalence of anti-nested or non-nested network structures is in sharp contrast to
326 observations on other types of plant–partner networks, which commonly show statistically

327 significant nested architecture [32]. Specifically, plant–pollinator and plant–seed disperser
328 interactions are generally characterized by nested network architecture in which overlap of
329 partner ranges within the same guild are expected to mitigate competition between plant
330 species [32-34]. Therefore, anti-nested structure of plant–fungus networks provides important
331 insights in terms of community ecological theory linking network structure and species
332 coexistence [34, 35, 68]. Given that below-ground fungi constitute one of the most
333 species-rich components of the terrestrial biosphere [3], understanding community-scale
334 properties of below-ground plant–fungus associations is a major step for disentangling
335 relationship among network structure, species coexistence, and community stability.

336 To overcome the inconsistency between theory and observations, we may need to take
337 into account basic biology of below-ground plant–fungus associations. We here highlight two
338 backgrounds that need more attention for deepening discussion on ecological networks and
339 species coexistence. First, in contrast to plant–pollinator or plant–seed disperser networks,
340 which are often assumed to consist only of mutualistic interactions, below-ground plant–
341 fungus networks can involve not only mutualistic but also antagonistic and commensalistic
342 interactions. This diversity of interaction type can lead to high stability of below-ground
343 fungal and their host plant communities. Specifically, while communities consisting
344 exclusively of mutualistic interactions are inherently unstable [69], involvement of a small
345 fraction of antagonistic interactions in those communities can dramatically enhance species
346 coexistence [70]. Second, because fungi can disperse long distances as spores [71, 72] (but
347 see [73]), their local species richness (alpha diversity) may be greatly impacted by
348 metacommunity processes [74]. Interestingly, a recent theoretical study on food webs
349 predicted that strong coupling of local communities within a metacommunity could result in
350 positive relationship between species richness and community stability [75]. Such theoretical
351 evaluation of metacommunity dynamics has been extended to systems involving mutualistic
352 interactions [76], providing platforms for considering how dispersal abilities of constituent
353 species determine local species richness/coexistence of different types of plant–partner
354 networks.

355 To apply a standardized criterion for plant–fungus associations, we did not perform any

356 data screening based on the "mycorrhizal types" of plant species. As a result, our data
357 included plant–fungus combinations that could not be classified into well-recognized
358 categories of mycorrhizal symbioses [8]. For example, ectomycorrhizal fungi were detected
359 not only from plant species in "ectomycorrhizal" families (e.g., Fagaceae, Pinaceae, and
360 Betulaceae) but also from other plant species (Fig. 2; Additional file 6: Data S6). In addition,
361 the data included network links between arbuscular mycorrhizal fungi and ectomycorrhizal
362 plant species (Additional file 6: Data S6) as reported previously [77]. Although such plant–
363 fungus associations that do not fall into classic categories of mycorrhizal symbioses seldom
364 attract attention and they are often removed from high-throughput sequencing datasets, some
365 of them may represent important ecological interactions. An ectomycorrhizal fungus in the
366 truffle genus (*Tuber melanosporum*), for instance, is known to cause severe necrosis in root
367 cortices of non-ectomycorrhizal herbaceous plants [78]. Thus, for the standardization of
368 plant–fungus network analyses inferred with high-throughput sequencing, we need to take
369 into account the possibility that network links can represent not only mutualistic but also
370 neutral and antagonistic interactions [15]. Given also that even well-known combinations of
371 plant–fungus mycorrhizal interactions can result in antagonistic interaction depending on soil
372 environmental conditions and host plant nutrition [79, 80], potential diversity of ecological
373 interactions within a network and its community-scale consequences [70] deserve intensive
374 research.

