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REGULAR ARTICLE

Colonization status and community structure of arbuscular mycorrhizal fungi in the coniferous tree, *Cryptomeria japonica*, with special reference to root orders

Yosuke Matsuda · Kohei Kita · Yudai Kitagami · Toko Tanikawa

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Abstract

Aims Arbuscular mycorrhizal (AM) fungi are intimately associated with fine roots and are involved in nutrient acquisition. However, little information is available on the links between roots of individual orders and fungus colonization and community structure. Our aim was to elucidate AM fungal communities in the fine root systems of the temperate coniferous tree species, *Cryptomeria japonica* (Cupressaceae).

Methods We characterized the morphological traits of AM fungi microscopically and determined the community structure of AM fungi using metabarcoding with an Ion Torrent Personal Genome Machine (PGM) focusing on lower-order roots from first to third order roots.

Results Paris-type and Arum-type AM morphologies were both generally more prevalent on first-order roots than on second- or third-order roots, but the colonization rates by the Paris type were higher than those by the Arum type. We found a total of 48 fungal operational taxonomic units dominated by Glomeraceae, and all the AM taxa detected on third order roots were also found on first and/or second order roots. In the case of the second and third orders, AM fungal communities were affected by soil conditions: electrical conductivity, pH, and N concentration.

Conclusion These results suggest that the abundance and species richness of AM fungi vary among lower root order systems, and that the AM community is sensitive to soil conditions and turns over as roots age.

Keywords Arbuscular mycorrhiza · Colonization · Community structure · Fine root order · Metabarcoding · Nestedness

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Introduction

Japanese cedar, *Cryptomeria japonica* (Cupressaceae), is an endemic coniferous tree species in Japan, with its natural distribution from the northern part of Honshu to southern part of Kyushu,

Yakushima island (Ohashi 2015). Extensive loggings in the past has reduced the natural population of the species that has been designated as near threatened by the IUCN Red List (Thomas et al. 2013). However, the species has been planted throughout Japan as well as in neighboring countries, and the timber of the species has been used widely since more than several hundred years ago as material in various ways, e.g. daily commodities, houses, and ships (Ohba 1993). Currently, *C. japonica* is one of the principal planted trees in Japan, which produces the largest volume of logs accounting for 58% among domestic supplies (Forestry Agency 2019). In terms of forest management, the water shortage caused by climate change is a concern for the accelerating decline of *C. japonica* forests in the future (Matsumoto et al. 2006). In addition, edaphic conditions, such as soil acidity and calcium content in the forests can, in turn, affect other edaphic and root growth conditions (Tanikawa et al. 2014; Wada et al. 2019). However, limited information is available on root-associating fungi, or mycorrhizal fungi, of this species (e.g. Yamato and Iwasaki 2002; Hishi et al. 2017), which are partly responsible for nutrient and water acquisition for plant growth (Smith and Read 2008).

Among mycorrhizal fungi, arbuscular mycorrhizal (AM) fungi are associated with the fine roots of a wide array of plant taxa covering as many as about 80% of land plants (Smith and Read 2008; Brundrett and Tedersoo 2018) that are distributed mainly in seasonal and warm tropical forests (Steidinger et al. 2019). Taxonomically, more than 200 fungal species or as many as 563 operational taxonomic units in the phylum Glomeromycota are involved in AM associations (Kivlin et al. 2011). Structurally, the roots associated with AM fungi are discriminated into either Arum or Paris types (Gallaud 1905; Smith and Read 2008), and the hyphal morphologies of these types within host cells are defined respectively as finely branched or coiled. These types sometimes occur simultaneously within one host plant, in which case, the type is defined as intermediate (Dickson et al. 2007). Although the functional significance of this morphological plasticity is not well understood, the formation of either Arum or Paris types on forest-floor plants might reflect the plants' levels of autotrophy (Giesemann et al. 2020) and may thus imply functional variations between these types of AM roots. In this respect, gaining an understanding of AM communities through the analysis of morphological types could enable us to identify possible functional plasticity within fine-root systems.

In natural habitats, AM fungi are accommodated within plant roots around the world, and AM taxa are shared among distant continents and biomes (Davison et al. 2015). At a regional scale, however, AM communities are likely affected by vegetation type (Neuenkamp et al. 2018) and host-plant turnover (Davison et al. 2016), as well as by abiotic factors, such as soil pH and N content (Neuenkamp et al. 2018), and climate (Kohout et al. 2015). Although the community structures of AM fungi have been clarified at larger spatial scales (e.g. Öpik et al., 2013; Davison et al., 2015; Pärtel et al., 2017), few studies have focused on the structure of AM communities in relation to the architecture of fine-root systems, which is likely to be intimately associated with plant nutrient uptake (McCormack et al. 2017; Freschet et al. 2021).

Root traits, including AM associations, have received less attention in woody plants than in herbaceous plants, and thus little information is available regarding the former (Eissenstat et al. 2015; Valverde-Barrantes et al. 2016; Ma et al. 2018). Among the traits, fine roots play a pivotal role in nutrient cycling via mineral acquisition from the soil (Freschet et al. 2021). The roots which are generally deemed to be 2 mm or less in diameter (McCormack et al. 2015) are intimately involved in nutrient acquisition in cooperation with mycorrhizal fungi (Guo et al. 2008; Smith and Read 2008). However, the function of roots may change with secondary development (Pregitzer et al. 2002; Guo et al. 2008; Yin et al. 2020), and the roots become less involved in nutrient absorption and more involved in nutrient translocation (Brundrett 2002; Valenzuela-Estrada et al. 2008). In this context, root order may be useful in distinguishing structural and functional differences within root systems (Pregitzer et al. 2002). Lower-order roots have a greater capacity than higher-order roots for nutrient acquisition with the aid of mycorrhizal fungi (Eissenstat et al. 2015; McCormack et al. 2015) and, therefore, one might expect the abundance of mycorrhizal fungi and the structure of their communities to differ depending on root order.

