Network hubs in root-associated fungal metacommunities

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This article includes 5 Figures, 1 Table, and 9 additional files.

Abstract

Background: Although a number of recent studies have uncovered remarkable diversity of microbes associated with plants, understanding and managing dynamics of plant microbiomes remain major scientific challenges. In this respect, network analytical methods have provided a basis for exploring "hub" microbial species, which potentially organize community-scale processes of plant–microbe interactions.

Methods: By compiling Illumina sequencing data of root-associated fungi in eight forest ecosystems across the Japanese Archipelago, we explored hubs within "metacommunity-scale" networks of plant–fungus associations. In total, the metadata included 8,080 fungal operational taxonomic units (OTUs) detected from 227 local populations of 150 plant species/taxa.

Results: Few fungal OTUs were common across all the eight forests. However, in each metacommunity-scale network representing northern four localities or southern four localities, diverse mycorrhizal, endophytic, and pathogenic fungi were classified as "metacommunity hubs", which could associate with diverse host plant taxa throughout a climatic region. Specifically, *Mortierella* (Mortierellales), *Cladophialophora* (Chaetothyriales), *Ilyonectria* (Hypocreales), *Pezicula* (Helotiales), and *Cadophora* (incertae sedis) had broad geographic and host ranges across the northern (cool-temperate) region, while *Saitozyma*/*Cryptococcus* (Tremellales/Trichosporonales) and *Mortierella* as well as some arbuscular mycorrhizal fungi were placed at the central positions of the metacommunity-scale network representing warm-temperate and subtropical forests in southern Japan.

Conclusions: The network theoretical framework presented in this study will help us explore prospective fungi and bacteria, which have high potentials for agricultural application to diverse plant species within each climatic region. As some of those fungal taxa with broad geographic and host ranges have been known to increase the growth and pathogen resistance of host plants, further studies elucidating their functional roles are awaited.

Keywords: biodiversity; community ecology; host specificity or preference; latitudinal gradients; metacommunities; microbial inoculation; microbiomes; network hubs; plant–

fungus interactions; mycorrhizal and endophytic symbiosis.

Background

Below-ground fungi associated with plants in the endosphere and rhizosphere are key drivers of terrestrial ecosystem processes [1-4]. Mycorrhizal fungi, for example, are important symbionts of most land plant species, enhancing nutritional conditions and pathogen resistance of host plants [5-7]. In reward for the essential physiological services, they receive ca. 20% of net photosynthetic products from plants [8, 9]. Recent studies have also indicated that diverse taxonomic groups of endophytic fungi (e.g., endophytic fungi in the ascomycete orders Helotiales and Chaetothyriales) commonly interact with plant roots, providing soil nitrogen/phosphorous to their hosts [10-14], converting organic nitrogen into inorganic forms in the rhizosphere [15], and increasing plants' resistance to environmental stresses [16-18]. Because of their fundamental roles, below-ground fungi have been considered as prospective sources of ecosystem-level functioning in forest management, agriculture, and ecosystem restoration [17-20]. However, due to the exceptional diversity of below-ground fungi [21-23] and the extraordinary complexity of below-ground plant–fungus interactions [24-26], we are still at an early stage of managing and manipulating plant-associated microbiomes [27-29].

In disentangling complex webs of below-ground plant–fungus associations, network analyses, which have been originally applied to human relations and the World-Wide Web [30, 31], provide crucial insights. By using network analytical tools, we can infer how plant species in a forest, grassland, or farmland are associated with diverse taxonomic and functional groups of fungi [24, 32-34]. Such information of network structure (topology) can be used to identify "hub" species, which are placed at the center of a network depicting multispecies host–symbiont associations [35] (cf. [34, 36, 37]). Those hubs with broad host/symbiont ranges are expected to play key roles by mediating otherwise discrete ecological processes within a community [19, 24]. For example, although arbuscular mycorrhizal and ectomycorrhizal symbioses have been considered to involve distinct sets of plant and fungal lineages [38] (but see [39, 40]), hub endophytic fungi with broad host ranges may mediate indirect interactions between arbuscular mycorrhizal and ectomycorrhizal plant species through below-ground mycelial connections. As information of plant-associated fungal communities is now easily available with high-throughput DNA sequencing

technologies [1, 21, 22], finding hub microbial species out of hundreds or thousands of species within a network has become an important basis for understanding and predicting ecosystem-scale phenomena.

Nonetheless, given that fungi can disperse long distances with spores, conidia, and propagules [41-44], information of local-scale networks alone does not provide thorough insights into below-ground plant–fungus interactions in the wild. In other words, no forests, grasslands, and farmlands are free from perturbations caused by fungi immigrating from other localities [45-49]. Therefore, to consider how local ecosystem processes are interlinked by dispersal of fungi, we need to take into account "metacommunity-scale" networks of plant–fungus associations [35]. Within a dataset of multiple local communities (e.g., [25]), fungal species that occur in multiple localities may interlink local networks of plant–fungus associations. Among them, some species that not only have broad geographic ranges but also are associated with diverse host plant species would be placed at the core positions of a metacommunity-scale network [35]. Such "metacommunity hub" fungi would be major drivers of the synchronization and restructuring of local ecosystem processes (*sensu* [50]), and hence their functional roles need to be investigated with priority [35]. Moreover, in the screening of mycorrhizal and endophytic fungi that can be used in agriculture and ecosystem restoration programs [17, 20, 51], analytical pipelines for identifying metacommunity hubs will help us explore species that are potentially applied (inoculated) to diverse plant species over broad geographic ranges of farmlands, forests, or grasslands. Nonetheless, despite the potential importance of metacommunity hubs in both basic and applied microbiology, few studies have examined metacommunity-level networks of plant–symbiont associations.

By compiling Illumina sequencing datasets of root-associated fungi [52], we herein inferred a metacommunity-level network of below-ground plant–fungus associations and thereby explored metacommunity hubs. Our metadata consisted of plant–fungus association data in eight forest localities across the entire range of the Japanese Archipelago, including 150 plant species/taxa and 8,080 fungal operational taxonomic units (OTUs) in temperate and subtropical regions. Based on the information of local- and metacommunity-level networks, each of the fungal OTUs was evaluated in light of its topological positions. We then

105 examined whether fungal OTUs placed at the core of local-level plant–fungus networks could
106 play key topological roles within the metacommunity-level network. Overall, this study
107 uncover how diverse taxonomic groups of mycorrhizal and endophytic fungi can form
108 metacommunity-scale networks of below-ground plant–fungus associations, providing a basis
109 for analyzing complex spatial processes of species-rich host–microbe systems.

