# Network hubs in root-associated fungal metacommunities

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## 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 were detected from 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:** agriculture; biodiversity; ecosystem restoration; host specificity or preference; latitudinal gradients; metacommunities; microbial inoculation; network hubs; plant–fungus

interactions; mycorrhizal and endophytic symbiosis.

## Background

Below-ground fungi in the endosphere and rhizosphere are key drivers of terrestrial ecosystem processes [1-4]. Mycorrhizal fungi, for example, are important partners 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

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

112

113 ## Methods

114 ### Terminology

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

128

129 ### Data

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

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

143 The Illumina sequencing reads of the fungal ITS1 region were processed using the
144 program Claidnet [55, 56] as detailed in the data-source study [52]: the Unix scripts used are
145 available as Additional file 1. The primers used were designed to target not only Ascomycota
146 and Basidiomycota but also diverse non-Dikarya (e.g., Glomeromycota) taxa [57]. In most
147 studies analyzing community structure of Ascomycota and Basidiomycota fungi, OTUs of the
148 ITS region are defined with a cut-off sequence similarity of 97% [22, 58, 59] (see also [60]).
149 Meanwhile, Glomeromycota fungi generally have much higher intraspecific ITS-sequence
150 variation than other taxonomic groups of fungi [61]. Consequently, we used 97% and 94%
151 cut-off sequence similarities for defining non-Glomeromycota and Glomeromycota fungal
152 OTUs, respectively [52]. The OTUs were then subjected to reference database search with the
153 query-centric auto-*k*-nearest-neighbor algorithm [55, 56] and subsequent taxonomic
154 assignment with the lowest common ancestor algorithm [62]. Based on the inferred taxonomy,
155 the functional group of each fungal OTU was inferred using the program FUNGuild 1.0 [63].

156 After a series of bioinformatics and rarefaction procedures, 1,000 fungal ITS reads were
157 obtained from each of the 240 samples collected in each forest locality (i.e., 1,000 reads × 240
158 samples × 8 sites). A sample (row) × fungal OTU (column) data matrix, in which a cell entry
159 depicted the number of sequencing reads of an OTU in a sample, was obtained for each local
160 forest ("sample-level" matrix) (Additional file 2: Data S2). Each local sample-level matrix
161 was then converted into a "species-level" matrix, in which a cell entry represented the number
162 of root samples from which associations of a plant species/taxa (row) and a fungal OTU

163 (columns) was observed: 17–55 plant species/taxa and 1,149–1,797 fungal OTUs were
164 detected from the local species-level matrices (Additional file 3: Data S3). In total, the
165 matrices included 150 plant species/taxa and 8,080 fungal OTUs (Additional file 4: Data S4).

166

167 **Local networks**

168 Among the eight forest localities, variation in the order-level taxonomic compositions were
169 examined with the permutational analysis of variance (PERMANOVA; [64]) and the
170 permutational analysis for the multivariate homogeneity of dispersions (PERMDISP; [65])
171 with the "adonis" and "betadisper" functions of the vegan 2.4-3 package [66] of R 3.4.1 [67],
172 respectively. The $\beta$-diversity values used in the PERMANOVA and PERMDISP analyses
173 were calculated with the "Bray-Curtis" metric based on the sample-level matrices (Additional
174 file 2: Data S2). Note that the "Raup-Crick" $\beta$-diversity metric [68], which controls
175 $\alpha$-diversity in community data but requires computationally intensive randomization, was not
176 applicable to our large metadata. Geographic variation in the compositions of fungal
177 functional groups was also evaluated by PERMANOVA and PERMDISP analyses. The R
178 scripts for the PERMANOVA and PERMDISP analyses are available as Additional file 5.

179 For each of the eight local forests, the network structure of below-ground plant–fungus
180 associations was visualized based on the species-level matrix (Additional file 3: Data S3)
181 using the program GePhi 0.9.1 [69] with the "ForceAtlas2" layout algorithm [70]. Within the
182 networks, the order-level taxonomy of fungal OTUs was highlighted.

183 To evaluate host ranges of each fungal OTU in each local forest, we first calculated the *d'*
184 metric of interaction specificity [71]. However, estimates of the *d'* metric varied considerably
185 among fungal OTUs observed from small numbers of root samples (Additional file 6; Figure
186 S1) presumably due to overestimation or underestimation of host preferences for those rare
187 OTUs. Therefore, we scored each fungal OTU based on their topological positions within
188 each local network by calculating network centrality indices (degree, closeness, betweenness,
189 and eigenvector centralities metrics of network centrality; [31]). Among the centrality metrics,
190 betweenness centrality, which measures the extent to which a given nodes (species) is located

191 within the shortest paths connecting pairs of other nodes in a network [72], is often used to
192 explore organisms with broad host or partner ranges [35]. Thus, in each local network, fungal
193 OTUs were ranked based on their betweenness centrality scores (local betweenness).