In forest ecosystems, AM associations have been studied extensively in the last several decades, but relatively few studies have focused on coniferous trees (e.g. Hart et al. 2016; Gorzelak et al. 2017; Li

et al. 2019). For *C. japonica*, AM associations were known since as early as the beginning of the twentieth century (Mimura 1917). Although the morphology of AM fungi and the colonization of roots by AM fungi have been studied (Fujimaki et al. 2001; Yamato and Iwasaki 2002; Hishi et al. 2017), the links between AM fungal morphological traits and AM fungal community patterns have not yet been clarified. Root traits of *C. japonica* may vary depending on the soil nutrient conditions such as inorganic nitrogen content (Wada et al. 2019). Moreover, lower-order roots in the species may vary anatomically and architecturally (Hishi et al. 2007; Tawa and Takeda 2015). Therefore, an understanding of AM fungal morphologies and of fungal associations with fine root systems consecutively from first- to third-order roots would provide us with information on community development of AM fungi in the field (Johnson 2015).

Our aim here was to characterize AM fungal communities in the fine root systems of *C. japonica*. We examined the colonization patterns and community structures of AM fungi on roots of different orders. For this purpose, we microscopically examined first- to third-order roots, separately, and evaluated their AM colonization rates and the morphological types of the fungi. We then subjected subsets of the roots to comprehensive DNA analyses to infer the AM taxa involved. We hypothesized that lower-order roots would be associated with more AM fungal taxa and with higher colonization rates. In addition, we expected that the morphological types and assemblage patterns of AM fungi would vary among different root orders because of the differences in ages and/or nutrition status of the orders.

Materials and Methods

Study sites

Study sites were established at seven *C. japonica* forests in five prefectures in central Japan (Fig. 1): Mayumi (MY, 34°31′N, 136°33′E) and Wakide (WK, 34°37′N, 136°54′E) in Mie Prefecture; Hoki (HK, 34°94′N, 135°60′E) in Osaka Prefecture; Kuroi (KR, 35°25′N, 135°6′E) in Hyogo Prefecture; and Maruoka (MR, 36°12′N, 136°32′E), Komatsu (KM, 36°36′N, 136°49′E), and Shimokarakawa (SM, 37°22′N, 136°76′E) in Ishikawa Prefecture (Table 1). The forests had been planted between 45 and 66 years earlier and the trees had diameters at breast height ranging from 26 ± 1.2 to 37 ± 0.7 cm (mean ± SE, Table 1). At all the sites, there was no history of fertilization and the sites are located on middle slopes facing south to south-west directions with moderate inclinations, i.e. sloping land (FAO 2006). The ground vegetation in the sites consisted of sporadic cover with unidentified fern species, as well as shrubs or *Quercus serrata* saplings (at KM), *Eurya japonica* (at KR, MY, and WK), and *Rhododendron dilatatum* (at KM). At all sites, soils were classified as Brown Forest Soil according to the classification system of forest soils in Japan (Forest Soil Division 1976), or as Inceptisol in US soil taxonomy (Soil Survey Staff 2010). Mean air temperatures and annual precipitation, recorded in 2016 and 2017 at the Japan Meteorological Agency weather stations closest to each site, are shown in Table 1.

Soil collection

In 2016 and 2017, to collect cedar roots in the forests, after measuring and then removing the litter layer we sampled soil blocks measuring 15 × 15 × 15 cm, where most fine roots are distributed (Konôpka et al. 2006). The blocks were brought back to the laboratory in a cooler box and kept at 4 °C until use in the following processes. To construct clone libraries of AM fungi associated with *C. japonica*, four blocks were collected at each corner of a 10 × 10-m plot at each site in June or July 2016, and the fine roots retrieved were used for DNA analyses. In 2017, we collected one soil block at three out of four corners of a 100 × 100-m plot at the same sites as in 2016 between June and August to explore wider areas of AM associations. At the time of the collections, soil temperature, soil water content, and electrical conductivity (EC) at 5 cm depth were measured with a soil moisture sensor (WET-2, Delta-T, Cambridge, UK) set on "organic" mode.