110

111 # Methods

112 ## Terminology

113 While a single type of plant–fungus interactions is targeted in each of most mycological
114 studies (e.g., arbuscular mycorrhizal symbiosis or ectomycorrhizal symbiosis), we herein
115 analyze the metadata including multiple categories of below-ground plant–fungus
116 associations [52]. Because arbuscular mycorrhizal, ectomycorrhizal, and endophytic fungi, for
117 example, vary in their microscopic structure within plant tissue [38], it is impossible to
118 develop a general criterion of mutualistic/antagonistic interactions for all those fungal
119 functional groups. Therefore, we used the phrase "associations" instead of "interactions"
120 throughout the manuscript when we discuss patterns detected based on the Illumina
121 sequencing metadata of root-associated fungi. Consequently, our results represented not only
122 mutualistic or antagonistic interactions but also neutral or commensalistic interactions [24, 53,
123 54]. Our aim in this study is to gain an overview of the metacommunity-scale plant–fungus
124 associations, while the nature of respective plant–fungus associations should be evaluated in
125 future inoculation experiments.

126

127 ## Data

128 We compiled the Illumina (MiSeq) sequencing data collected in a previous study [52], in
129 which community-scale statistical properties of below-ground plant–fungus associations were
130 compared among eight forest localities (four cool-temperate, one warm-temperate, and three
131 subtropical forests) across the entire range of the Japanese Archipelago (45.042–24.407 ºN;

132 Fig. 1) (DDBJ Sequence Read Archives accession: DRA006339). In each forest, 2-cm
133 segment of terminal roots were sampled from 3-cm below the soil surface at 1-m horizontal
134 intervals [52]. Those root samples were collected irrespective of their morphology and
135 mycorrhizal type: hence, the samples as a whole represented below-ground relative
136 abundance of plant species in each forest community. Based on the sequences of the genes
137 encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*) and the internal
138 transcribed spacer 1 (ITS1) of the ribosomal RNA region, host plant species were identified,
139 although there were plant root samples that could not be identified to species with the *rbcL*
140 and ITS1 regions [52].

141 The Illumina sequencing reads of the fungal ITS1 region were processed as detailed in
142 the data-source study [52]. The primers used were designed to target not only Ascomycota
143 and Basidiomycota but also diverse non-Dikarya (e.g., Glomeromycota) taxa [55]. In most
144 studies analyzing community structure of Ascomycota and Basidiomycota fungi, OTUs of the
145 ITS region are defined with a cut-off sequence similarity of 97% [22, 56, 57] (see also [58]).
146 Meanwhile, Glomeromycota fungi generally have much higher intraspecific ITS-sequence
147 variation than other taxonomic groups of fungi [59]. Consequently, we used 97% and 94%
148 cut-off sequence similarities for defining non-Glomeromycota and Glomeromycota fungal
149 OTUs, respectively [52]. The OTUs were then subjected to reference database search with the
150 query-centric auto-*k*-nearest-neighbor algorithm [60, 61] and subsequent taxonomic
151 assignment with the lowest common ancestor algorithm [62]. Based on the inferred taxonomy,
152 the functional group of each fungal OTU was inferred using the program FUNGuild 1.0 [63].

153 After a series of bioinformatics and rarefaction procedures, 1,000 fungal ITS reads were
154 obtained from each of the 240 samples collected in each forest locality (i.e., 1,000 reads × 240
155 samples × 8 sites). A sample (row) × fungal OTU (column) data matrix, in which a cell entry
156 depicted the number of sequencing reads of an OTU in a sample, was obtained for each local
157 forest ("sample-level" matrix) (Additional file 1: Data S1). Each local sample-level matrix
158 was then converted into a "species-level" matrix, in which a cell entry represented the number
159 of root samples from which associations of a plant species/taxa (row) and a fungal OTU
160 (columns) was observed: 17–55 plant species/taxa and 1,149–1,797 fungal OTUs were

161 detected from the local species-level matrices (Additional file 2: Data S2). In total, the
162 matrices included 150 plant species/taxa and 8,080 fungal OTUs (Additional file 3: Data S3).

163

164 **Local networks**

165 Among the eight forest localities, variation in the order-level taxonomic compositions were
166 examined with the permutational analysis of variance (PERMANOVA; [64]) and the
167 permutational analysis for the multivariate homogeneity of dispersions (PERMDISP; [65])
168 with the "adonis" and "betadisper" functions of the vegan 2.4-3 package [66] of R 3.4.1 [67],
169 respectively. The β-diversity values used in the PERMANOVA and PERMDISP analyses
170 were calculated with the "Bray-Curtis" metric based on the sample-level matrices (Additional
171 file 1: Data S1). Note that the "Raup-Crick" β-diversity metric [68], which controls
172 α-diversity in community data but requires computationally intensive randomization, was not
173 applicable to our large metadata. Geographic variation in the compositions of fungal
174 functional groups was also evaluated by PERMANOVA and PERMDISP analyses.

175 For each of the eight local forests, the network structure of below-ground plant–fungus
176 associations was visualized based on the species-level matrix (Additional file 2: Data S2)
177 using the program GePhi 0.9.1 [69] with the "ForceAtlas2" layout algorithm [70]. Within the
178 networks, the order-level taxonomy of fungal OTUs was highlighted.

179 To evaluate host ranges of each fungal OTU in each local forest, we first calculated the *d'*
180 metric of interaction specificity [71]. However, estimates of the *d'* metric varied considerably
181 among fungal OTUs observed from small numbers of root samples (Additional file 4; Figure
182 S1) presumably due to overestimation or underestimation of host preferences for those rare
183 OTUs. Therefore, we scored each fungal OTU based on their topological positions within
184 each local network by calculating network centrality indices (degree, closeness, betweenness,
185 and eigenvector centralities metrics of network centrality; [31]). Among the centrality metrics,
186 betweenness centrality, which measures the extent to which a given nodes (species) is located
187 within the shortest paths connecting pairs of other nodes in a network [72], is often used to
188 explore organisms with broad host or partner ranges [35]. Thus, in each local network, fungal

189 OTUs were ranked based on their betweenness centrality scores (local betweenness).

190

191 **Metacommunity-scale network**

192 By compiling the species-level matrices of the eight local forests, the topology of the
193 metacommunity-scale network of plant–fungus associations was inferred. In general, species
194 interaction (association) networks of local communities can be interconnected by species that
195 appear in two or more local networks, thereby merged into a metacommunity-scale network
196 [35]. In our data across the eight local forests, 2,109 OTUs out of the 8,080 fungal OTUs
197 appeared in two or more localities. Therefore, we could infer the topology of a
198 metacommunity-scale network, in which the eight local networks were combined by the
199 2,109 fungal OTUs. In the metacommunity-scale network, plant species/taxa observed in
200 different localities were treated as different network nodes because our purpose in this study
201 was to explore fungi that potentially play key roles in synchronizing local ecosystem
202 processes [35]. In total, 227 plant nodes representing local populations of 150 plant
203 species/taxa were included in the metacommunity-scale network.