375 Our community-scale comparative analysis targeting a broad latitudinal range from
376 cool-temperate to subtropical regions has some implications for geographic diversity patterns
377 of plant-associated fungi, although careful interpretation is required given the small number
378 of study sites. The number of detected ectomycorrhizal fungal OTUs was lower in subtropical
379 than in temperate forests (Fig. 3a), presumably reflecting geographic variation in the relative
380 abundance of Fagaceae, Pinaceae, and Betulaceae plants in plant communities as discussed in
381 previous studies [81-84] (see also [85]). In contrast, the number of arbuscular mycorrhizal
382 fungal OTUs increased towards south in our data, while a previous meta-analysis detected no
383 latitudinal diversity gradient regarding the fungal functional group [86] (see also [87]). The
384 total number of fungal OTUs was also higher in subtropical forests, peaked in the

southernmost site. Interestingly, unlike other study sites, the southernmost sampling site was characterized by low levels of network-scale interaction specificity and host plant differentiation as well as by the absence of anti-nested network architecture. Although some pioneering studies have investigated host preferences of tropical fungi [88-90], it remains a major challenge to examine whether the observed latitudinal gradient in plant–fungus network structure is extended to tropical regions.

Conclusions

Based on the large datasets of root-associated fungi, we herein showed how plant–fungus network architecture vary across a latitudinal gradient across the Japanese Archipelago. For further understanding the diversity of below-ground pant–fungus associations, more comparative studies of community-scale characteristics are required especially in the tropics. Moreover, further data of networks consisting of pathogenic fungi and their host plants are awaited to discuss community-scale properties of negative plant–soil feedbacks [91]. Given that the number of pathogenic fungi included in our present analysis was too few to evaluate statistical features of their networks, selective sampling of pathogen infected plant individuals may be necessary. Improving reference databases of fungal functions is also an important challenge towards better understanding of the roles of fungal communities. More macroecological studies of plant–fungus interactions [73, 92, 93], along with experimental studies testing functions of poorly characterized fungi [11, 13, 14], will reorganize our knowledge of terrestrial ecosystem processes.

Abbreviations

ANOVA: analysis of variance; DDBJ: DNA Data Bank of Japan; ITS: internal transcribed spacer; LCA: lowest common ancestor; OTU: Operational taxonomic unit; QCauto method: query-centric auto-*k*-nearest neighbor method.

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Availability of data and materials

The Illumina sequencing data were deposited to DNA Data Bank of Japan (DDBJ Sequence Read Archive: DRA006339). The raw data of fungal community structure and the fungal community matrices analyzed are available as Additional files 1-6.

Authors' contributions

HT designed the work. HT, HS, SY, and AST conducted fieldwork. HT, HS, and SY performed the molecular experiments. HT wrote the manuscript with HS, SY, and AST.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

Additional files

Additional file 1: Data S1. Information of study sites, taxonomic and functional-group information of the fungal OTUs detected.

Additional file 2: Data S2. Sample-level matrices of plant–fungus associations.

Additional file 3: Data S3. Sequences of the non-glomeromycete fungal OTUs detected.

Additional file 4: Data S4. Sequences of the glomeromycete fungal OTUs detected.

Additional file 5: Data S5. Species-level matrices of plant–fungus associations.

Additional file 6: Data S6. Network data matrices.

Additional file 7: Data S7. Results of the randomization analysis.

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688
689

690 **Table 1** Potential factors contributing to variation in plant–fungus network structure. For each
691 response variable representing network structure, an ANOVA model including the number of
692 plant species/taxa, that of fungal OTUs, network connectance, sampling locality, and fungal
693 functional groups (i.e., categories of plant–fungus networks) was constructed. *P* values
694 significant after a Bonferroni correction are shown in bold for ANOVA model.

695

Response variable	Explanatory variable	df	*F*	*P*
Relative interaction specificity	No. plant species/taxa	1	4.6	0.0426
	No. fungal OTUs	1	96.9	**< 0.0001**
	Connectance	1	10.1	**0.0040**
	Locality	7	4.0	**0.0048**
	Functional group	4	2.6	0.0572
Relative nestedness	No. plant species/taxa	1	5.1	0.0322
	No. fungal OTUs	1	39.3	**< 0.0001**
	Connectance	1	2.4	0.1308
	Locality	7	1.5	0.2216
	Functional group	4	2.1	0.1179
Relative checkerboard score	No. plant species/taxa	1	1.0	0.3182
	No. fungal OTUs	1	62.9	**< 0.0001**
	Connectance	1	5.6	0.0262
	Locality	7	1.3	0.2772
	Functional group	4	4.0	**0.0121**