194

195 **Metacommunity-scale network**

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

208 We then screened for fungal OTUs with broad geographic and host ranges based on the
209 betweenness centrality scores of respective fungal OTUs within the metacommunity network
210 (metacommunity betweenness, $B_{\mathrm{meta}}$). In general, species with highest metacommunity
211 betweenness scores not only occur in local communities over broad biotic/abiotic
212 environmental conditions but also are associated with broad ranges of host/partner species
213 [35]. Possible relationship between local- and metacommunity-scale topological roles was
214 then examined by plotting local and metacommunity betweenness scores ($B_{\mathrm{local}}$ and $B_{\mathrm{meta}}$) of
215 each fungal OTUs on a two-dimensional surface. To make the betweenness scores vary from
216 0 to 1, betweenness centrality of a fungal OTU *i* was standardized in each of the local- and
217 metacommunity-scale networks as follows:

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

where $B_{\text{local},i}$ and $B_{\text{meta},i}$ 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 ($\overline{B}'_{\text{local},i}$): 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 ($\overline{B}'_{\text{local},i} \geq 0.5$; $B'_{\text{meta},i} \geq 0.5$), metacommunity connectors that had broad geographic ranges but displayed low local betweenness ($\overline{B}'_{\text{local},i} < 0.5$; $B'_{\text{meta},i} \geq 0.5$), local hubs that had high betweenness in local networks but not in the metacommunity-scale network ($\overline{B}'_{\text{local},i} \geq 0.5$; $B'_{\text{meta},i} < 0.5$), and peripherals with low betweenness at both local and metacommunity levels ($\overline{B}'_{\text{local},i} < 0.5$; $B'_{\text{meta},i} < 0.5$) [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; $F_{\text{model}} = 35.7$, $P < 0.001$), while the differentiation of

244 community structure was attributed at least partly to geographic variation in among-sample
245 dispersion (PERMDISP; $F = 13.2$, $P < 0.001$) (Fig. 2a). Compositions of fungal functional
246 groups were also differentiated among the eight localities (PERMANOVA; $F_{model} = 34.9$, $P <$
247 $0.001$), while within-site dispersion was significantly varied geographically (PERMDISP; $F =$
248 $9.2$, $P < 0.001$) (Fig. 2b). The proportion of ectomycorrhizal fungal orders, such as Russulales,
249 Thelephorales, and Sebacinales, was higher in temperate forests than in subtropical forests,
250 while that of arbuscular mycorrhizal fungi increased in subtropical localities (Fig. 2). The
251 proportion of the ascomycete order Helotiales, which has been known to include not only
252 ectomycorrhizal but also endophytic, saprotrophic, and ericoid mycorrhizal fungi [73], was
253 higher in northern localities. In contrast, Diaporthales, which has been considered as
254 predominantly plant pathogenic taxon [74] (but see [75]), was common in subtropical forests
255 but not in others.

256 In each of the eight local networks depicting plant–fungus associations, some fungal
257 OTUs were located at the central positions of the network, while others are distributed at
258 peripheral positions (Additional file 7; Figure S2). Specifically, fungal OTUs belonging to the
259 ascomycete orders Chaetothyriales (e.g., *Cladophialophora* and *Exophiala*) and Helotiales
260 (e.g., *Rhizodermea*, *Pezicula*, *Rhizoscyphus*, and *Leptodontidium*) as well as some *Mortierella*
261 OTUs had high betweenness centrality scores in each of the cool-temperate forests (Fig. 3a-b).
262 In contrast, arbuscular mycorrhizal fungi (Glomeromycota) were common among OTUs with
263 highest betweenness scores in subtropical forests (Fig. 3a-c). Some fungi in the ascomycete
264 order Hypocreales (e.g., *Trichoderma*, *Ilyonectria*, *Simplicillium*, and *Calonectria*) also had
265 high betweenness scores in some temperate and subtropical forests (Fig. 3b).

266

267 **Metacommunity-scale network**

268 In the metacommunity-scale network representing the connections among the eight local
269 networks, not only arbuscular mycorrhizal but also saprotrophic/endophytic fungi were placed
270 at the central topological positions (Fig. 4; Additional file 8; Figure S3). Among
271 non-Glomeromycota OTUs, *Mortierella* (Mortierellales), *Cryptococcus* (Trichosporonales;

the Blast top-hit fungus in the NCBI database was recently moved to *Saitozyma* (Tremellales); [76]), *Malassezia* (Malasseziales), *Oidiodendron* (incertae sedis), *Trichoderma* (Hypocreales), and a fungus distantly allied to *Melanconiella* (Diaporthales) displayed highest metacommunity betweenness (Table 1). Among the OTUs with high metacommunity betweenness, only a *Mortierella* OTU was designated as a metacommunity hub (i.e., $\overline{B}'_{\mathrm{local},i} \geq 0.5$; $B'_{\mathrm{meta},i} \geq 0.5$) and others had low betweenness scores at the local community level ($\overline{B}'_{\mathrm{local},i} < 0.5$; Fig. 5a).

