1 # Network hubs in root-associated fungal

2 # metacommunities

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18

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

47   interactions; mycorrhizal and endophytic symbiosis.

48

Background

50   Below-ground fungi in the endosphere and rhizosphere are key drivers of terrestrial

51   ecosystem processes [1-4]. Mycorrhizal fungi, for example, are important partners of most

52   land plant species, enhancing nutritional conditions and pathogen resistance of host plants

53   [5-7]. In reward for the essential physiological services, they receive ca. 20% of net

54   photosynthetic products from plants [8, 9]. Recent studies have also indicated that diverse

55   taxonomic groups of endophytic fungi (e.g., endophytic fungi in the ascomycete orders

56   Helotiales and Chaetothyriales) commonly interact with plant roots, providing soil

57   nitrogen/phosphorous to their hosts [10-14], converting organic nitrogen into inorganic forms

58   in the rhizosphere [15], and increasing plants' resistance to environmental stresses [16-18].

59   Because of their fundamental roles, below-ground fungi have been considered as prospective

60   sources of ecosystem-level functioning in forest management, agriculture, and ecosystem

61   restoration [17-20]. However, due to the exceptional diversity of below-ground fungi [21-23]

62   and the extraordinary complexity of below-ground plant–fungus interactions [24-26], we are

63   still at an early stage of managing and manipulating plant-associated microbiomes [27-29].

64   In disentangling complex webs of below-ground plant–fungus associations, network

65   analyses, which have been originally applied to human relations and the World-Wide Web

66   [30, 31], provide crucial insights. By using network analytical tools, we can infer how plant

67   species in a forest, grassland, or farmland are associated with diverse taxonomic and

68   functional groups of fungi [24, 32-34]. Such information of network structure (topology) can

69   be used to identify "hub" species, which are placed at the center of a network depicting

70   multispecies host–symbiont associations [35] (cf. [34, 36, 37]). Those hubs with broad

71   host/symbiont ranges are expected to play key roles by mediating otherwise discrete

72   ecological processes within a community [19, 24]. For example, although arbuscular

73   mycorrhizal and ectomycorrhizal symbioses have been considered to involve distinct sets of

74   plant and fungal lineages [38] (but see [39, 40]), hub endophytic fungi with broad host ranges

75   may mediate indirect interactions between arbuscular mycorrhizal and ectomycorrhizal plant

76   species through below-ground mycelial connections. As information of plant-associated

77   fungal communities is now easily available with high-throughput DNA sequencing

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78   technologies [1, 21, 22], finding hub microbial species out of hundreds or thousands of

79   species within a network has become an important basis for understanding and predicting

80   ecosystem-scale phenomena.

81   Nonetheless, given that fungi can disperse long distances with spores, conidia, and

82   propagules [41-44], information of local-scale networks alone does not provide thorough

83   insights into below-ground plant–fungus interactions in the wild. In other words, no forests,

84   grasslands, and farmlands are free from perturbations caused by fungi immigrating from other

85   localities [45-49]. Therefore, to consider how local ecosystem processes are interlinked by

86   dispersal of fungi, we need to take into account "metacommunity-scale" networks of plant–

87   fungus associations [35]. Within a dataset of multiple local communities (e.g., [25]), fungal

88   species that occur in multiple localities may interlink local networks of plant–fungus

89   associations. Among them, some species that not only have broad geographic ranges but also

90   are associated with diverse host plant species would be placed at the core positions of a

91   metacommunity-scale network [35]. Such "metacommunity hub" fungi would be major

92   drivers of the synchronization and restructuring of local ecosystem processes (*sensu* [50]),

93   and hence their functional roles need to be investigated with priority [35]. Moreover, in the

94   screening of mycorrhizal and endophytic fungi that can be used in agriculture and ecosystem

95   restoration programs [17, 20, 51], analytical pipelines for identifying metacommunity hubs

96   will help us explore species that are potentially applied (inoculated) to diverse plant species

97   over broad geographic ranges of farmlands, forests, or grasslands. Nonetheless, despite the

98   potential importance of metacommunity hubs in both basic and applied microbiology, few

99   studies have examined metacommunity-level networks of plant–symbiont associations.