696

697

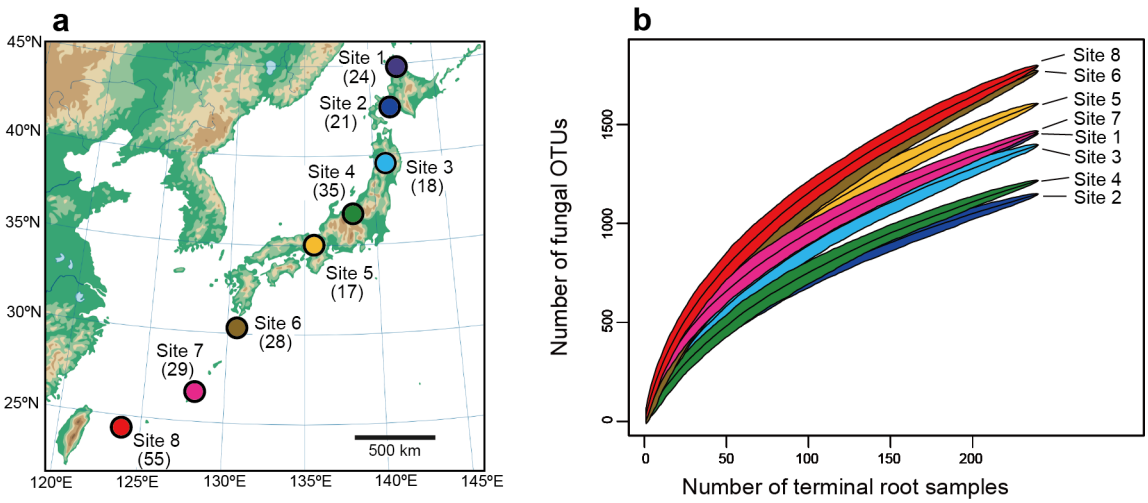
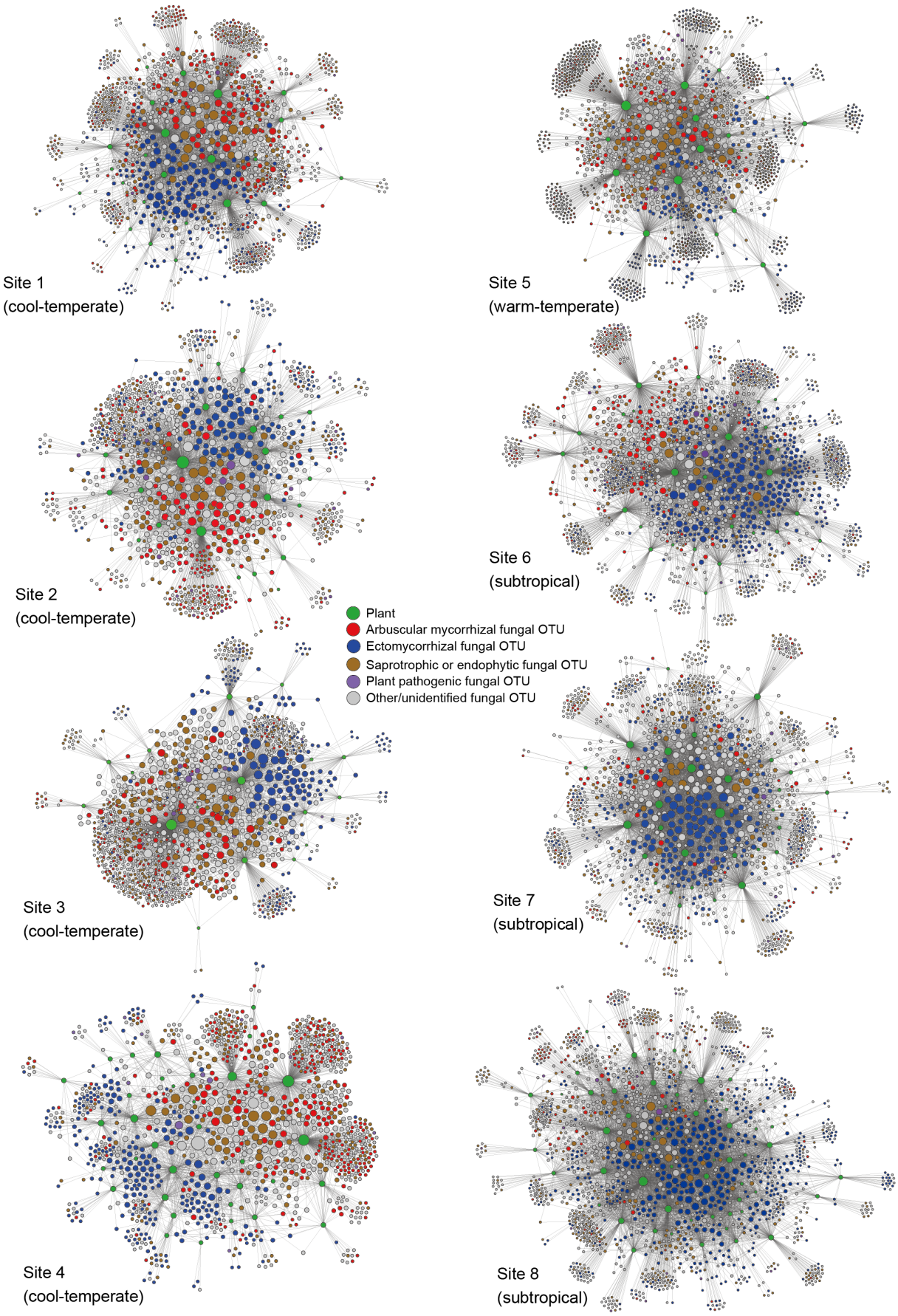