In the metacommunity-scale network representing the four cool-temperate forests (sites 1–4), many saprotrophic/endophytic fungal OTUs were associated with diverse plant species/taxa, located at the central topological positions within the network topology (Additional file 9; Figure S4; Fig. 5b). The list of these fungi with high metacommunity betweenness involved OTUs in the genera *Mortierella*, *Cladophialophora* (Chaetothyriales), *Pezicula* (Helotiales), and *Oidiodendron* as well as OTUs allied to *Ilyonectria protearum* (Nectriales) and *Cadophora orchidicola* (Helotiales) (Table 1). Most of those fungal OTUs also had high metacommunity betweenness, designated as metacommunity hubs (Fig. 5b).

In the metacommunity-scale network consisting of the warm-temperate and subtropical forests (sites 5–8), arbuscular mycorrhizal and saprotrophic/endophytic fungi were placed at the hub positions (Additional file 10; Figure S5; Fig. 5c). The list of non-Glomeromycota OTUs with highest metacommunity betweenness included *Saitozyma* (*Cryptococcus*), *Mortierella*, *Trichoderma*, and *Tomentella* as well as OTUs allied to *Cladophialophora*, *Scleropezicula* (Helotiales), *Melanconiella* (Diaporthales), and *Rhexodenticula* (incertae sedis) (Table 1). Among the taxa, *Saitozyma* and *Mortierella* included OTUs classified as metacommunity hubs (Fig. 5c; Table 1). In an additional analysis of a metacommunity-scale network including only the three subtropical forests (sites 6-8), similar sets of fungal taxa were highlighted (Additional file 11; Table S1). The detailed information of the network index scores examined in this study is provided in Data S3 (Additional file 4: Data S4).

## Discussion

300 Based on the metadata of root-associated fungi across the Japanese Archipelago, we herein
301 inferred the structure of a network representing metacommunity-scale associations of 150
302 plant species/taxa and 8,080 fungal OTUs. Our analysis targeted diverse functional groups of
303 fungi such as arbuscular mycorrhizal, ectomycorrhizal, ericoid-mycorrhizal,
304 saprotrophic/endophytic, and pathogenic fungi, which have been analyzed separately in most
305 previous studies on plant–fungus networks. The comprehensive analysis of below-ground
306 plant–fungus associations allowed us to explore metacommunity hub fungi, which not only
307 occurred over broad geographic ranges but also had broad host ranges in respective local
308 communities. Consequently, this study highlights several taxonomic groups of fungi
309 potentially playing key roles in synchronizing metacommunity-scale processes of temperate
310 and/or subtropical forests.

311 In the metacommunity-scale network representing all the eight local forests (Fig. 4),
312 fungi in several saprotrophic or endophytic taxa showed higher betweenness centrality scores
313 than other fungi (Table 1). *Mortierella* is generally considered as a saprotrophic lineage [77]
314 but it also includes fungi contributing to the growth and pathogen resistance of plants [78-80].
315 A phosphate solubilizing strain of *Mortierella*, for example, increases shoot and root growth
316 of host plants under salt stress, especially when co-inoculated with an arbuscular mycorrhizal
317 fungus [78]. In addition, polyunsaturated fatty acids produced by some *Mortierella* species
318 are known to increase resistance of plants against phytopathogens [79, 80]. Fungi in the genus
319 *Trichoderma* are commonly detected and isolated from the rhizosphere [77, 81]. Many of
320 them inhibit the growth of other fungi, often used in the biological control of phytopathogens
321 [82-84]. Some of them are also reported to suppress root-knot nematodes [85] or to promote
322 root growth [86]. The analysis also highlighted basidiomycete yeasts in the genus *Saitozyma*
323 or *Cryptococcus* (teleomorph = *Filobasidiella*), which are often isolated from soil [22, 87] as
324 well as both above-ground and below-ground parts of plants [88-91].