Root systems in soil blocks were washed in a 2-mm-mesh sieve under running tap water to remove adhering soil particles. The root systems were transferred to a tray filled with distilled water, and were examined for fine root morphologies carefully, e.g. surface color, diameter and branching

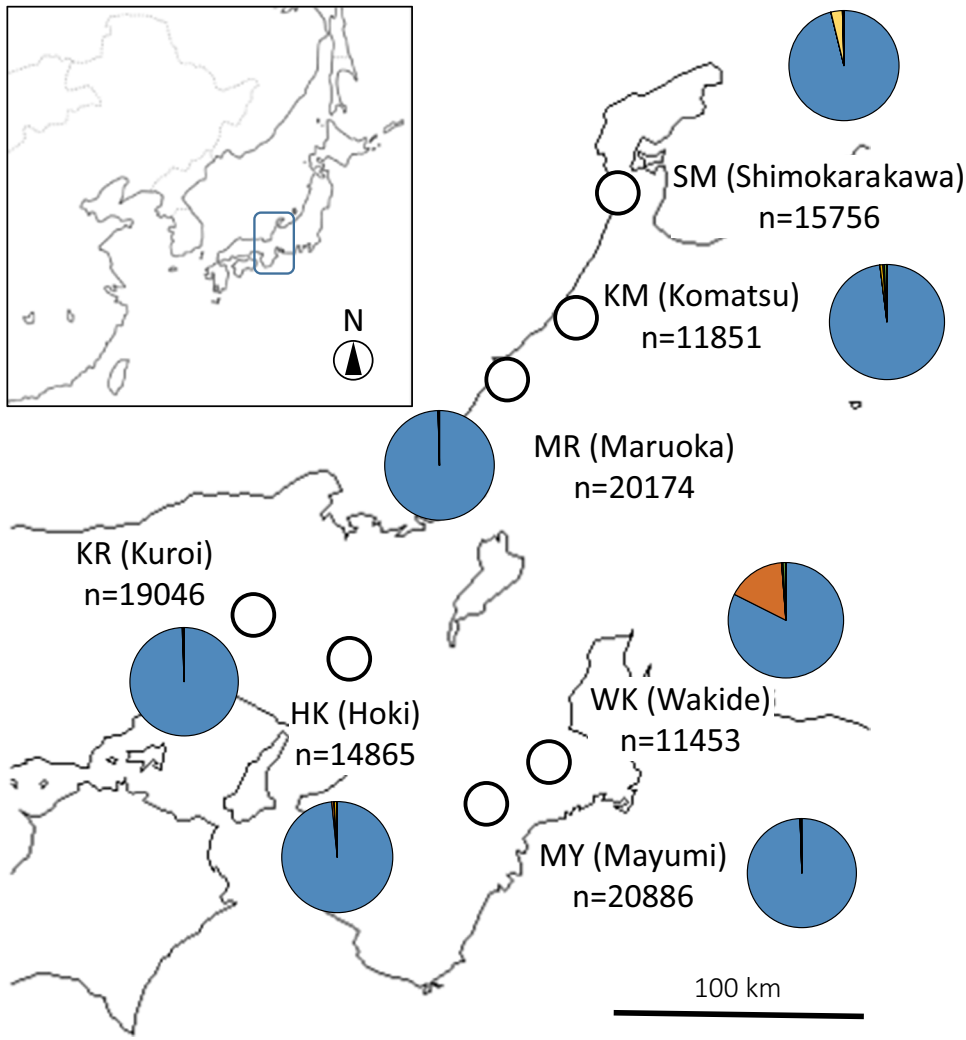

Fig. 1 Study sites in seven *Cryptomeria japonica* forests in central Japan and abundance of arbuscular mycorrhizal fungal taxa. Pie charts next to site names indicate the percentages of inferred AM taxa on roots of *Cryptomeria japonica* of all three orders. The pie charts in blue, orange and yellow parts are of Glomeraceae, Acaulosporaceae and Diversisporaceae families, respectively. Numbers below site codes indicate sequence reads obtained by next-generation sequencing

patterns, to retrieve *C. japonica* roots while avoiding roots of other species. For clone library construction from the 2016 samples, overall 5 cm in length of first-order roots and 5 cm of second-order roots were chosen from each sample block, i.e. 28 samples in each order root, and preserved in 2-ml centrifuge tubes at –20 °C for DNA analysis. In the case of the 2017 samples, first-, second-, and third-order roots were separately preserved in 2-ml centrifuge tubes for C/N ratio analyses, light microscopy, and next-generation sequencing (NGS). For the latter two analyses, we used a total of 5 cm of root length per order per sample block. About 50 mg of each order roots per site was used for the C/N analysis by using an elemental analyzer (vario EL cube, Elementar Japan Ltd., Yokohama, Japan).

Subsets of soil samples collected in 2017, which were air-dried soils that were passed through a 2-mm sieve were assessed for soil pH (H_2O) with a soil: H_2O ratio of 1:2.5 (w:v) by using a pH meter (MP220, Mettler-Toledo, Greifensee, Switzerland). The air-dried soil samples, as well as preserved root samples that had been dried at 105 °C for 24 h, were separately ground for the analysis of total C