204 We then screened for fungal OTUs with broad geographic and host ranges based on the
205 betweenness centrality scores of respective fungal OTUs within the metacommunity network
206 (metacommunity betweenness, B_{meta}). In general, species with highest metacommunity
207 betweenness scores not only occur in local communities over broad biotic/abiotic
208 environmental conditions but also are associated with broad ranges of host/partner species
209 [35]. Possible relationship between local- and metacommunity-scale topological roles was
210 then examined by plotting local and metacommunity betweenness scores (B_{local} and B_{meta}) of
211 each fungal OTUs on a two-dimensional surface. To make the betweenness scores vary from
212 0 to 1, betweenness centrality of a fungal OTU *i* was standardized in each of the local- and
213 metacommunity-scale networks as follows:

214 $$B'_{\text{local},\,i} = \frac{B_{\text{local},\,i} - \min(B_{\text{local}})}{\max(B_{\text{local}}) - \min(B_{\text{local}})} \quad \text{and} \quad B'_{\text{meta},\,i} = \frac{B_{\text{meta},\,i} - \min(B_{\text{meta}})}{\max(B_{\text{meta}}) - \min(B_{\text{meta}})},$$

where $B_{\mathrm{local},i}$ and $B_{\mathrm{meta},i}$ were raw estimates of local- and metacommunity-scale betweenness of a fungal OTU *i*, and min() and max() indicated minimum and maximum values, respectively. For local betweenness of each OTU, a mean value across local networks was subsequently calculated ($\overline{B}'_{\mathrm{local},i}$): the local communities from which a target OTU was absent was omitted in the calculation of mean local betweenness. On the two-dimensional surface, the OTUs were then classified into four categories: metacommunity hubs having high betweenness in both local- and metacommunity-scale networks ($\overline{B}'_{\mathrm{local},i} \geq 0.5$; $B'_{\mathrm{meta},i} \geq 0.5$), metacommunity connectors that had broad geographic ranges but displayed low local betweenness ($\overline{B}'_{\mathrm{local},i} < 0.5$; $B'_{\mathrm{meta},i} \geq 0.5$), local hubs that had high betweenness in local networks but not in the metacommunity-scale network ($\overline{B}'_{\mathrm{local},i} \geq 0.5$; $B'_{\mathrm{meta},i} < 0.5$), and peripherals with low betweenness at both local and metacommunity levels ($\overline{B}'_{\mathrm{local},i} < 0.5$; $B'_{\mathrm{meta},i} < 0.5$) [35]. Approximately, 1–2% of fungal OTUs show betweenness scores higher than 0.5 in each local or metacommunity network, while the threshold value can be changed depending on the purpose of each study [35].

In addition to metacommunity hubs within the metacommunity-scale network representing all the eight localities, those within the metacommunity-scale network representing northern (sites 1–4) or southern (sites 5–8) four localities were also explored. This additional analysis allowed us to screen for fungal OTUs that potentially adapted to broad ranges of biotic and abiotic environments within northern (cool-temperate) or southern (warm-temperate or subtropical) part of Japan.

Results

Local networks

Among the eight forest localities, order-level taxonomic compositions of fungi varied significantly (PERMANOVA; $F_{\mathrm{model}} = 35.7$, $P < 0.001$), while the differentiation of community structure was attributed at least partly to geographic variation in among-sample dispersion (PERMDISP; $F = 13.2$, $P < 0.001$) (Fig. 2a). Compositions of fungal functional groups were also differentiated among the eight localities (PERMANOVA; $F_{\mathrm{model}} = 34.9$, $P <$

243 0.001), while within-site dispersion was significantly varied geographically (PERMDISP; F =
244 9.2, $P < 0.001$) (Fig. 2b). The proportion of ectomycorrhizal fungal orders, such as Russulales,
245 Thelephorales, and Sebacinales, was higher in temperate forests than in subtropical forests,
246 while that of arbuscular mycorrhizal fungi increased in subtropical localities (Fig. 2). The
247 proportion of the ascomycete order Helotiales, which has been known to include not only
248 ectomycorrhizal but also endophytic, saprotrophic, and ericoid mycorrhizal fungi [73], was
249 higher in northern localities. In contrast, Diaporthales, which has been considered as
250 predominantly plant pathogenic taxon [74] (but see [75]), was common in subtropical forests
251 but not in others.

252 In each of the eight local networks depicting plant–fungus associations, some fungal
253 OTUs were located at the central positions of the network, while others are distributed at
254 peripheral positions (Additional file 5; Figure S2). Specifically, fungal OTUs belonging to the
255 ascomycete orders Chaetothyriales (e.g., *Cladophialophora* and *Exophiala*) and Helotiales
256 (e.g., *Rhizodermea*, *Pezicula*, *Rhizoscyphus*, and *Leptodontidium*) as well as some *Mortierella*
257 OTUs had high betweenness centrality scores in each of the cool-temperate forests (Fig. 3a-b).
258 In contrast, arbuscular mycorrhizal fungi (Glomeromycota) were common among OTUs with
259 highest betweenness scores in subtropical forests (Fig. 3a-c). Some fungi in the ascomycete
260 order Hypocreales (e.g., *Trichoderma*, *Ilyonectria*, *Simplicillium*, and *Calonectria*) also had
261 high betweenness scores in some temperate and subtropical forests (Fig. 3b).

262

263 **Metacommunity-scale network**

264 In the metacommunity-scale network representing the connections among the eight local
265 networks, not only arbuscular mycorrhizal but also saprotrophic/endophytic fungi were placed
266 at the central topological positions (Fig. 4; Additional file 6; Figure S3). Among
267 non-Glomeromycota OTUs, *Mortierella* (Mortierellales), *Cryptococcus* (Trichosporonales;
268 the Blast top-hit fungus in the NCBI database was recently moved to *Saitozyma*
269 (Tremellales); [76]), *Malassezia* (Malasseziales), *Oidiodendron* (incertae sedis), *Trichoderma*
270 (Hypocreales), and a fungus distantly allied to *Melanconiella* (Diaporthales) displayed highest

271 metacommunity betweenness (Table 1). Among the OTUs with high metacommunity
272 betweenness, only a *Mortierella* OTU was designated as a metacommunity hub (i.e., $\overline{B}'_{\mathrm{local},i}$
273 ≥ 0.5; $B'_{\mathrm{meta},i} \geq 0.5$) and others had low betweenness scores at the local community level
274 ($\overline{B}'_{\mathrm{local},i} < 0.5$; Fig. 5a).

275 In the metacommunity-scale network representing the four cool-temperate forests (sites
276 1–4), many saprotrophic/endophytic fungal OTUs were associated with diverse plant
277 species/taxa, located at the central topological positions within the network topology
278 (Additional file 7; Figure S4; Fig. 5b). The list of these fungi with high metacommunity
279 betweenness involved OTUs in the genera *Mortierella*, *Cladophialophora* (Chaetothyriales),
280 *Pezicula* (Helotiales), and *Oidiodendron* as well as OTUs allied to *Ilyonectria protearum*
281 (Nectriales) and *Cadophora orchidicola* (Helotiales) (Table 1). Most of those fungal OTUs
282 also had high metacommunity betweenness, designated as metacommunity hubs (Fig. 5b).