325 Along with those possibly saprotrophic or endophytic taxa, ericoid mycorrhizal and
326 phytopathogenic taxa of fungi displayed relatively high betweenness scores within the
327 metacommunity-scale network representing all the eight local forests (Table 1). Specifically,
328 *Oidiodendron* (teleomorph = *Myxotrichum*) is a taxon represented by possibly ericoid

329 mycorrhizal species (*O. maius* and *O. griseum*) [92, 93], although fungi in the genus are found
330 also from roots of non-ericaceous plants and soil [94]. On the other hand, fungi in the family
331 Nectriaceae are known to cause black foot disease [95], often having serious damage on
332 economically important woody plants [96, 97]. Although we collected seemingly benign roots
333 in the study forests, some samples may be damaged by those pathogens. Alternatively, some
334 lineages of Nectriaceae fungi may be associated with plant hosts non-symptomatically,
335 having adverse effects context-dependently.

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

344 Therefore, we also explored fungi with broad geographic and host ranges within the
345 metacommunities representing northern (cool-temperate) and southern (warm-temperate and
346 subtropical) regions of Japan. In the metacommunity consisting of the four cool-temperate
347 forests (Additional file 9; Figure S4), fungal OTUs in the genera *Mortierella*,
348 *Cladophialophora*, and *Pezicula* as well as those allied to *Ilyonectria* and *Cadophora* had
349 highest betweenness at both local and metacommunity levels, classified as metacommunity
350 hubs (Fig. 5b; Table 1). Among them, *Cladophialophora* is of particular interest because it
351 has been known as a lineage of "dark septate endophytes" [98-100] (*sensu* [14, 15, 101]). A
352 species within the genus, *C. chaetospira* (= *Heteroconium chaetospira*), to which
353 high-betweenness OTUs in our data were closely allied, has been known not only to provide
354 nitrogen to host plants but also to suppress pathogens [12, 16, 102]. Likewise, the Helotiales
355 genus *Pezicula* (anamorph = *Cryptosporiopsis*) includes endophytic fungi [103-105], some of
356 which produce secondary metabolites suppressing other microbes in the rhizosphere [106,
357 107]. Our finding that some of *Cladophialophora* and *Pezicula* fungi could be associated with

358 various taxonomic groups of plants over broad geographic ranges highlights potentially
359 important physiological and ecological roles of those endophytes at the community and
360 metacommunity levels.

361 In the southern metacommunity networks consisting of warm-temperate and subtropical
362 forests (Additional file 10; Figure S5), some arbuscular mycorrhizal OTUs and *Saitozyma*
363 (*Cryptococcus*) and *Mortierella* OTUs had high betweenness scores at both local and
364 metacommunity levels, designated as metacommunity hubs (Fig. 5c; Table 1). Given the
365 above-mentioned prevalence of fungal OTUs allied to *Cladophialophora chaetospira* in the
366 cool-temperate metacommunity, the contrasting list of metacommunity hubs in the southern
367 (warm-temperate–subtropical) metacommunity implies that different taxonomic and
368 functional groups of fungi play major metacommunity-scale roles in different climatic regions.
369 This working hypothesis is partially supported by previous studies indicating endemism and
370 vicariance in the biogeography of fungi and bacteria [108, 109], promoting conceptual
371 advances beyond the classic belief that every microbe is everywhere but the environment
372 selects microbes colonizing respective local communities [110].

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

387 programs [20, 51], exploring fungi with highest potentials in each climatic/biogeographic
388 region will be a promising direction of research in conservation biology.

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

403 For more advanced applications in conservation biology and agriculture, continual
404 improvements of methods for analyzing metacommunity-scale networks are necessary. First,
405 while the fungal OTUs in our network analysis was defined based on the cut-off sequence
406 similarities used in other studies targeting "species-level" diversity of fungi [59, 61],
407 physiological functions can vary greatly within fungal species or species groups [14, 118].
408 Given that bioinformatic tools that potentially help us detect single-nucleotide-level variation
409 are becoming available [119], the resolution of network analyses may be greatly improved in
410 the near future. Second, although some computer programs allow us to infer functions of
411 respective microbial OTUs within network data [63, 120], the database information of
412 microbial functions remains scarce. To increase the coverage and accuracy of automatic
413 annotations of microbial functions, studies describing the physiology, ecology, and genomes
414 of microbes should be accelerated. With improved reference databases, more insights into the
415 metacommunity-scale organization of plant–fungus associations will be obtained by

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

435

436 **Conclusions**

437 By compiling datasets of below-ground plant–fungus associations in temperate and
438 subtropical forest ecosystems, we explored metacommunity-hub fungi, which were
439 characterized by broad geographic and host ranges. Such metacommunity-scale analyses are
440 expected to provide bird's-eye views of complex plant–microbe associations, highlighting
441 plant-growth-promoting microbes that can be applied to diverse plant taxa in various
442 environments. Given that endophytic fungi promoting the growth and pathogen resistance of
443 host plants can be isolated from forest soil (e.g., *Cladophialophora chaetospira* [99]), the list

of metacommunity-hub endophytic fungi featured in this study itself may include prospective species to be used in agriculture. By extending the targets of such network analyses to diverse 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 2-4, respectively. The Unix scripts for the bioinformatic pipeline and the R scripts for the

PERMANOVA and PERMDISP analyses are available as Additional files 1 and 4, 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.** Unix scripts for the bioinformatic pipeline.