Table 1 Study site conditions in seven *Cryptomeria japonica* forests

Study sites (code, prefecture)	Latitude	Longitude	MAP (mm) *		MAT (°C) *		Tree ages	DBH (cm)**	Soil conditions***				
			2016	2017	2016	2017			EC (S/m)	pH (H_2O)	C (%w)	N (%w)	C/N
Mayumi (MY, Mie)	34°31′N	136°33′E	2889	2584	16.6	15.8	61	34 ± 1.0	18.7 ± 0.6	5.2 ± 0.4	12.7 ± 3.7	0.8 ± 0.2	15.2 ± 1.3
Wakide (WK, Mie)	34°37′N	136°54′E	2317	2128	16.6	15.7	51	37 ± 0.7	21.7 ± 20.2	4.6 ± 0.3	10.1 ± 1.8	0.7 ± 0.2	15.6 ± 1.2
Hoki (HK, Osaka)	34°94′N	135°60′E	1529	1436	16.9	15.8	56	32 ± 1.2	43.7 ± 7.1	5.8 ± 0.7	7.3 ± 3.9	0.5 ± 0.2	14.4 ± 1.2
Kuroi (KR, Hyogo)	35°25′N	135°06′E	1959	1903	15.2	14.2	66	30 ± 1.0	16.7 ± 8.0	5.0 ± 0.2	11.8 ± 3.2	0.8 ± 0.1	15.0 ± 1.6
Maruoka (MR, Fukui)	36°12′N	136°32′E	2027	2507	15.6	14.6	60	26 ± 1.2	45.0 ± 19.3	5.5 ± 1.2	4.0 ± 0.9	0.3 ± 0.1	15.6 ± 0.8
Komatsu (KM, Ishikawa)	36°36′N	136°49′E	2127	2253	15.3	14.4	49	28 ± 1.3	35.0 ± 9.5	5.2 ± 0.8	7.7 ± 4.4	0.4 ± 0.2	17.3 ± 3.4
Shimokarakawa (SM, Ishikawa)	37°22′N	136°76′E	1873	2140	12.7	11.8	45	34 ± 1.8	38.7 ± 3.2	5.2 ± 0.4	6.1 ± 1.4	0.4 ± 0.1	13.5 ± 0.9

*Mean annual precipitation, MAP, and mean air temperature, MAT, were recorded at the closest AMeDAS weather stations (Japan Meteorological Agency) from each site

**Diameter at breast height is shown, means (n = 10) ± standard errors

***Soil conditions of electric conductivity (EC), pH, carbon (C), nitrogen (N) and CN are shown, means (n = 3) ± standard errors

and total N concentrations by using the elemental analyzer.

Microscopy of fine roots

Light microscopic examination of the AM structures of fine roots followed the method of McGonigle et al. (1990), with slight modifications. Each root sample was removed from its 2-ml centrifuge tube and placed in a perfluoroalkoxy alkane container (PF-90, Maruemu, Japan) which is thermo-, chemical- and pressure-tolerant. It was then immersed in a 10% (w/v) potassium hydroxide solution and decolorized by heat treatment at 121 °C for 15 min. The samples were then stained with either 0.05% trypan blue solution or 0.03% chlorazol black E solution. To improve the clarity of the microscopic images of cortical cells, the stele parts were manually excluded under a stereomicroscope. The remaining cortical cells were further broken into pieces in 1.5 ml tubes by pestles and spread over glass slides. AM fungal structures were observed under a light microscope (BX51, Olympus, Tokyo) with a maximum of 400 × magnification. Hyphal structures found within the host plant cells were assigned as either hyphal coils, arbusculate coils, or arbuscules until the cell count reached 500. AM fungal colonization was defined as the percentage of AM structures (i.e. hyphal coils, arbusculate coils, and arbuscules) among the total number of host cells examined. In addition, AM with either hyphal coils or arbusculate coils were defined as Paris types, and AM with arbuscules only were defined as Arum types.

DNA analyses of fine roots

Genomic DNA was extracted from root tips kept at –20 °C by using a DNeasy Plant Mini Kit (Qiagen, Tokyo) in accordance with the manufacturer's instructions. PCR amplification was conducted on the 2016 samples, with a primer set of AMV4.5NF (5′-AAGCTCGTAGTTGAATTTCG-3′) (Sato et al. 2005) and AML2 (5′- GAACCCAAACACTTTGGT TTCC-3′) (Lee et al. 2008), by using TaKaRa Ex Taq (TaKaRa, Ohtsu, Japan) in accordance with the manufacturer's recommendations. PCR was performed with a TaKaRa PCR Thermal Cycler Dice (Model TP600, TaKaRa) with 30 cycles at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s. A negative control

was used in each PCR run. Positive DNA bands were subsequently subjected to cloning analyses, which were conducted by using a TA-Enhancer Cloning Kit (Nippon Gene, Toyama, Japan) in accordance with the manufacturer's instructions. For each positive sample, 15 to 27 colonies were subjected to PCR using an EmeraldAmp MAX PCR Master Mix Kit (TaKaRa) with the same primer set as above. Positive PCR samples were digested with Exo-SAP-IT PCR product cleanup reagent (Affymetrix, Tokyo, Japan), labeled with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), and sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

For NGS, fusion PCR was conducted with the primer set AMV4.5NF and AMDGR (5′-CCCAAC TATCCCTATTAATCA-3′) (Sato et al. 2005). To discriminate each sample, Primer A-key + Ion Xpress Barcode and Primer P1-key provided by the Ion Torrent System (Thermo Fisher Scientific, Waltham, MA, USA) were added to the 5′-ends of AMV4.5NF and AMDGR, respectively. The 10-μl aliquots of PCR products derived from roots of the same order at each site were pooled together to make one sample. The PCR thermal cycling conditions were one cycle at 94 °C for 5 min, followed by 30 cycles at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s. DNA concentrations in the purified PCR samples were measured by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, USA), and all the samples were calibrated against the lowest DNA concentration, i.e. 436.9 pM. They were sent to the Mie University Center for Molecular Biology and Genetics and analyzed by using NGS with an Ion Torrent PGM (Personal Genome Machine) system and an Ion 318 Chip Kit (Life Technologies). The raw sequence data are available in the DNA Data Bank of Japan (DDBJ) under accession number DRA011250.