283 In the metacommunity-scale network consisting of the warm-temperate and subtropical
284 forests (sites 5–8), arbuscular mycorrhizal and saprotrophic/endophytic fungi were placed at
285 the hub positions (Additional file 8; Figure S5; Fig. 5c). The list of non-Glomeromycota
286 OTUs with highest metacommunity betweenness included *Saitozyma* (*Cryptococcus*),
287 *Mortierella*, *Trichoderma*, and *Tomentella* as well as OTUs allied to *Cladophialophora*,
288 *Scleropezicula* (Helotiales), *Melanconiella* (Diaporthales), and *Rhexodenticula* (incertae
289 sedis) (Table 1). Among the taxa, *Saitozyma* and *Mortierella* included OTUs classified as
290 metacommunity hubs (Fig. 5c; Table 1). In an additional analysis of a metacommunity-scale
291 network including only the three subtropical forests (sites 6-8), similar sets of fungal taxa
292 were highlighted (Additional file 9; Table S1). The detailed information of the network index
293 scores examined in this study is provided in Data S3 (Additional file 3: Data S3).

294

295 **Discussion**

296 Based on the metadata of root-associated fungi across the Japanese Archipelago, we herein
297 inferred the structure of a network representing metacommunity-scale associations of 150
298 plant species/taxa and 8,080 fungal OTUs. Our analysis targeted diverse functional groups of

fungi such as arbuscular mycorrhizal, ectomycorrhizal, ericoid-mycorrhizal, saprotrophic/endophytic, and pathogenic fungi, which have been analyzed separately in most previous studies on plant–fungus networks. The comprehensive analysis of below-ground plant–fungus associations allowed us to explore metacommunity hub fungi, which not only occurred over broad geographic ranges but also had broad host ranges in respective local communities. Consequently, this study highlights several taxonomic groups of fungi potentially playing key roles in synchronizing metacommunity-scale processes of temperate and/or subtropical forests.

In the metacommunity-scale network representing all the eight local forests (Fig. 4), fungi in several saprotrophic or endophytic taxa showed higher betweenness centrality scores than other fungi (Table 1). *Mortierella* is generally considered as a saprotrophic lineage [77] but it also includes fungi contributing to the growth and pathogen resistance of plants [78-80]. A phosphate solubilizing strain of *Mortierella*, for example, increases shoot and root growth of host plants under salt stress, especially when co-inoculated with an arbuscular mycorrhizal fungus [78]. In addition, polyunsaturated fatty acids produced by some *Mortierella* species are known to increase resistance of plants against phytopathogens [79, 80]. Fungi in the genus *Trichoderma* are commonly detected and isolated from the rhizosphere [77, 81]. Many of them inhibit the growth of other fungi, often used in the biological control of phytopathogens [82-84]. Some of them are also reported to suppress root-knot nematodes [85] or to promote root growth [86]. The analysis also highlighted basidiomycete yeasts in the genus *Saitozyma* or *Cryptococcus* (teleomorph = *Filobasidiella*), which are often isolated from soil [22, 87] as well as both above-ground and below-ground parts of plants [88-91].

Along with those possibly saprotrophic or endophytic taxa, ericoid mycorrhizal and phytopathogenic taxa of fungi displayed relatively high betweenness scores within the metacommunity-scale network representing all the eight local forests (Table 1). Specifically, *Oidiodendron* (teleomorph = *Myxotrichum*) is a taxon represented by possibly ericoid mycorrhizal species (*O. maius* and *O. griseum*) [92, 93], although fungi in the genus are found also from roots of non-ericaceous plants and soil [94]. On the other hand, fungi in the family Nectriaceae are known to cause black foot disease [95], often having serious damage on

328 economically important woody plants [96, 97]. Although we collected seemingly benign roots
329 in the study forests, some samples may be damaged by those pathogens. Alternatively, some
330 lineages of Nectriaceae fungi may be associated with plant hosts non-symptomatically,
331 having adverse effects context-dependently.

332 Although these fungi were candidates of metacommunity hubs, which are characterized
333 by broad geographic ranges and host plant ranges, none except but a *Mortierella* OTU had
334 high betweenness scores at both local and metacommunity levels (Fig. 5a). This result
335 suggests that even if some fungi have broad geographic ranges across the Japanese
336 Archipelago, few played important topological roles in each of the local networks
337 representing plant–fungus associations. In other words, fungi that can adapt to biotic and
338 abiotic environments in forest ecosystems throughout cool-temperate, warm-temperate, and
339 subtropical regions are rare.

340 Therefore, we also explored fungi with broad geographic and host ranges within the
341 metacommunities representing northern (cool-temperate) and southern (warm-temperate and
342 subtropical) regions of Japan. In the metacommunity consisting of the four cool-temperate
343 forests (Additional file 7; Figure S4), fungal OTUs in the genera *Mortierella*,
344 *Cladophialophora*, and *Pezicula* as well as those allied to *Ilyonectria* and *Cadophora* had
345 highest betweenness at both local and metacommunity levels, classified as metacommunity
346 hubs (Fig. 5b; Table 1). Among them, *Cladophialophora* is of particular interest because it
347 has been known as a lineage of "dark septate endophytes" [98-100] (*sensu* [14, 15, 101]). A
348 species within the genus, *C. chaetospira* (= *Heteroconium chaetospira*), to which
349 high-betweenness OTUs in our data were closely allied, has been known not only to provide
350 nitrogen to host plants but also to suppress pathogens [12, 16, 102]. Likewise, the Helotiales
351 genus *Pezicula* (anamorph = *Cryptosporiopsis*) includes endophytic fungi [103-105], some of
352 which produce secondary metabolites suppressing other microbes in the rhizosphere [106,
353 107]. Our finding that some of *Cladophialophora* and *Pezicula* fungi could be associated with
354 various taxonomic groups of plants over broad geographic ranges highlights potentially
355 important physiological and ecological roles of those endophytes at the community and
356 metacommunity levels.

357 In the southern metacommunity networks consisting of warm-temperate and subtropical
358 forests (Additional file 8; Figure S5), some arbuscular mycorrhizal OTUs and *Saitozyma*
359 (*Cryptococcus*) and *Mortierella* OTUs had high betweenness scores at both local and
360 metacommunity levels, designated as metacommunity hubs (Fig. 5c; Table 1). Given the
361 above-mentioned prevalence of fungal OTUs allied to *Cladophialophora chaetospira* in the
362 cool-temperate metacommunity, the contrasting list of metacommunity hubs in the southern
363 (warm-temperate–subtropical) metacommunity implies that different taxonomic and
364 functional groups of fungi play major metacommunity-scale roles in different climatic regions.
365 This working hypothesis is partially supported by previous studies indicating endemism and
366 vicariance in the biogeography of fungi and bacteria [108, 109], promoting conceptual
367 advances beyond the classic belief that every microbe is everywhere but the environment
368 selects microbes colonizing respective local communities [110].