**Additional file 2: Data S2.** Sample-level matrices of the eight forests examined.

**Additional file 3: Data S3.** Species-level matrices of plant–fungus associations.

**Additional file 4: Data S4.** Information of 8080 fungal OTUs analyzed.

**Additional file 5: Data S5.** R scripts for the PERMANOVA and PERMDISP analyses.

494 **Additional file 6: Figure S1.** Number of sequencing reads, interaction specificity, and local
495 betweenness.

496 **Additional file 7: Figure S2.** Structure of plant–fungus networks in each local forest.

497 **Additional file 8 Figure S3.** Locality information within the full metacommunity-scale
498 network.

499 **Additional file 9: Figure S4.** Metacommunity-scale network of cool-temperate forests.

500 **Additional file 10: Figure S5.** Metacommunity-scale network of warm-temperate and
501 subtropical forests.

502 **Additional file 11: Table S1.** Top-10 list of non-Glomeromycota OTUs with highest
503 betweenness within the subtropical metacommunity network.

504

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**Table 1** Top-10 list of non-Glomeromycota OTUs with highest betweenness within the metacommunity networks. In each of the three metacommunity-scale networks examined (full, cool-temperate, and warm-temperate/subtropical), fungal OTUs were ranked based on their betweenness centrality scores. As taxonomic information of Glomeromycota OTUs with high betweenness scores was redundant (e.g., *Glomus* spp. or Glomeraceae spp.), the top-10 list of non-Glomeromycota OTUs is shown. Taxonomy information of each OTU was inferred based on the query-centric auto-*k*-nearest-neighbor algorithm of reference database search [55, 56] and subsequent taxonomic assignment with the lowest common ancestor algorithm [62]. The results of the NCBI nucleotide Blast are also shown. For simplicity, the functional groups of fungi inferred with the program FUNGuild [63] were organized into several categories. See Data S4 (Additional file 4) for details of the categories and for full results including Glomeromycota and other fungal OTUs.

| 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% |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Northen 4 sites (cool-temperate) | | | | | | | | | | |
| F_0042* | - | - | Mortierellales | Mortierellaceae | *Mortierella* | Saprotroph/Endophyte | *Mortierella humilis* | KP714537 | 100% | 100% |
| F_0034* | Ascomycota | Eurotiomycetes | Chaetothyriales | Herpotrichiellaceae | *Cladophialophora* | Saprotroph/Endophyte | *Cladophialophora chaetospira* | KF359558 | 100% | 99% |
| 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*

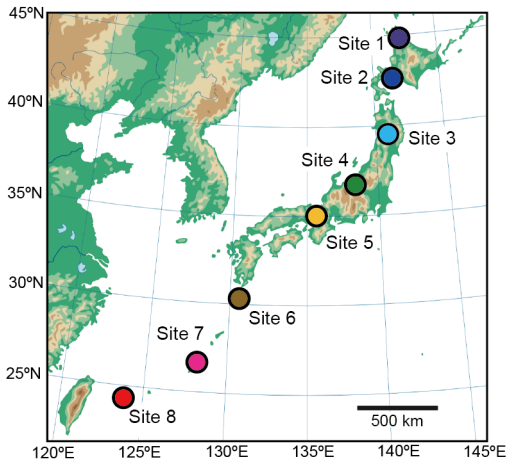


**Fig. 1** Study sites examined in this study. Across the entire range of the Japanese Archipelago, root samples were collected in four cool-temperate forests (sites 1–4), one warm-temperate forest (site 5), and three subtropical forests (sites 6–8).

