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

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

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

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

165

166 **Local networks**

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

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

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

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

192

193 **Metacommunity-scale network**

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

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

216 $$B'_{\text{local},\,i} = \frac{B_{\text{local},\,i} - \min(B_{\text{local}})}{\max(B_{\text{local}}) - \min(B_{\text{local}})} \quad \text{and} \quad B'_{\text{meta},\,i} = \frac{B_{\text{meta},\,i} - \min(B_{\text{meta}})}{\max(B_{\text{meta}}) - \min(B_{\text{meta}})},$$

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; $F_{\mathrm{model}} = 34.9$, $P <$

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

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

264

265 **Metacommunity-scale network**

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

273 metacommunity betweenness (Table 1). Among the OTUs with high metacommunity
274 betweenness, only a *Mortierella* OTU was designated as a metacommunity hub (i.e., $\overline{B}'_{\mathrm{local},i}$
275 ≥ 0.5; $B'_{\mathrm{meta},i} \geq 0.5$) and others had low betweenness scores at the local community level
276 ($\overline{B}'_{\mathrm{local},i} < 0.5$; Fig. 5a).

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

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

296

297 **Discussion**

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

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

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

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

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

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

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

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

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

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

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

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

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

433

434 **Conclusions**

435 By compiling datasets of below-ground plant–fungus associations in temperate and
436 subtropical forest ecosystems, we explored metacommunity-hub fungi, which were
437 characterized by broad geographic and host ranges. Such metacommunity-scale analyses are
438 expected to provide bird's-eye views of complex plant–microbe associations, highlighting
439 plant-growth-promoting microbes that can be applied to diverse plant taxa in various
440 environments. Given that endophytic fungi promoting the growth and pathogen resistance of
441 host plants can be isolated from forest soil (e.g., *Cladophialophora chaetospira* [99]), the list
442 of metacommunity-hub endophytic fungi featured in this study itself may include prospective
443 species to be used in agriculture. By extending the targets of such network analyses to diverse
444 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.

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

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

498

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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 [60, 61] 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 S3 (Additional file 3) 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*

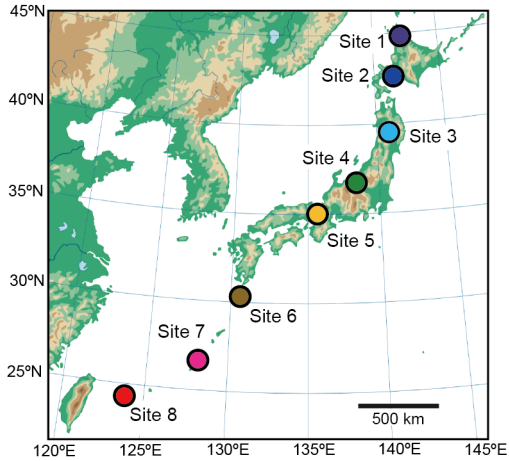

Fig. 1 Study sites examined in this study. Across the entire range of the Japanese Archipelago, root samples were collected in four cool-temperate forests (sites 1–4), one warm-temperate forest (site 5), and three subtropical forests (sites 6–8).