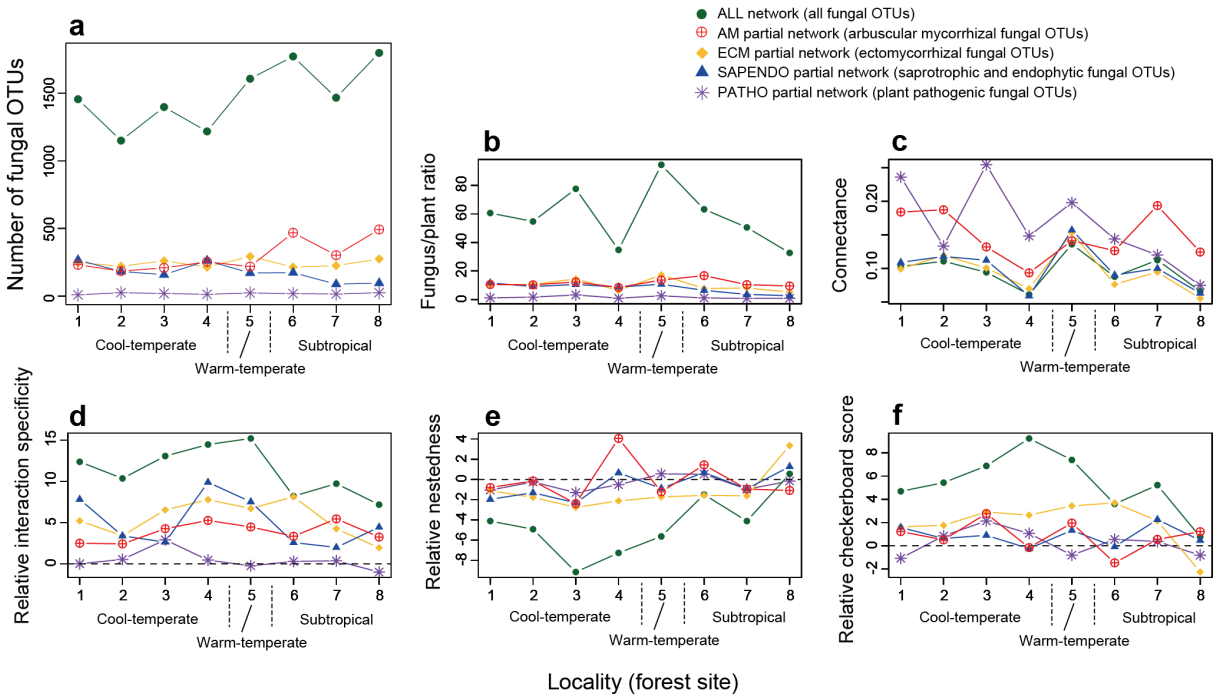
Fig. 1 Study sites. **a** Map of study sites. In each forest site, a number in a parenthesis indicates the number of plant species/taxa observed in the 240 terminal root samples from which sequencing data were successfully obtained. **b** Relationship between the number of samples and that of plant species/taxa observed. A rarefaction curve obtained from 240 terminal-root samples is shown for each study site.