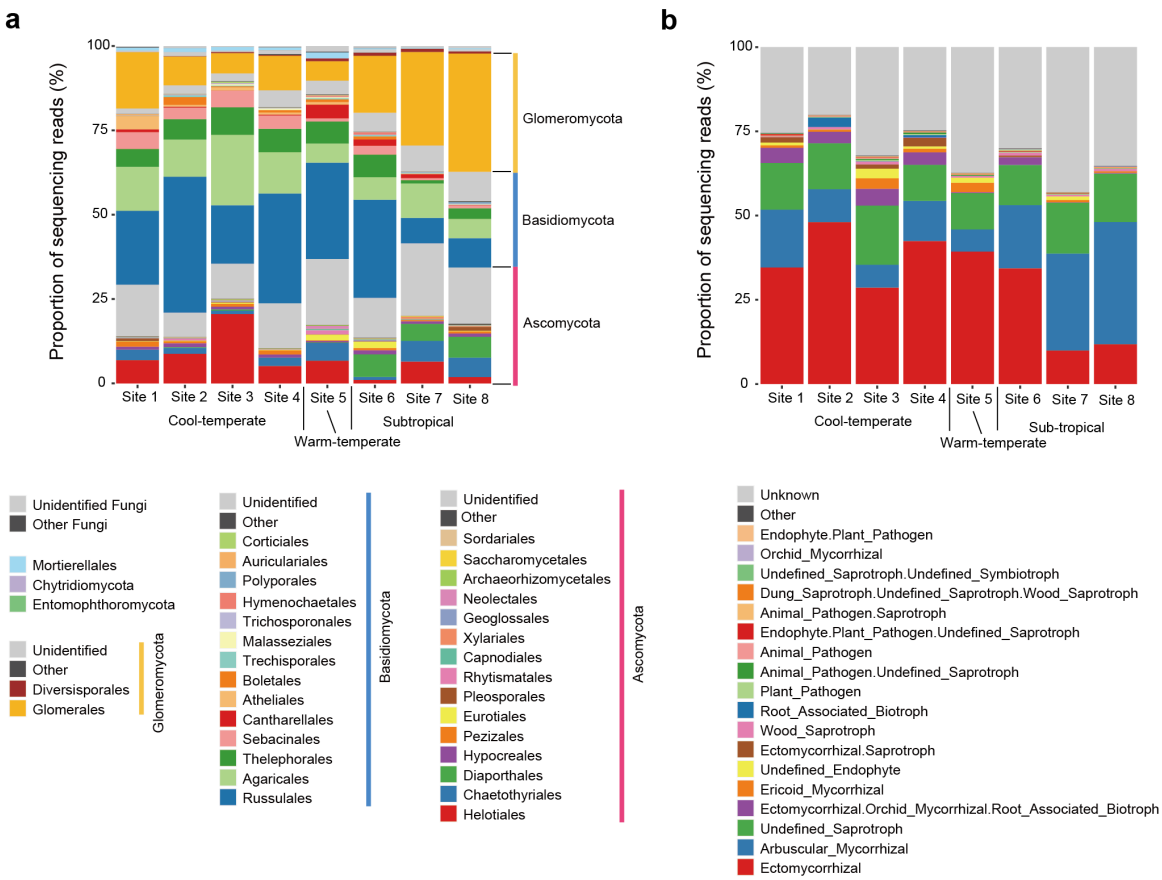


**Fig. 2** Compositions of fungal taxa and functional groups in each forest. **a** Order-level taxonomic composition of fungal OTUs in each locality. The number of fungal OTUs detected is shown in a parenthesis for each forest. **b** Functional-group composition. The fungal functional groups were inferred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