Data analyses

AM colonization rates were analyzed using two-way analysis of variance (ANOVA) with study sites as a random effect and root order as a fixed effect. Pearson's product-moment correlation was used to assess the relationships between AM colonization and C/N ratio and N concentration by using R software version 3.5.0 (R Core Team 2018).

For Sanger sequencing, ambiguous loci were adjusted manually with the aid of MEGA ver. 7.0.21 (Kumar et al. 2016). Sequences greater than 300 bp were clustered at 97% sequence similarities as molecular operational taxonomic units, OTUs. Representative sequences in the OTUs were subjected to BLAST analyses by using the Maarj*AM* database (Öpik et al. 2010) or information from the National Center for Biotechnology Information (NCBI, Altschul et al., 1997) to infer taxonomic assignments. Sequences that matched with those of the Glomeromycota were deposited in DDBJ/EMBL/NCBI under accession numbers LC550571 to LC551791.

The NGS data were analyzed with mothur ver. 1.41.3 software (Schloss et al. 2009). The sequences obtained after removing those less than 200 bp long and with Q values < 25 were subjected to UCHIME (Edgar et al. 2011) to detect chimeric sequences. The remaining sequences were clustered at 97% sequence similarities and the sequence clusters with more than 11 sequence reads were defined as tentative OTUs. Representative OTUs as determined by the 'get.oturep' command were subjected to BLAST analysis by referring to the SILVA 132 database (Quast et al. 2013). Taxonomic information, but only of Glomeromycota sequences, was refined by applying BLAST analyses provided by either the Maarj*AM* database or NCBI. According to the BLAST inference, differences in the occurrence frequency of AM fungal taxa at the family level were detected with the χ^2-test. Samples were rarefied to the median number of sequence reads (de Cárcer et al. 2011) by using the "rarefy" function in the R package VEGAN (Oksanen et al. 2018). Following this, the OTU richness for each root order at the study sites was estimated by using the Shannon diversity index, and significant differences in the richness were detected by one-way ANOVA following Tukey's HSD test. To test the validity of the sampling scheme, the mean OTU richness as an estimated taxon richness (S_{est}) was evaluated on the basis of the frequency of OTU sequences per root sample by using the POOLACCUM function in the VEGAN package. The expected number of OTUs (taxa) was based on 1000 random samplings of each study site, without replacement. A non-metric multidimensional scaling (NMDS) scatter-plot based on the Chao dissimilarity index was constructed, and a permutational multivariate analysis of variance (PERMANOVA) was conducted to test whether the AM communities were structured in accordance with root order or study site. The effects of

soil properties, i.e. water content, EC, litter thickness, pH, C, N and C/N, on the communities on either entire roots or roots of each order were examined by using the "envfit" function and by conducting 9999 permutations using the VEGAN package. As soil pH was significantly ordinated with the OTU clusterings in the second- and third-order roots (See *Results*), an OTU network based on the degree centrality (i.e. the number of links incident upon a node) was depicted by using the R package IGRAPH (Csardi and Nepusz 2006). Moreover, the edge numbers of root orders were compared by nonparametric multiple comparison using the Steel–Dwass test and the NSM3 package in R (Schneider et al. 2020).

Overlap of OTUs among root orders was detected and depicted with a Venn diagram constructed using the GPLOTS package (Warnes et al. 2016). By simulation with null models executing the "oecosimu function" in the VEGAN package, nestedness analysis was conducted if AM OTUs detected in the higher root orders were subsets within the lower root orders. We used the NODF (nestedness metric based on overlap and decreased fill) metric proposed by Almeida-Neto et al. (2008); this metric is based on decreasing fill and paired overlap. We applied the swapping method "r00", which constructs null models with the observed OTU richness (i.e. maintaining the OTU richness of individual sampling units), drawing individual OTUs at random from the OTU pool with 9999 randomizations and discarding the first 1000 times as burn-in.

Indicator species analysis by calculating an indicator value (IndVal) was applied to determine the extent of pairwise associations between root orders or study sites and the detected OTUs by using the "multipatt" function in the R package INDICSPECIES (De Cáceres and Legendre 2009). To test the significance of the observed IndVals, we recalculated 9999 values following the permutation of all possible combinations. For all analyses, the significance level was set at $P < 0.05$ unless otherwise stated.

Results

AM fungal colonization of roots of different orders

We found both Arum and Paris types of AM roots at all study sites (Figs. S1a, b). Other than AM fungi, we also detected mycelia and microsclerotia of dark septate endophytic fungi (Figs. S1c, d). Generally, AM colonization tended to decrease from first- to third-order roots, with colonization rates of 5.3% to 0% for Arum types (Fig. 2a) and 42.7% to 11.9% for Paris types (Fig. 2b). A significant interaction ($P < 0.05$) was detected between study site and root order, suggesting that these factors affected AM colonization rates. Arum types were relatively more frequent on first-order roots than on second- or third-order roots at most sites. Paris types were found on roots of all orders, but their colonization rates were higher on first-order roots than on those other orders, except at the HK site where the rates of second-order roots were higher.

When the results for the roots of all orders were pooled, AM colonization rate was significantly and negatively correlated with root C/N ratio ($r = -0.66$, $P = 0.001$, Fig. 3a) and significantly and positively correlated with root N concentration ($r = 0.62$, $P = 0.003$, Fig. 3b). First-order roots tended to be plotted at lower C/N ratios and higher N concentrations. No significant correlations between AM colonization rate and soil pH were found for roots of any order ($P > 0.05$, data not shown).