Site 1
(cool-temperate)
Site 2
(cool-temperate)
Site 3
(cool-temperate)
Site 4
(cool-temperate)
Site 5
(warm-temperate)
Site 6
(subtropical)
Site 7
(subtropical)
Site 8
(subtropical)
Plant
Arbuscular mycorrhizal fungal OTU
Ectomycorrhizal fungal OTU
Saprotrophic or endophytic fungal OTU
Plant pathogenic fungal OTU
Other/unidentified fungal OTU

706

707

Fig. 2 Below-ground plant–fungus networks. The "ALL" network involving all the root-associated fungal OTUs detected and their host plant species/taxa is shown for each forest. The OTUs/species in the networks are arranged with the "ForceAtlas2" layout algorithm [60]. Size of circles represents betweenness centrality scores compared within plant/fungal community.



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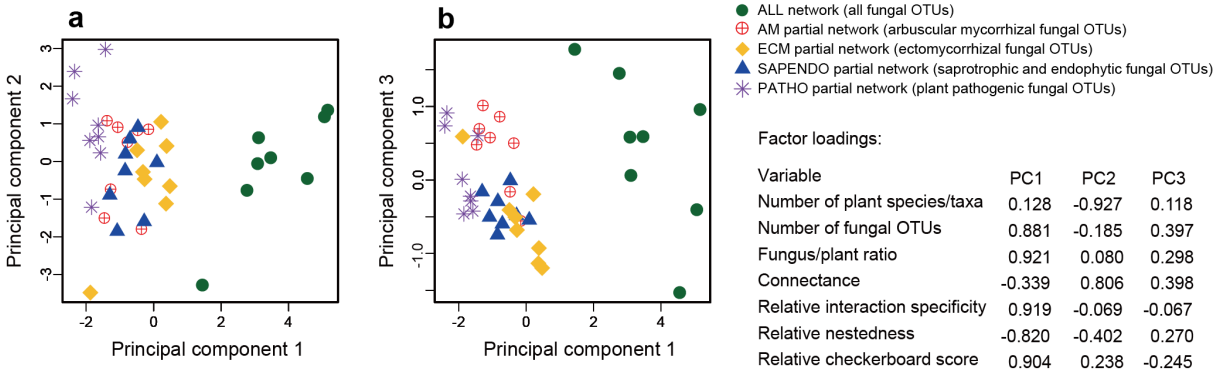
715

716 **Fig. 3** Network properties. The index scores representing the architecture of plant fungus
717 networks/partial networks are shown across the eight forests examined. **a** The number of
718 fungal OTUs. The code numbers of forest sites correspond to those shown in Figure 1. **b** The
719 ratio of the number of fungal OTUs to that of the plant species/taxa involved in each
720 network/partial network. **c** Connectance (the proportion of non-zero entries in a species-level
721 matrix). **d** Network-level interaction specificity (relative H_2'). **e** Nestedness (relative weighted
722 NODF nestedness). **f** Host range differentiation (relative checkerboard score). For relative
723 interaction specificity, relative nestedness, and relative checkerboard score (**d-f**), scores
724 higher/lower than 2 roughly indicate that observed network index values are higher/lower
725 than expected by chance (see Additional file 7: Data S7 for detailed results of the
726 randomization test).

727



Variable	PC1	PC2	PC3
Number of plant species/taxa	0.128	-0.927	0.118
Number of fungal OTUs	0.881	-0.185	0.397
Fungus/plant ratio	0.921	0.080	0.298
Connectance	-0.339	0.806	0.398
Relative interaction specificity	0.919	-0.069	-0.067
Relative nestedness	-0.820	-0.402	0.270
Relative checkerboard score	0.904	0.238	-0.245

Fig. 4 Principal component analysis of network properties. **a** Principal component 1 vs. principal component 2. Factor loadings of the examined variables are shown on the right. **b** Principal component 1 vs. principal component 3.