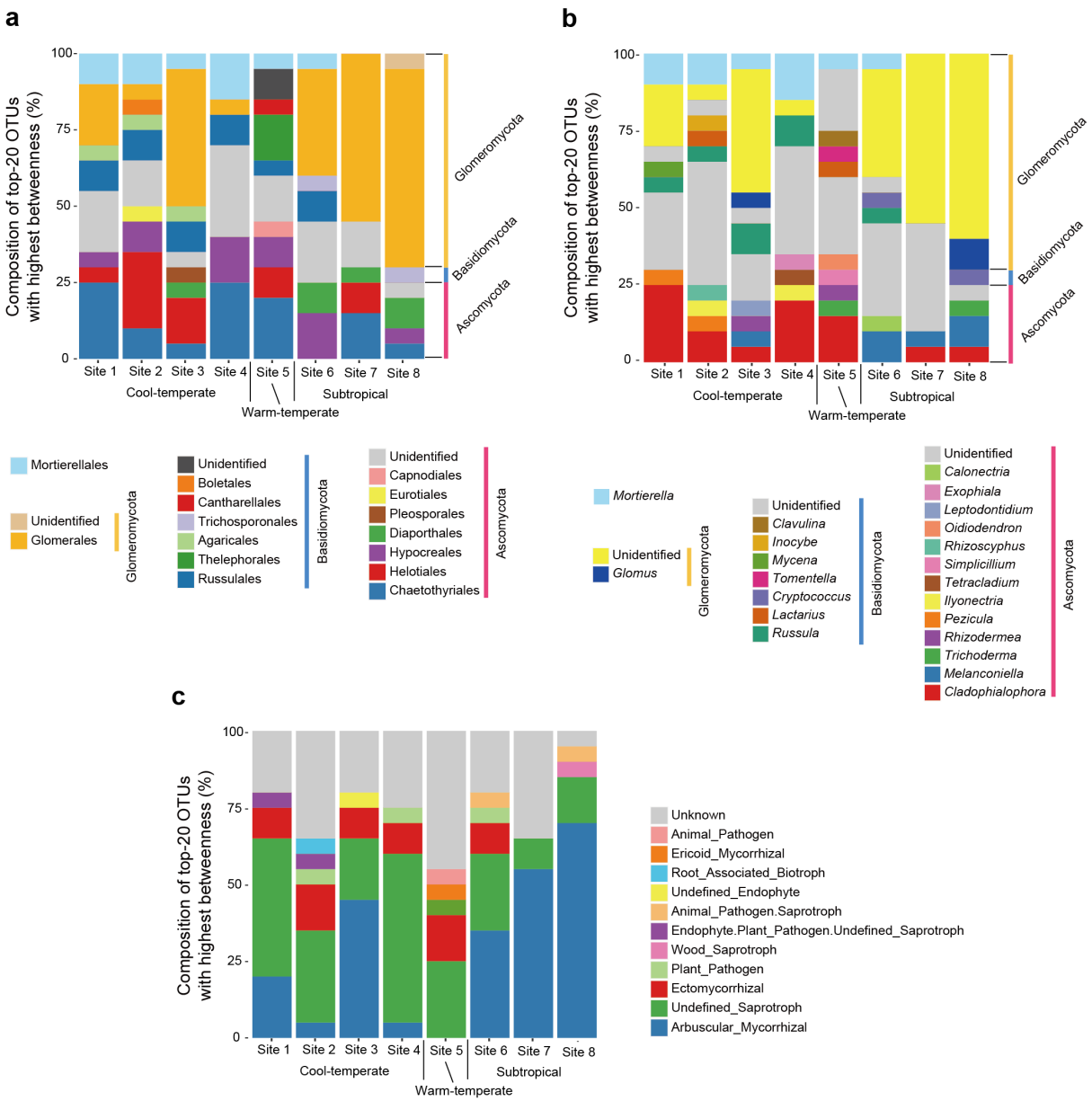


**Fig. 3** Fungal OTUs with highest local betweenness. **a** Order-level taxonomic composition of top-20 OTUs with highest local betweenness in each forest. See Data S4 (Additional file 4) for betweenness scores of all fungal OTUs in respective local forests. **b** Genus-level taxonomic composition of top-20 OTUs with highest local betweenness. **c** Functional-group composition of top-20 OTUs with highest local betweenness.

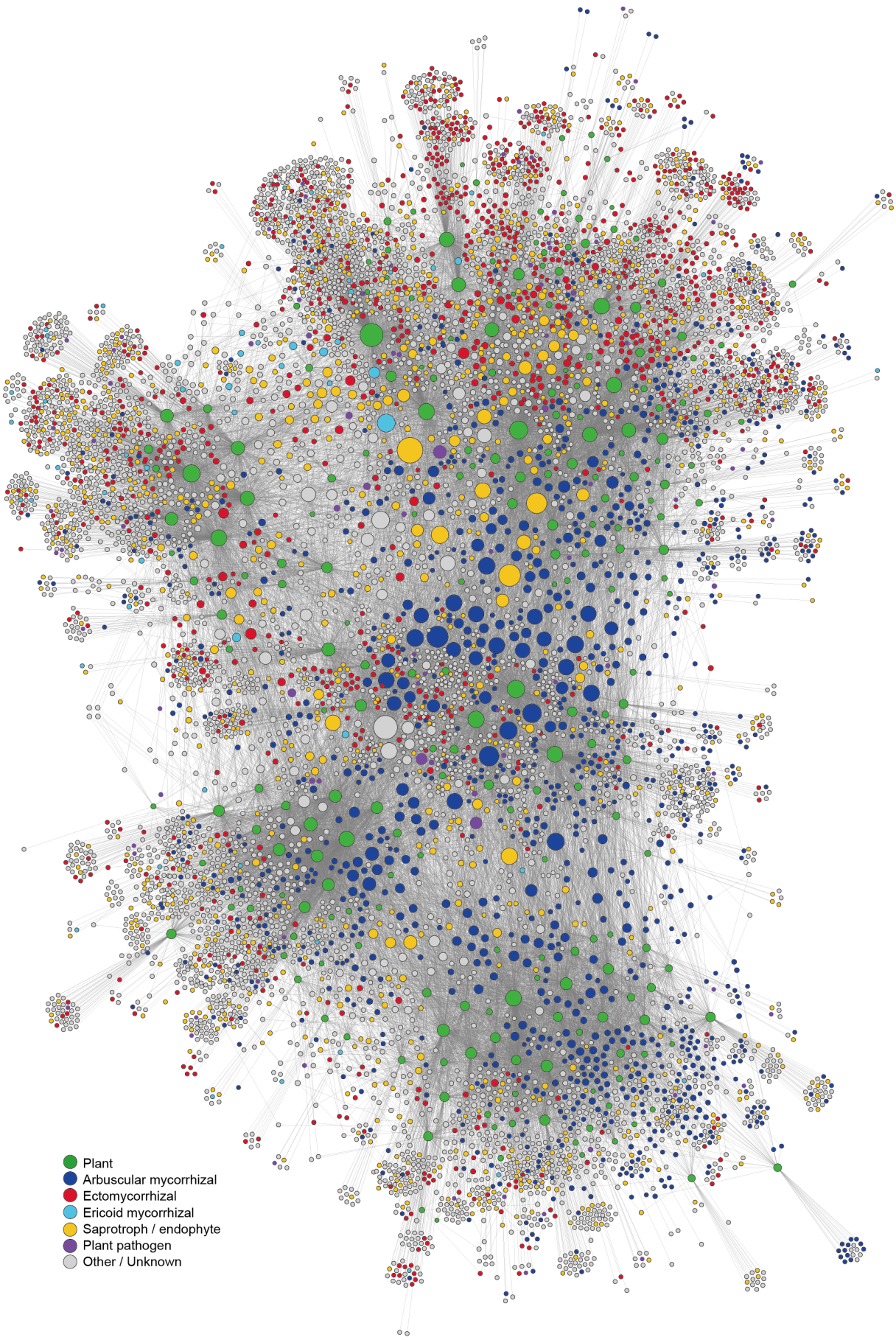
Plant
Arbuscular mycorrhizal
Ectomycorrhizal
Ericoid mycorrhizal
Saprotroph / endophyte
Plant pathogen
Other / Unknown