369 The roles of those metacommunity hubs detected in this study are of particular interest
370 from the aspect of theoretical ecology. Hub species connected to many other species in an
371 ecosystem often integrate "energy channels" [111] within species interaction networks,
372 having great impacts on biodiversity and productivity of the ecosystems [35]. The concept of
373 "keystone" or "foundation" species [112, 113] can be extended to the metacommunity level,
374 thereby promoting studies exploring species that restructure and synchronize ecological (and
375 evolutionary) dynamics over broad geographic ranges [35]. Given that below-ground plant–
376 fungus symbioses are key components of the terrestrial biosphere [1, 2], identifying fungal
377 species that potentially have great impacts on the metacommunity-scale processes of such
378 below-ground interactions will provide crucial insights into the conservation and restoration
379 of forests and grasslands. We here showed that the list of metacommunity hubs could involve
380 various lineages of endophytic fungi, whose ecosystem-scale functions have been
381 underappreciated compared to those of mycorrhizal fungi. As those endophytic fungi are
382 potentially used as inoculants when we reintroduce plant seedlings in ecosystem restoration
383 programs [20, 51], exploring fungi with highest potentials in each climatic/biogeographic
384 region will be a promising direction of research in conservation biology.

385 The finding that compositions of metacommunity hubs could vary depending on climatic

386 regions also gives key implications for the application of endophytes in agriculture. Although
387 a number of studies have tried to use endophytic fungi and/or bacteria as microbial inoculants
388 in agriculture [17, 18, 114], such microbes introduced to agroecosystems are often
389 outcompeted and replaced by indigenous (resident) microbes [115, 116]. Moreover, even if an
390 endophytic species or strain increases plant growth in pot experiments under controlled
391 environmental conditions, its effects in the field often vary considerably depending on biotic
392 and abiotic contexts of local agroecosystems [17] (see also [117]). Therefore, in the screening
393 of endophytes that can be used in broad ranges of biotic and abiotic environmental conditions,
394 the metacommunity-scale network analysis outlined in this study will help us find promising
395 candidates out of thousands or tens of thousands microbial species in the wild. Consequently,
396 to find promising microbes whose inocula can persist in agroecosystems for long time periods,
397 exploration of metacommunity hubs needs to be performed in respective climatic or
398 biogeographic regions.

399 For more advanced applications in conservation biology and agriculture, continual
400 improvements of methods for analyzing metacommunity-scale networks are necessary. First,
401 while the fungal OTUs in our network analysis was defined based on the cut-off sequence
402 similarities used in other studies targeting "species-level" diversity of fungi [57, 59],
403 physiological functions can vary greatly within fungal species or species groups [14, 118].
404 Given that bioinformatic tools that potentially help us detect single-nucleotide-level variation
405 are becoming available [119], the resolution of network analyses may be greatly improved in
406 the near future. Second, although some computer programs allow us to infer functions of
407 respective microbial OTUs within network data [63, 120], the database information of
408 microbial functions remains scarce. To increase the coverage and accuracy of automatic
409 annotations of microbial functions, studies describing the physiology, ecology, and genomes
410 of microbes should be accelerated. With improved reference databases, more insights into the
411 metacommunity-scale organization of plant–fungus associations will be obtained by
412 reanalyzing the network data based on enhanced information of fungal functional groups.
413 Third, as the diversity and compositions of plant–fungus associations included in a network
414 can depend on how we process raw samples, special care is required in the selection of

415 methods for washing and preparing root (or soil) samples. By sterilizing root samples with
416 NaClO [121], for example, we may be able to exclude fungi or bacteria that are merely
417 adhering to root surfaces. Meanwhile, some of those fungi and bacteria on root surfaces may
418 play pivotal physiological roles in the growth and survival of plants [122]. Accordingly, it
419 would be productive to compare network topologies of plant–microbe associations among
420 different source materials by partitioning endosphere, rhizoplane, and rhizosphere microbial
421 samples with a series of sample cleaning processes using ultrasonic devices [123]. Fourth,
422 although this study targeted fungi associated with roots, our methods can be easily extended
423 to network analyses involving other groups of microbes. By simultaneously analyzing the
424 prokaryote 16S rRNA region [123-125] with the fungal ITS region, we can examine how
425 bacteria, archaea, and fungi are involved in below-ground webs of symbioses. Fifth, not only
426 plant–microbe associations but also microbe–microbe interactions can be estimated with
427 network analytical frameworks. Various statistical pipelines have been proposed to infer how
428 microbes interact with each other in facilitative or competitive ways within host
429 macroorganisms [37, 126, 127]. Overall, those directions of analytical extensions will
430 enhance our understanding of plant microbiome dynamics in nature.

431

432 **Conclusions**

433 By compiling datasets of below-ground plant–fungus associations in temperate and
434 subtropical forest ecosystems, we explored metacommunity-hub fungi, which were
435 characterized by broad geographic and host ranges. Such metacommunity-scale analyses are
436 expected to provide bird's-eye views of complex plant–microbe associations, highlighting
437 plant-growth-promoting microbes that can be applied to diverse plant taxa in various
438 environments. Given that endophytic fungi promoting the growth and pathogen resistance of
439 host plants can be isolated from forest soil (e.g., *Cladophialophora chaetospira* [99]), the list
440 of metacommunity-hub endophytic fungi featured in this study itself may include prospective
441 species to be used in agriculture. By extending the targets of such network analyses to diverse
442 types of plant-associated microbes (e.g., phyllosphere fungi and bacteria [75, 124, 128]) in

various climatic/biogeographic regions, a solid basis for managing plant microbiomes will be developed.

Abbreviations

DDBJ: DNA Data Bank of Japan; ITS: internal transcribed spacer; OTU: Operational taxonomic unit; PERMANOVA: permutational analysis of variance; PERMDISP: permutational analysis for the multivariate homogeneity of dispersions; rRNA: ribosomal ribonucleic acid.

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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 with the source study [52] and Additional files 1-3, respectively.

Authors' contributions

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

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. Sample-level matrices of the eight forests examined.

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

Additional file 3: Data S3. Information of 8080 fungal OTUs analyzed.

Additional file 4: Figure S1. Number of sequencing reads, interaction specificity, and local betweenness.

Additional file 5: Figure S2. Structure of plant–fungus networks in each local forest.

Additional file 6: Figure S3. Locality information within the full metacommunity-scale network.

Additional file 7: Figure S4. Metacommunity-scale network of cool-temperate forests.

492 **Additional file 8: Figure S5.** Metacommunity-scale network of warm-temperate and
493 subtropical forests.

494 **Additional file 9: Table S1.** Top-10 list of non-Glomeromycota OTUs with highest
495 betweenness within the subtropical metacommunity network.