100   By compiling Illumina sequencing datasets of root-associated fungi [52], we herein

101   inferred a metacommunity-level network of below-ground plant–fungus associations and

102   thereby explored metacommunity hubs. Our metadata consisted of plant–fungus association

103   data in eight forest localities across the entire range of the Japanese Archipelago, including

104   150 plant species/taxa and 8,080 fungal operational taxonomic units (OTUs) in temperate and

105   subtropical regions. Based on the information of local- and metacommunity-level networks,

106   each of the fungal OTUs was evaluated in light of its topological positions. We then

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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.

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Methods

113 **Methods**

Terminology

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

Data

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;

6

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

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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

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

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

192

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

217   where $B_{\text{local},i}$ and $B_{\text{meta},i}$ were raw estimates of local- and metacommunity-scale

218   betweenness of a fungal OTU i, and min() and max() indicated minimum and maximum

219   values, respectively. For local betweenness of each OTU, a mean value across local networks

220   was subsequently calculated ($\bar{B}'_{\text{local},i}$): the local communities from which a target OTU was

221   absent was omitted in the calculation of mean local betweenness. On the two-dimensional

222   surface, the OTUs were then classified into four categories: metacommunity hubs having high

223   betweenness in both local- and metacommunity-scale networks ($\bar{B}'_{\text{local},i} \geq 0.5$; $B'_{\text{meta},i} \geq$

224   0.5), metacommunity connectors that had broad geographic ranges but displayed low local

225   betweenness ($\bar{B}'_{\text{local},i} < 0.5$; $B'_{\text{meta},i} \geq 0.5$), local hubs that had high betweenness in local

226   networks but not in the metacommunity-scale network ($\bar{B}'_{\text{local},i} \geq 0.5$; $B'_{\text{meta},i} < 0.5$), and

227   peripherals with low betweenness at both local and metacommunity levels ($\bar{B}'_{\text{local},i} < 0.5$;

228   $B'_{\text{meta},i} < 0.5$) [35]. Approximately, 1–2% of fungal OTUs show betweenness scores higher

229   than 0.5 in each local or metacommunity network, while the threshold value can be changed

230   depending on the purpose of each study [35].

231   In addition to metacommunity hubs within the metacommunity-scale network

232   representing all the eight localities, those within the metacommunity-scale network

233   representing northern (sites 1–4) or southern (sites 5–8) four localities were also explored.

234   This additional analysis allowed us to screen for fungal OTUs that potentially adapted to

235   broad ranges of biotic and abiotic environments within northern (cool-temperate) or southern

236   (warm-temperate or subtropical) part of Japan.

237

Results

Local networks

240   Among the eight forest localities, order-level taxonomic compositions of fungi varied

241   significantly (PERMANOVA; $F_{\text{model}} = 35.7$, $P < 0.001$), while the differentiation of

242   community structure was attributed at least partly to geographic variation in among-sample

243   dispersion (PERMDISP; $F = 13.2$, $P < 0.001$) (Fig. 2a). Compositions of fungal functional

244   groups were also differentiated among the eight localities (PERMANOVA; $F_{\text{model}} = 34.9$, $P <$

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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

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

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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., $\overline{B}'_{\text{local},i}$

275   ≥ 0.5; $B'_{\text{meta},i} \geq 0.5$) and others had low betweenness scores at the local community level

276   ($\overline{B}'_{\text{local},i} < 0.5$; 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

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

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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

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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.

14

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

15

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

16

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

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

17

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

446   developed.

447

Abbreviations

448

449   DDBJ: DNA Data Bank of Japan; ITS: internal transcribed spacer; OTU: Operational

450   taxonomic unit; PERMANOVA: permutational analysis of variance; PERMDISP:

451   permutational analysis for the multivariate homogeneity of dispersions; rRNA: ribosomal

452   ribonucleic acid.