Clone libraries of AM fungi

For Sanger sequencing, clone libraries in each order root were established from all soil blocks at all study sites. We successfully obtained sequences for a total of 94.2% (1221/1296) of clones. The sequences were divided into 129 OTUs on the basis of a 97% identity cutoff (Fig. S2, Table S1). The family Glomeraceae was the most frequent (116/129 OTUs) and abundant (1160/1221 clones) taxon. Other minor taxa such as Acaulosporaceae and Diversisporaceae were also detected. The most dominant taxon, OTU001, accounting for 38.4% (469/1221) of clones, had the highest identity to VT (virtual taxon) 166, followed by OTU002 (10.2%, 124/1221 clones) with a high degree of identity to VT84 (Tables S1, S2).

Community structure of AM fungi

For the NGS analyses, PCR amplifications derived separately from first- to third-order roots were successful at all study sites. Among 477,696 raw sequences, 82.3% (393,308 sequences) remained after quality checks and chimera screening. Of

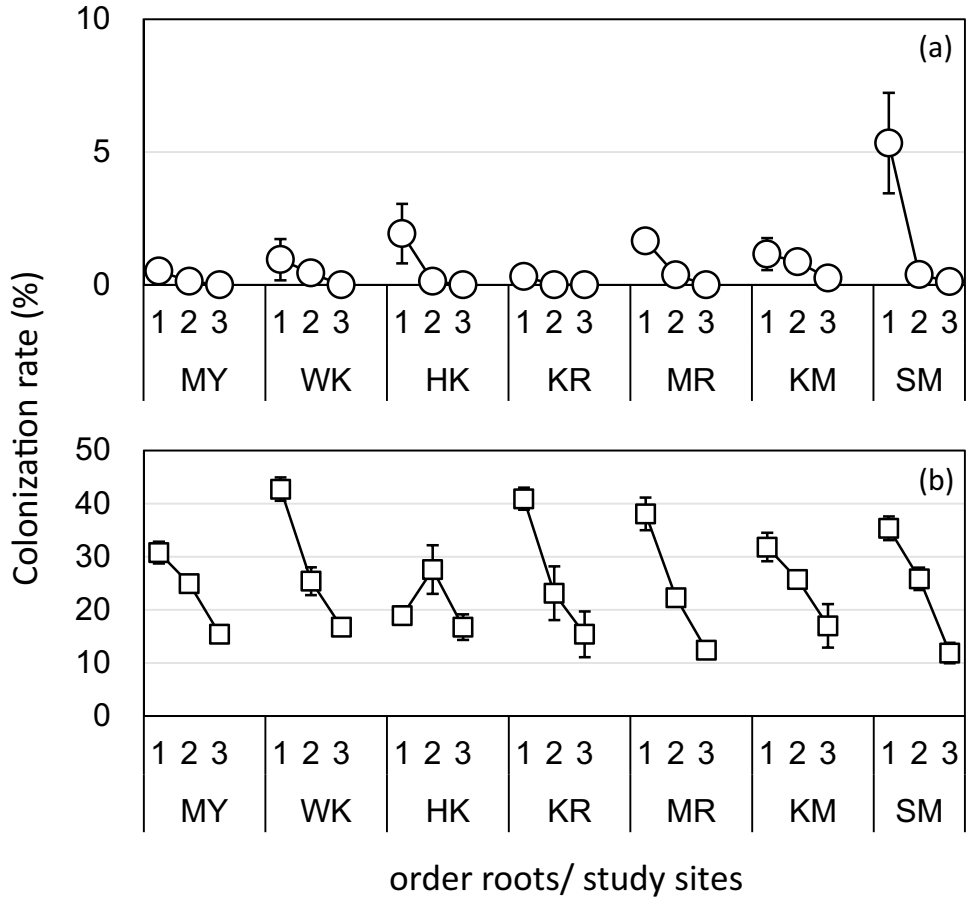

Fig. 2 Rates of colonization of arbuscular mycorrhizal roots of (a) Arum and (b) Paris types on first-, second-, and third-order roots collected from seven *Cryptomeria japonica* forests in 2017. Data are shown as means ($n=3$) ± SE. Numbers on the *x*-axis indicate root orders and letters are study site codes (see Table 1). A significant interaction between root order and study site was detected (two-way ANOVA, $P<0.05$)

these, 57.7% (i.e., 226,771 sequences) were matched with AM fungal taxa and, finally, 114,031 rarefied sequences were used for the community analyses (Table S3). The sequences were categorized into 48 representative OTUs (Fig. S2), ranging from 9 to 21 OTUs per sample (Table S3). The number of OTUs increased with the number of study sites and then approached a plateau (Fig. S3). Among the OTUs assigned, Glomeraceae was the most abundant taxon, accounting for 58.3% (28/48) of OTUs, followed by Acaulosporaceae (20.8%, 10 OTUs) (Fig. 4). The numbers of OTUs detected in the first-order roots, together with the Fisher index (16.7 ± 3.1 and 2.17 ± 0.39, respectively; means ± SD), were significantly higher than those in the third-order roots (11.4 ± 2.7 and 1.43 ± 0.31), and no significant differences in OTU numbers or diversity indexes were found among the study sites (Table S4).