496

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832 **Table 1** Top-10 list of non-Glomeromycota OTUs with highest betweenness within the metacommunity networks. In each of the three
833 metacommunity-scale networks examined (full, cool-temperate, and warm-temperate/subtropical), fungal OTUs were ranked based on their
834 betweenness centrality scores. As taxonomic information of Glomeromycota OTUs with high betweenness scores was redundant (e.g., *Glomus*
835 spp. or Glomeraceae spp.), the top-10 list of non-Glomeromycota OTUs is shown. Taxonomy information of each OTU was inferred based on
836 the query-centric auto-*k*-nearest-neighbor algorithm of reference database search [60, 61] and subsequent taxonomic assignment with the lowest
837 common ancestor algorithm [62]. The results of the NCBI nucleotide Blast are also shown. For simplicity, the functional groups of fungi inferred
838 with the program FUNGuild [63] were organized into several categories. See Data S3 (Additional file 3) for details of the categories and for full
839 results including Glomeromycota and other fungal OTUs.
840

OTU	Phylum	Class	Order	Family	Genus	Category	NCBI Blast top hit	Accession	Cover	Identity
Full (8sites)										
F_0042*	-	-	Mortierellales	Mortierellaceae	*Mortierella*	Saprotroph/Endophyte	*Mortierella humilis*	KP714537	100%	100%
F_0381	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cryptococcus*	Others_Unknown	*Saitozyma podzolica*†	KY320605	92%	99%
F_0079	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	-	Saprotroph/Endophyte	*Ilyonectria protearum*	NR_152890	99%	100%
F_0489	-	-	Mortierellales	Mortierellaceae	*Mortierella*	Saprotroph/Endophyte	*Mortierella* sp.	KM113754	100%	100%
F_0010	Ascomycota	Leotiomycetes	-	Myxotrichaceae	*Oidiodendron*	Ericoid_Mycorrhizal	*Oidiodendron maius*	LC206669	100%	100%
F_0368	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	Others_Unknown	*Malassezia restricta*	KT809059	100%	100%
F_0623	-	-	Mortierellales	Mortierellaceae	*Mortierella*	Saprotroph/Endophyte	*Mortierella gamsii*	KY305027	100%	100%
F_1188	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cryptococcus*	Others_Unknown	*Saitozyma podzolica*†	KY320605	92%	99%
F_0007	Ascomycota	Sordariomycetes	Diaporthales	Melanconidaceae	*Melanconiella*	Saprotroph/Endophyte	*Melanconiella elegans*	KJ173701	100%	85%
F_0485	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	Saprotroph/Endophyte	*Trichoderma* sp.	HG008760	100%	100%

F_0079*	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	-	Saprotroph/Endophyte	*Ilyonectria protearum*	NR_152890	99%	100%
F_0015*	Ascomycota	-	-	-	-	Others_Unknown	*Cadophora orchidicola*	KX611558	100%	99%
F_0202*	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	Saprotroph/Endophyte	*Cladophialophora chaetospira*	HQ871875	100%	99%
F_0195*	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	Saprotroph/Endophyte	*Cladophialophora chaetospira*	EU035405	100%	100%
F_0181*	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	*Pezicula*	Endophyte	*Pezicula melanigena*	LC206665	100%	99%
F_0010	Ascomycota	Leotiomycetes	-	Myxotrichaceae	*Oidiodendron*	Ericoid_Mycorrhizal	*Oidiodendron maius*	LC206669	100%	100%
F_0103*	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora*	Saprotroph/Endophyte	*Cladophialophora chaetospira*	EU035403	100%	97%
F_0489*	-	-	Mortierellales	Mortierellaceae	*Mortierella*	Saprotroph/Endophyte	*Mortierella* sp.	KM113754	100%	100%
Southern 4 sites (warm-temperate and subtropical)										
F_0381*	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cryptococcus*	Others_Unknown	*Saitozyma podzolica*†	KY320605	92%	99%
F_0042*	-	-	Mortierellales	Mortierellaceae	*Mortierella*	Saprotroph/Endophyte	*Mortierella humilis*	KP714537	100%	100%
F_0610*	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	Saprotroph/Endophyte	*Trichoderma spirale*	KU948158	100%	100%
F_1188*	Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cryptococcus*	Others_Unknown	*Saitozyma podzolica*†	KY320605	92%	99%
F_0029	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	-	Others_Unknown	*Cladophialophora* sp.	LC189029	100%	99%
F_0017	Ascomycota	-	-	-	-	Others_Unknown	*Scleropezicula* sp.	KT809119	100%	98%
F_0007	Ascomycota	Sordariomycetes	Diaporthales	Melanconidaceae	*Melanconiella*	Saprotroph/Endophyte	*Melanconiella elegans*	KJ173701	100%	85%
F_0485	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	Saprotroph/Endophyte	*Trichoderma* sp.	HG008760	100%	100%
F_0112	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	*Tomentella*	Ectomycorrhizal	*Tomentella stuposa*	KR019860	100%	98%
F_0073	Ascomycota	Sordariomycetes	-	-	-	Others_Unknown	*Rhexodenticula acaciae*	KY173442	94%	95%

*Fungal OTUs classified as metacommunity hubs (mean local betweenness > 0.5; metacommunity betweenness > 0.5)

†Synonym, *Cryptcoccus podzolica*

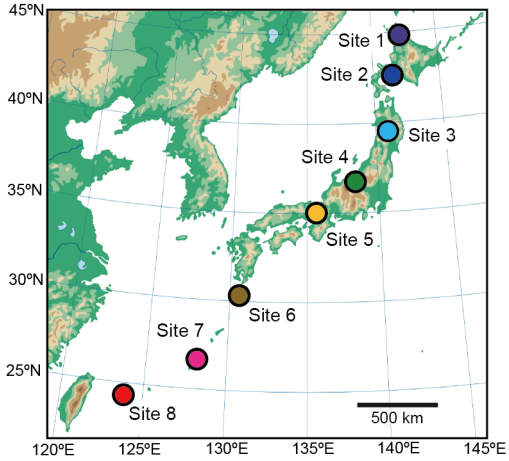


Fig. 1 Study sites examined in this study. Across the entire range of the Japanese root samples were collected in four cool-temperate forests (sites 1–4), one warm-forest (site 5), and three subtropical forests (sites 6–8).