453

464

Availability of data and materials

465

466   The Illumina sequencing data were deposited to DNA Data Bank of Japan (DDBJ Sequence

467   Read Archive: DRA006339). The raw data of fungal community structure and the fungal

468   community matrices analyzed are available with the source study [52] and Additional files

469   1-3, respectively.

470

Authors' contributions

471

472   HT designed the work. HT, AST, and HS conducted fieldwork. HT performed the molecular

473   experiments. HT wrote the manuscript with AST and HS.

474

475   **Competing interests**

476   The authors declare that they have no competing interests.

477

478   **Consent for publication**

479   Not applicable

480

481   **Ethics approval and consent to participate**

482   Not applicable

483

484   **Additional files**

485   **Additional file 1: Data S1.** Sample-level matrices of the eight forests examined.

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

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

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

489   betweenness.

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

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

492   network.

493   **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)
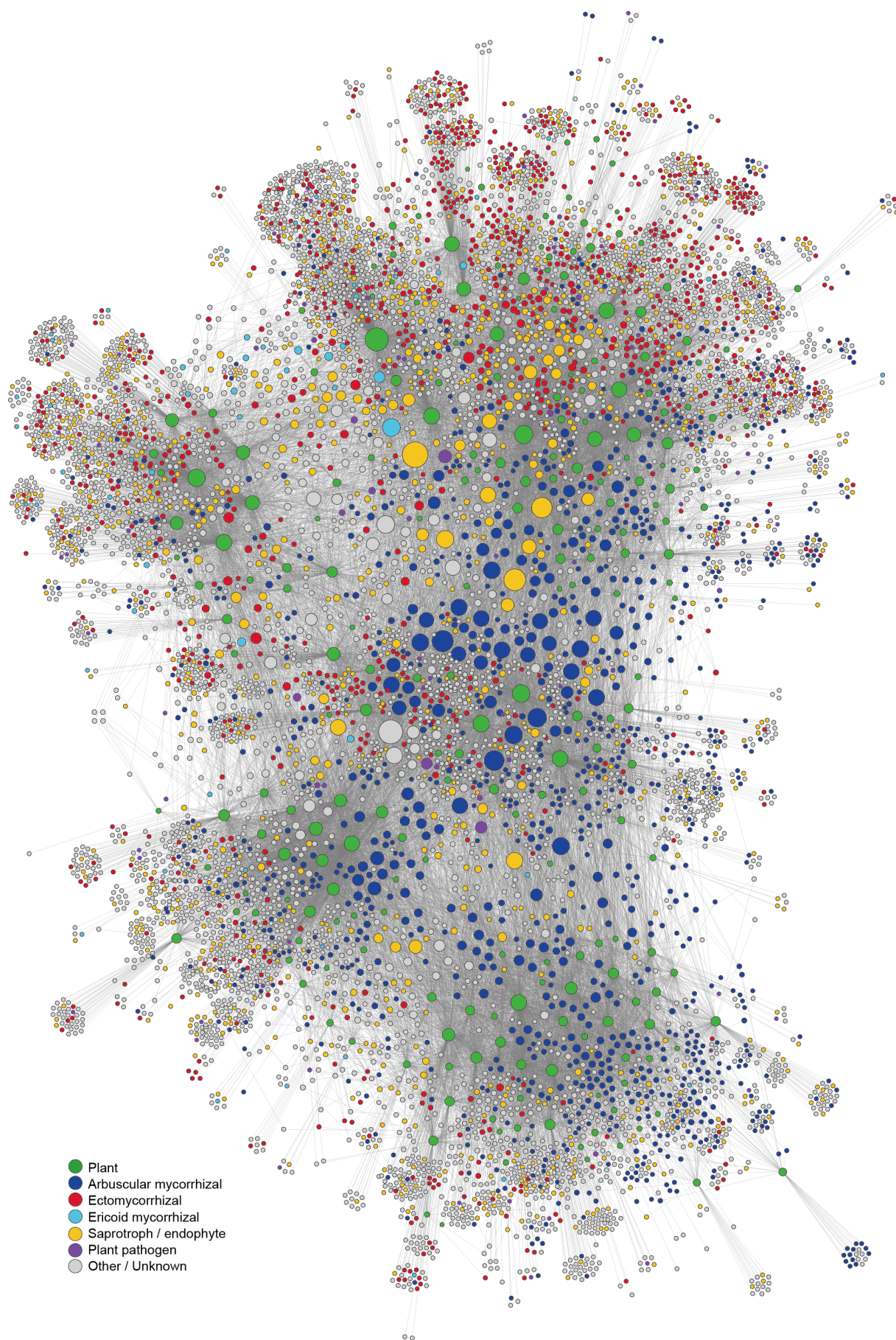
†Synonym, *Cryptcoccus podzolica*
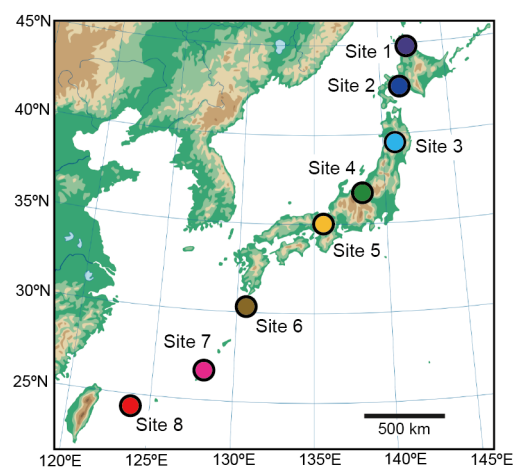