In the present study, more than 97% of sequence reads was assigned into the family Glomeraceae, and the three most dominant OTUs were assigned to members of this family (Table S3). The most dominant two taxa were Glomeraceae sp. 1 and Glomeraceae sp. 2, accounting for 64.4% and 27.5%, respectively, of sequence reads; they were best matched with VT84 and VT166, accordingly. At all the sites, members of the Glomeraceae dominated, accounting for 82.4% to 99.6% of sequence reads, but the measurable detection of Acaulosporaceae (16.4%) was found at site WK (Fig. 1).

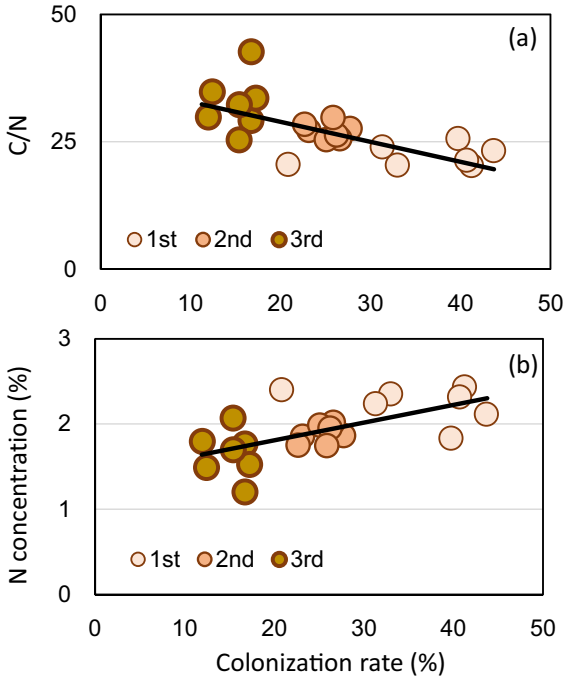

Fig. 3 Relationships between colonization rates of arbuscular mycorrhizal fungi on first-, second-, and third-order roots and (a)root C/N ratio and (b) root N concentration. When the data from roots of all orders were pooled together, significant correlations were found between colonization rate and C/N ratio (Pearson's product-moment correlation test, r = -0.66, $P = 0.001$) and N concentration (Pearson's product-moment correlation test, r = 0.62, $P = 0.003$)

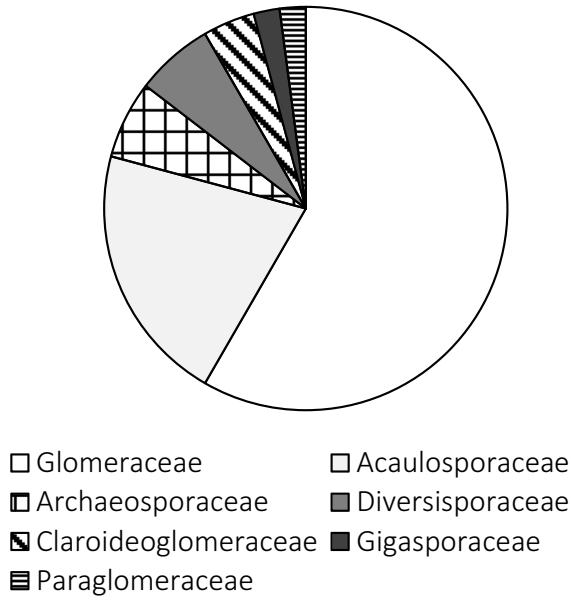

Fig. 4 Occurrence frequencies of arbuscular mycorrhizal fungal taxa detected in seven *Cryptomeria japonica* forests. Data were based on 48 operational taxonomic units derived from quality-filtered next-generation sequence data. The numbers of arbuscular mycorrhizal (AM) taxa detected differed significantly among AM families ($\chi^2 = 84.4$, df = 6, $P < 0.001$)

Indicator species analyses showed that four Glomeraceae taxa, namely spp. 5, 11, 12, and 15 (IndVals = 0.961, 0.939, 0.961, and 0.882, respectively) were detected significantly at only one site (HK, MR, or KR), and Glomeraceae sp. 6 was found significantly at only two sites, WK and KR (IndVal = 0.996, Table S3). Diversisporaceae sp. 1 was detected significantly on both first- and second-order roots (IndVal = 0.957) (Table S3).

The number of OTUs in roots of each order reached a plateau as the number of study sites increased (Fig. 5). Half (24/48 OTUs) of the taxa were shared among roots of all orders, and there were no OTUs unique to third-order roots, unlike the case with first- and second-order roots (Fig. 6a), and OTUs in third-order roots were all found with those in first- and second-order roots (Fig. 6b). In the presence or absence matrix of OTUs, the calculated NODF was 29.9, which was nested significantly more than in the null model (C-score = 40.84, $P < 0.001$). There was no

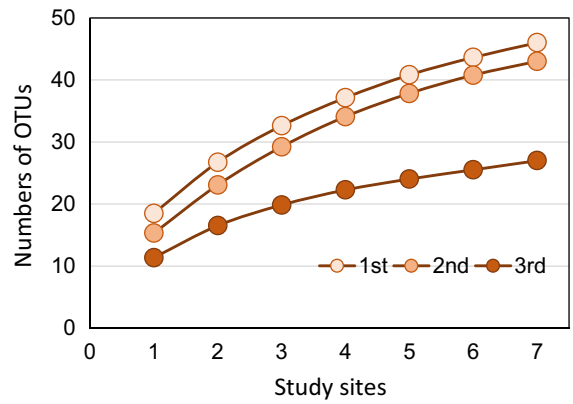

Fig. 5 Rarefaction curves of arbuscular mycorrhizal fungal taxa in roots of different orders in seven *Cryptomeria japonica* forests. Observed numbers of operational taxonomic units (OTUs) in roots of each order, with resampling 1000 times, are shown on the basis of quality-filtered next-generation sequence data