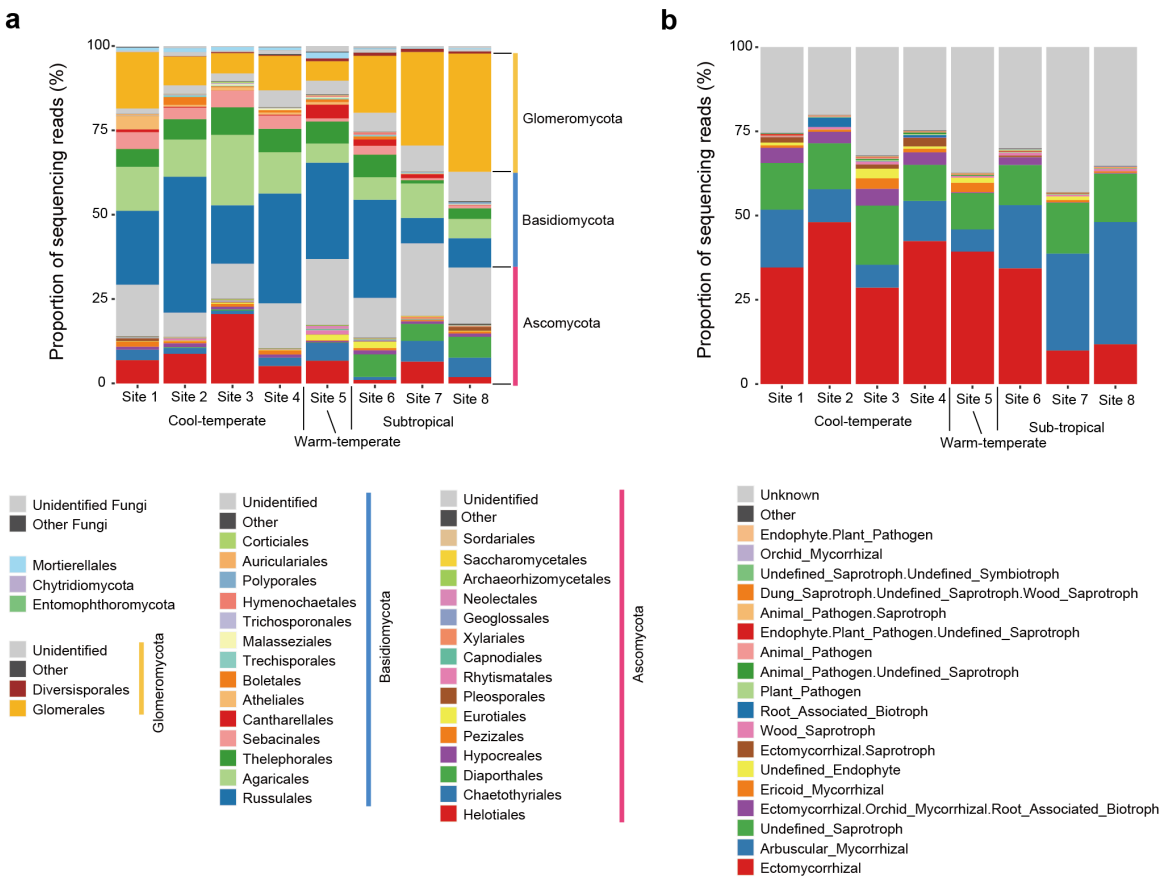


Fig. 2 Compositions of fungal taxa and functional groups in each forest. **a** Order-level taxonomic composition of fungal OTUs in each locality. The number of fungal OTUs detected is shown in a parenthesis for each forest. **b** Functional-group composition. The fungal functional groups were inferred by the program FUNGuild [63].