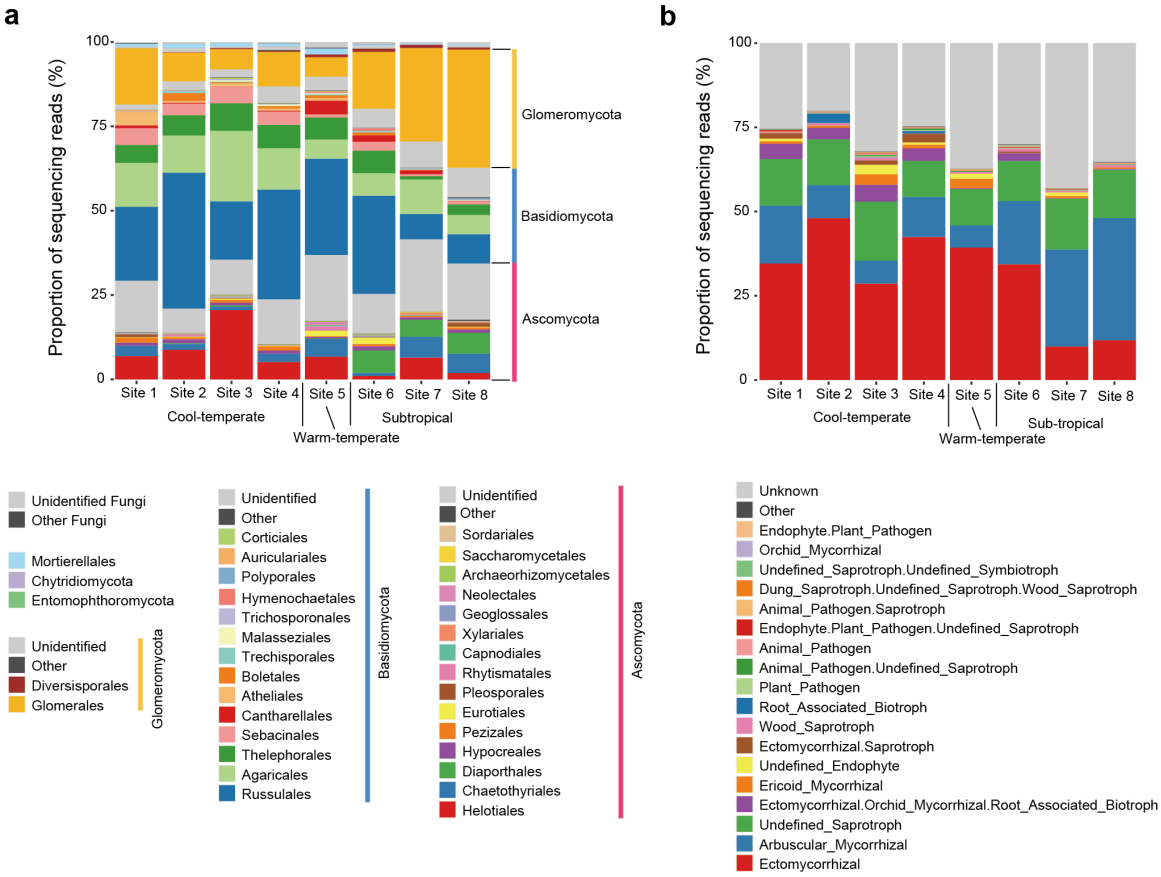

Fig. 2 Compositions of fungal taxa and functional groups in each forest. **a** Order-level taxonomic composition of fungal OTUs in each locality. The number of fungal OTUs detected is shown in a parenthesis for each forest. **b** Functional-group composition. The fungal functional groups were inferred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