Fig. 1 Study sites examined in this study. Across the entire range of the Japanese Archipelago, root samples were collected in four cool-temperate forests (sites 1–4), one warm-temperate forest (site 5), and three subtropical forests (sites 6–8).
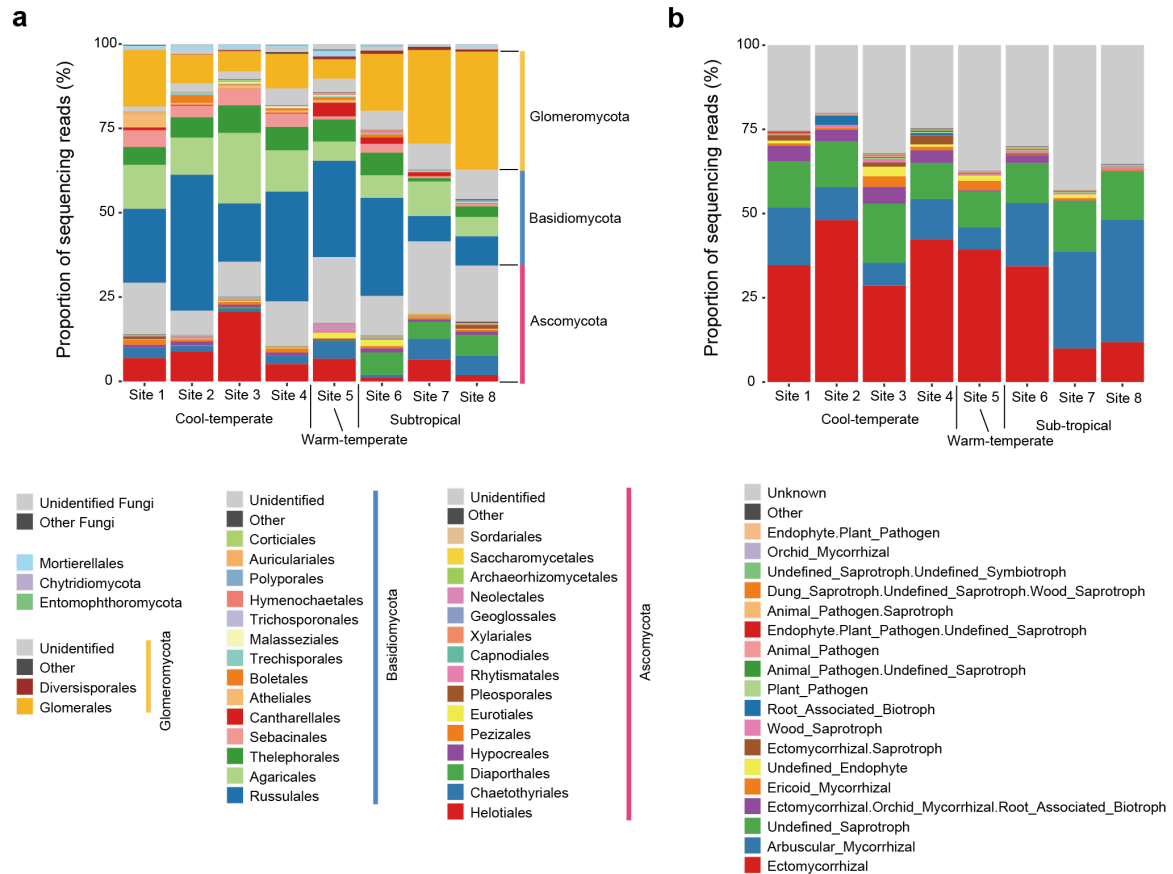
Fig. 2 Compositions of fungal taxa and functional groups in each forest. **a** Order-level taxonomic composition of fungal OTUs in each locality. The number of fungal OTUs detected is shown in a parenthesis for each forest. **b** Functional-group composition. The fungal functional groups were inferred by the program FUNGuild [63].
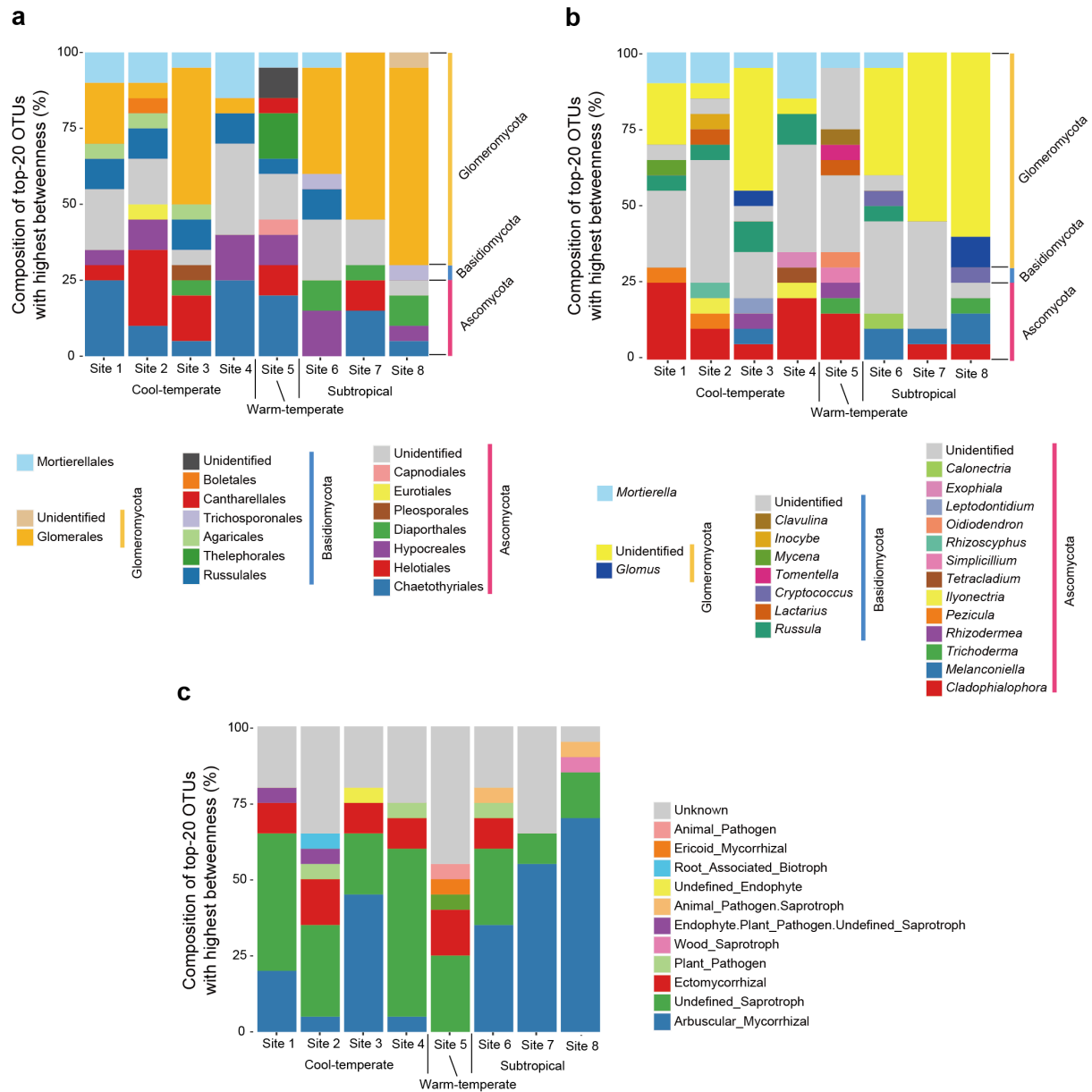