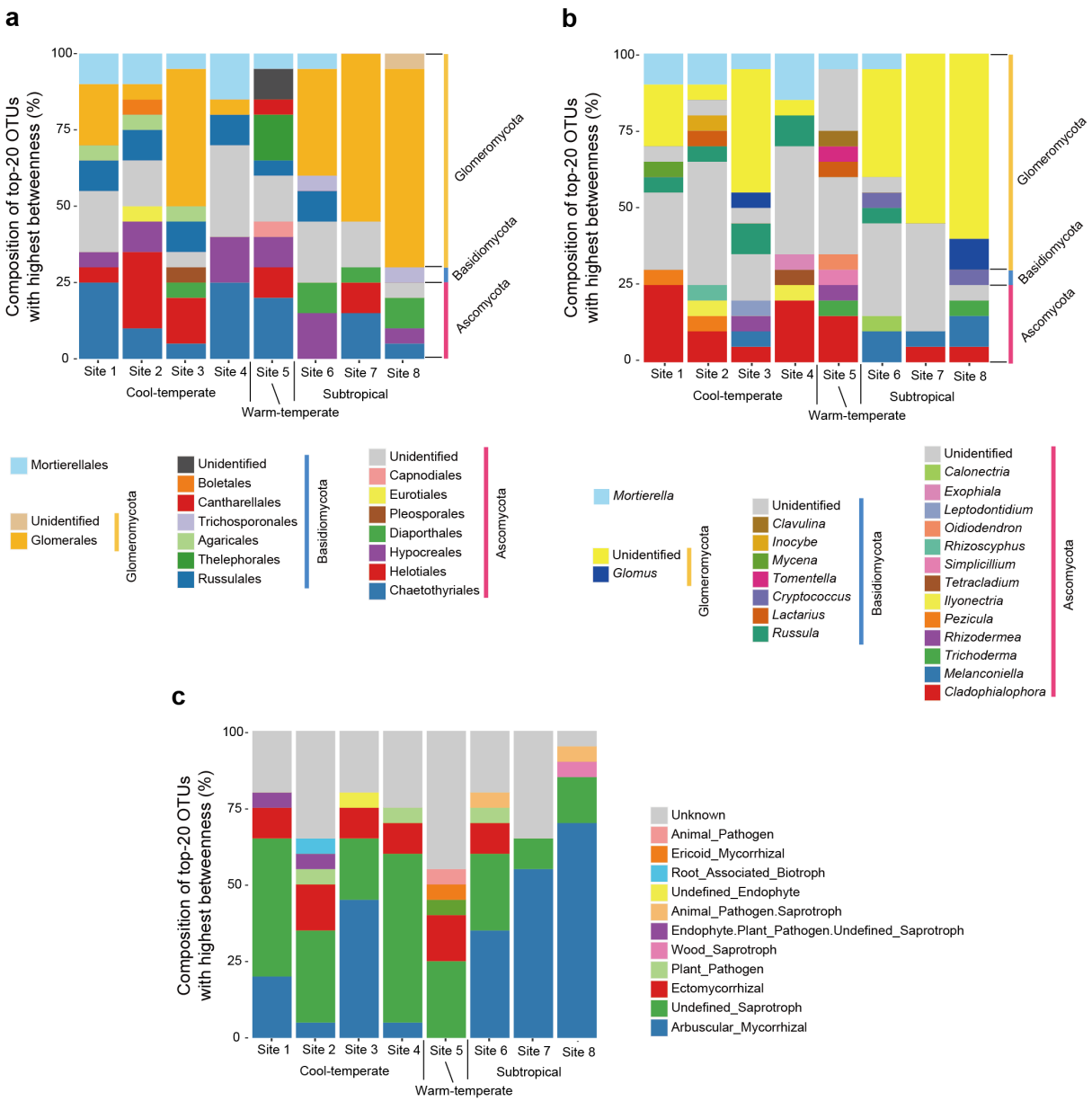
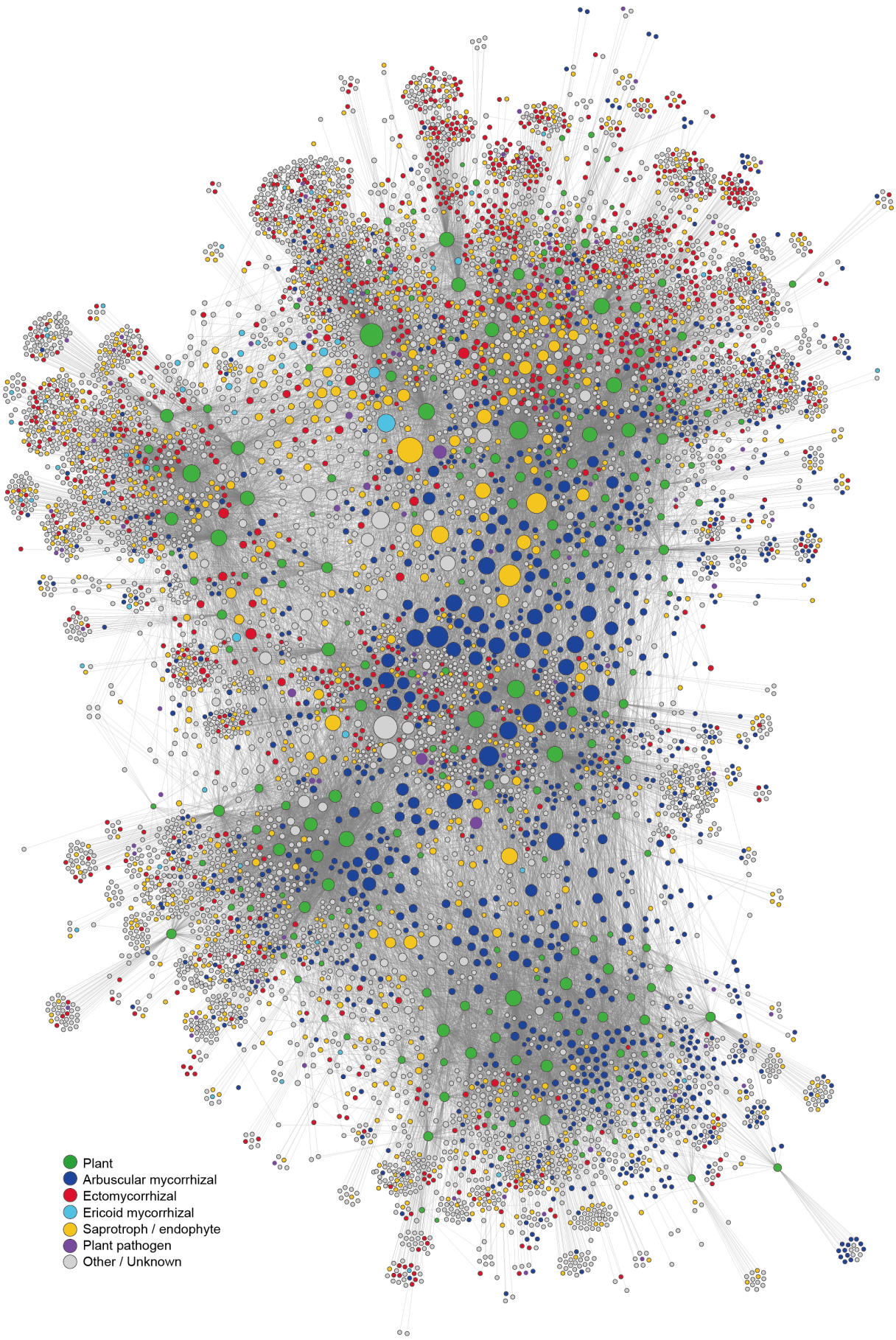
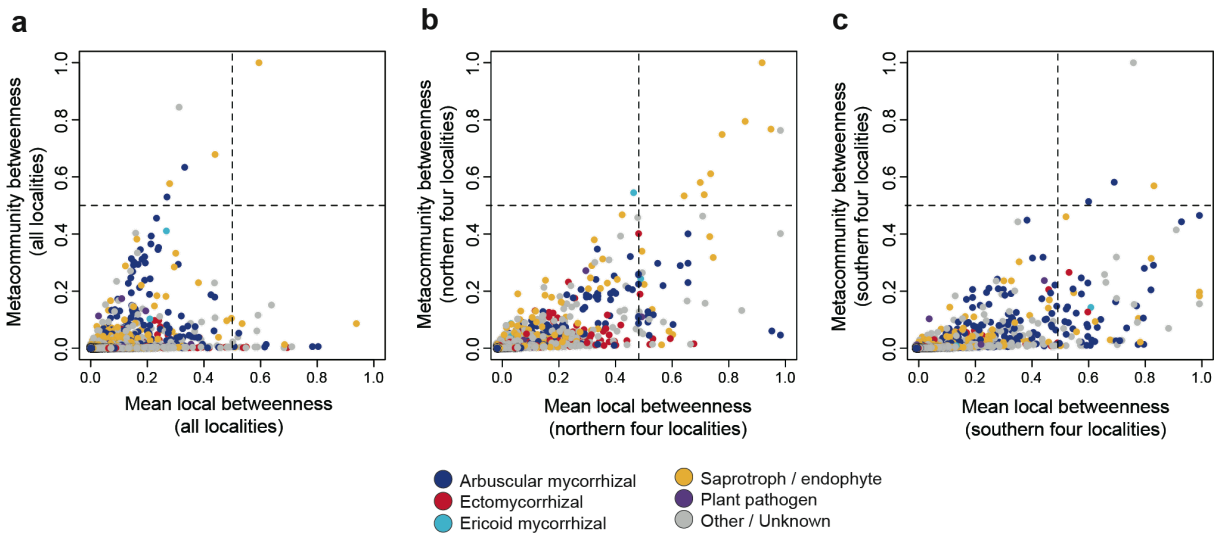


Fig. 3 Fungal OTUs with highest local betweenness. **a** Order-level taxonomic composition of top-20 OTUs with highest local betweenness in each forest. See Data S3 (Additional file 3) for betweenness scores of all fungal OTUs in respective local forests. **b** Genus-level taxonomic composition of top-20 OTUs with highest local betweenness. **c** Functional-group composition of top-20 OTUs with highest local betweenness.


Plant
Arbuscular mycorrhizal
Ectomycorrhizal
Ericoid mycorrhizal
Saprotroph / endophyte
Plant pathogen
Other / Unknown

865

Fig. 4 Metacommunity-scale network including all the eight local forests. The size of circles roughly represents relative scores of betweenness centrality. The functional groups of fungi inferred with the program FUNGuild [63] were organized into six categories: i.e., arbuscular mycorrhizal (bue), ectomycorrhizal (red), ericoid mycorrhizal (skyblue), saprotrophic/endophytic (yellow), plant pathogenic (purple), and other/unknown fungi (grey) (Additional file 3; Data S3). For plant species/taxa (green), the geographic information of source populations is indicated in Additional file 6 (Figure S3).



875

876

877 **Fig. 5** Relationship between local- and metacommunity-level betweenness. **a** Full
878 meatcommunity. On the horizontal axis, the mean values of betweenness centrality scores
879 across all the eight local forests are shown for respective fungal OTUs. On the vertical axis,
880 the betweenness scores within the metacommunity-scale network consisting of the eight
881 localities (Fig. 4) are shown for respective OTUs. **b** Metacommunity of cool-temperate
882 forests. For the sub-dataset consisting of the four cool-temperate forests (Additional file 7:
883 Figure S4), mean local betweenness and metacommunity betweenness are shown on the
884 horizontal and vertical axes, respectively. **c** Metacommunity of warm-temperate and
885 subtropical forests. For the sub-dataset consisting of the warm-temperate forest and the three
886 subtropical forests (Additional file 8: Figure S5), mean local betweenness and
887 metacommunity betweenness are shown on the horizontal and vertical axes, respectively.