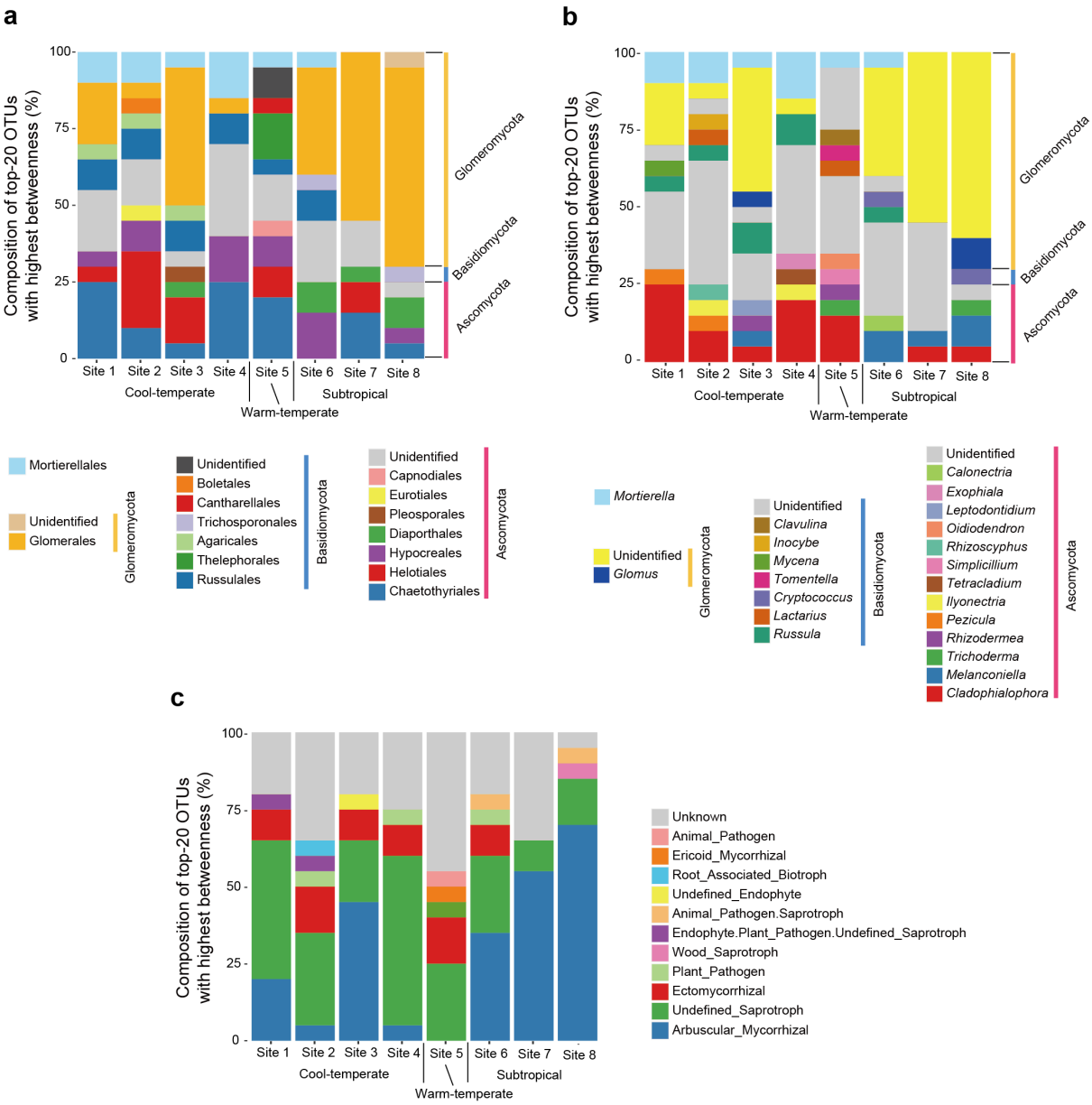

Fig. 3 Fungal OTUs with highest local betweenness. **a** Order-level taxonomic composition of top-20 OTUs with highest local betweenness in each forest. See Data S3 (Additional file 3) for betweenness scores of all fungal OTUs in respective local forests. **b** Genus-level taxonomic composition of top-20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

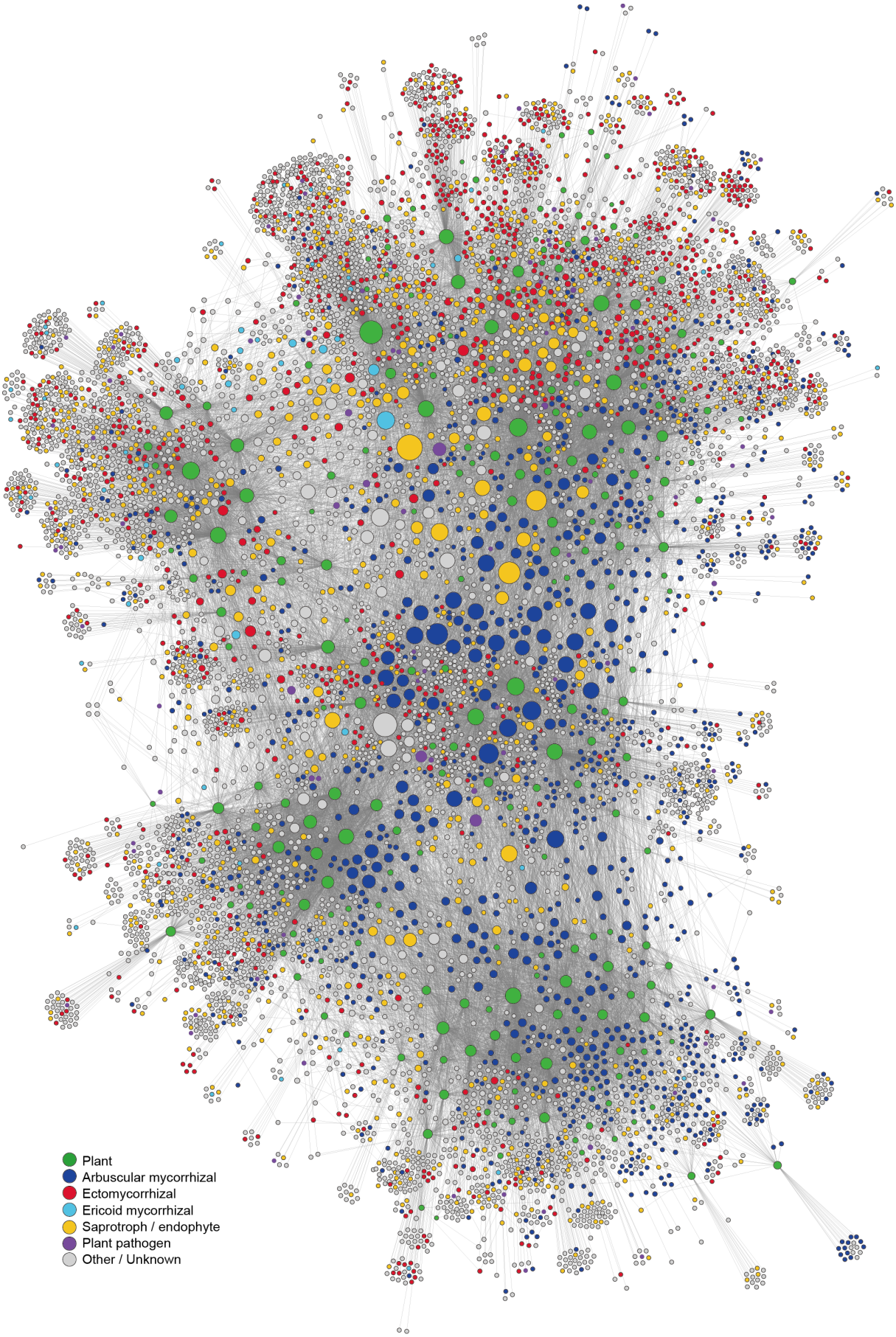
Plant
Arbuscular mycorrhizal
Ectomycorrhizal
Ericoid mycorrhizal
Saprotroph / endophyte
Plant pathogen
Other / Unknown