**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 4; Data S4). For plant species/taxa (green), the geographic information of source populations is indicated in Additional file 8 (Figure S3).

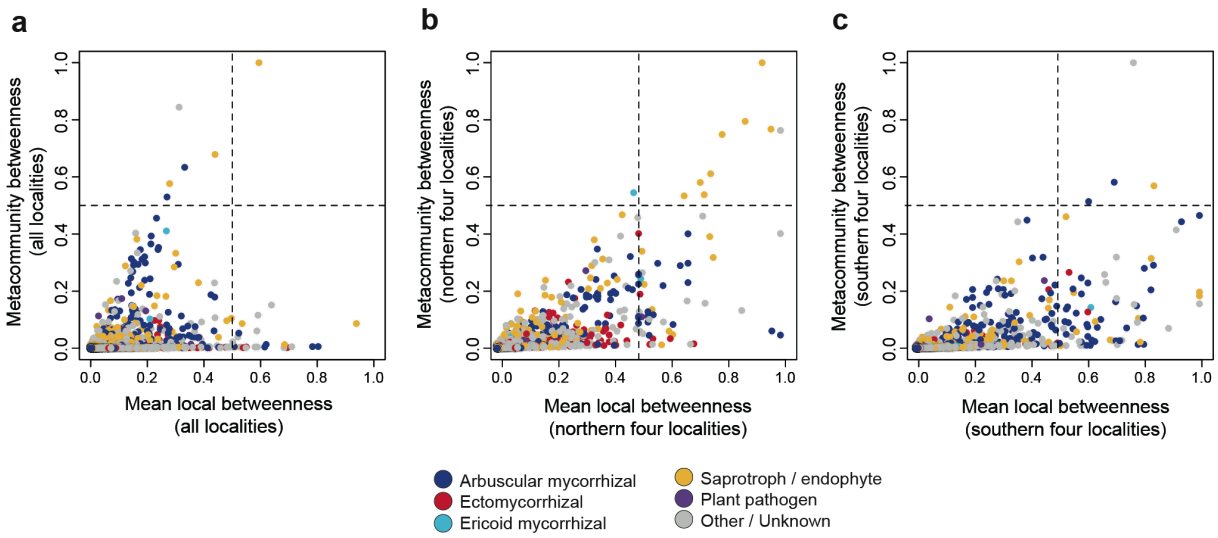


**Fig. 5** Relationship between local- and metacommunity-level betweenness. **a** Full meatcommunity. On the horizontal axis, the mean values of betweenness centrality scores across all the eight local forests are shown for respective fungal OTUs. On the vertical axis, the betweenness scores within the metacommunity-scale network consisting of the eight localities (Fig. 4) are shown for respective OTUs. **b** Metacommunity of cool-temperate forests. For the sub-dataset consisting of the four cool-temperate forests (Additional file 9: Figure S4), mean local betweenness and metacommunity betweenness are shown on the horizontal and vertical axes, respectively. **c** Metacommunity of warm-temperate and subtropical forests. For the sub-dataset consisting of the warm-temperate forest and the three subtropical forests (Additional file 10: Figure S5), mean local betweenness and metacommunity betweenness are shown on the horizontal and vertical axes, respectively.