Fig. 3 Fungal OTUs with highest local betweenness. **a** Order-level taxonomic composition of top-20 OTUs with highest local betweenness in each forest. See Data S3 (Additional file 3) for betweenness scores of all fungal OTUs in respective local forests. **b** Genus-level taxonomic composition of top-20 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).
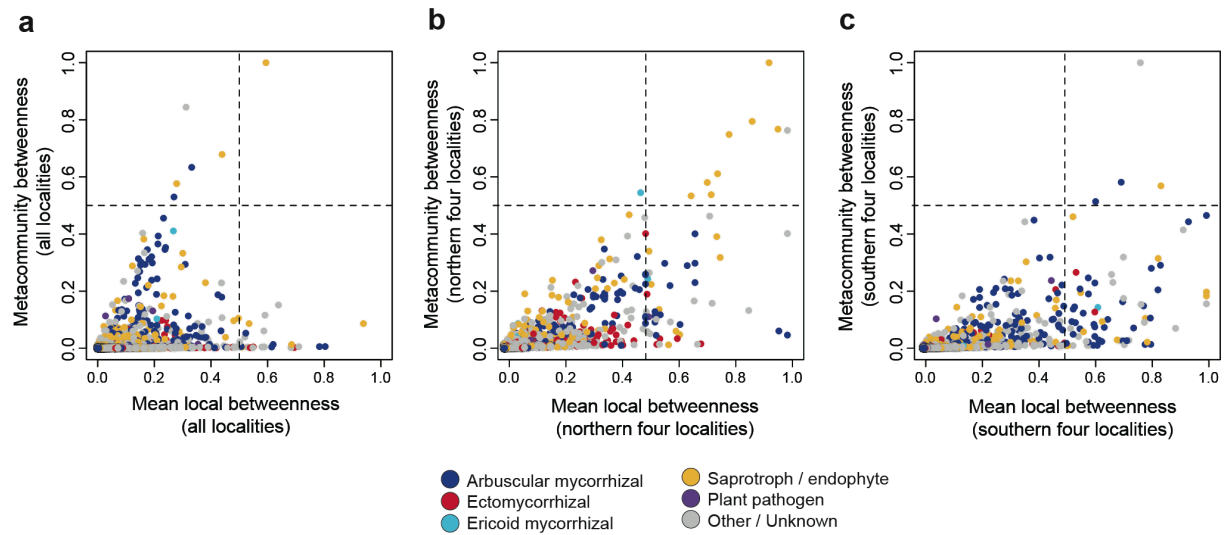
Fig. 5 Relationship between local- and metacommunity-level betweenness. **a** Full meatcommunity. On the horizontal axis, the mean values of betweenness centrality scores across all the eight local forests are shown for respective fungal OTUs. On the vertical axis, the betweenness scores within the metacommunity-scale 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.