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).

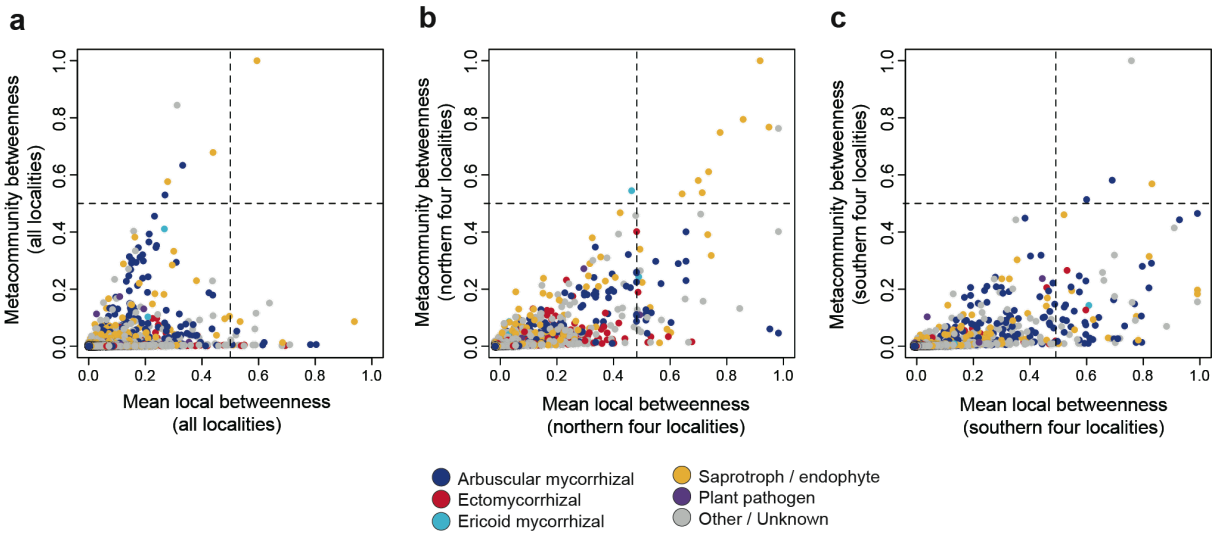

Fig. 5 Relationship between local- and metacommunity-level betweenness. **a** Full meatcommunity. On the horizontal axis, the mean values of betweenness centrality scores across all the eight local forests are shown for respective fungal OTUs. On the vertical axis, the betweenness scores within the metacommunity-scale 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 7: 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 8: Figure S5), mean local betweenness and metacommunity betweenness are shown on the horizontal and vertical axes, respectively.