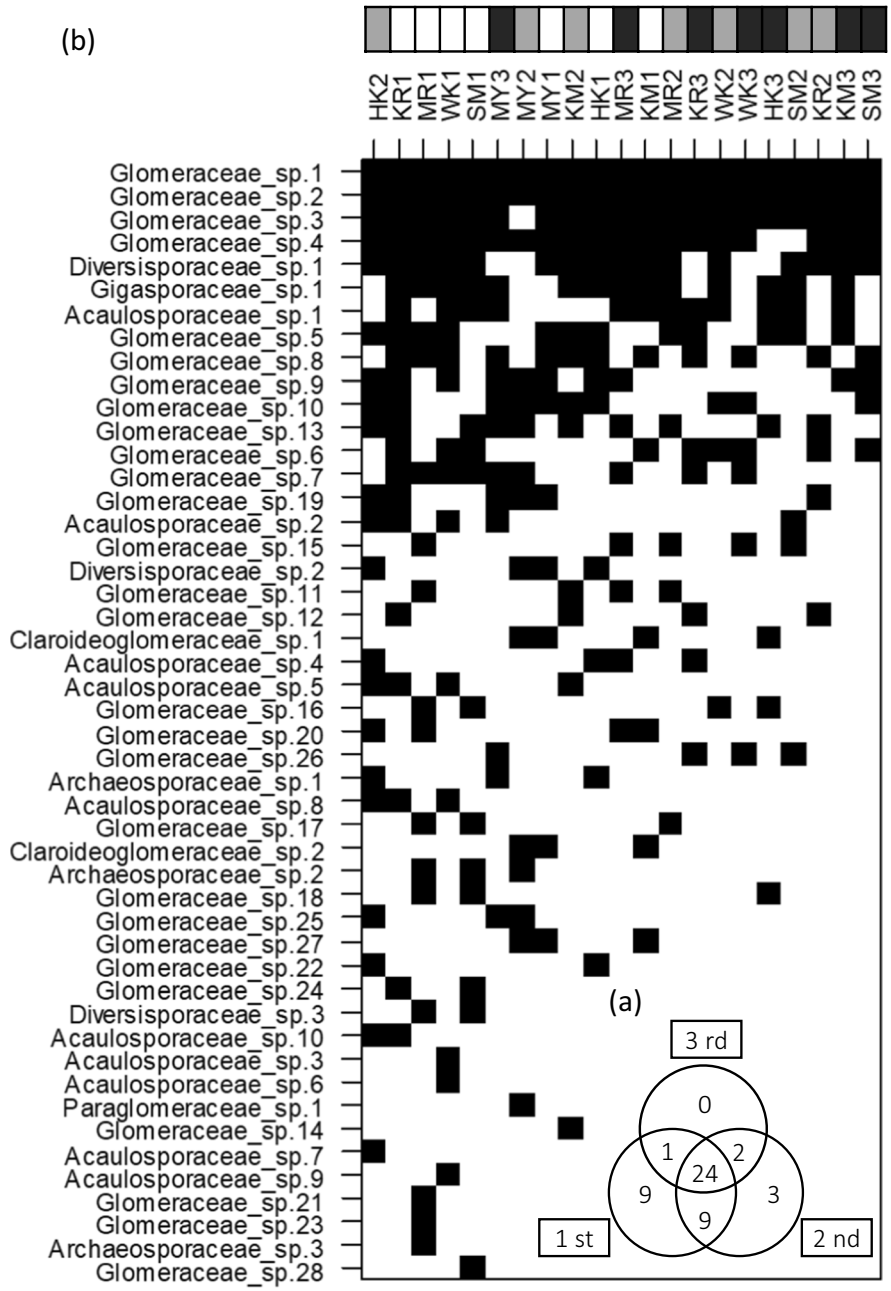

Fig. 6 Occurrence patterns of arbuscular mycorrhizal (AM) fungal taxa in roots of different orders in seven *Cryptomeria japonica* forests. (a) Venn diagram of AM taxa based on operational taxonomic units (OTUs) that were unique to, or shared among, roots of different orders. (b) Inferred taxa of OTUs (Table S2) are indicated on the left of the matrix. Matrix of OTU communities detected on first- (■), second- (■), or third- (■) order roots in the seven forests is sorted into columns. Filled (■) or empty (□) cells indicate the presence or absence, respectively, of the OTUs. Significant nestedness was observed among the columns (NODF [Nestedness metric based on Overlap and Decreased Fill] = 29.9, C-score = 40.84, $P < 0.001$)

significant NMDS clustering of OTU communities among study sites (PERMANOVA, $P = 0.83$; Fig S4a) or root orders (PERMANOVA, $P = 0.86$), but soil N and C concentration were significantly ordinated. OTU communities detected on both second- and third-order roots showed significant ordination with soil environmental conditions such as EC, pH, and N concentration (Figs. S4c, d). In a structural analysis based on degree centrality, the number of edge connections in first-order roots was significantly higher than that in third-order roots (Fig. 7) depicting that lower-order roots under lower soil pH (e.g. at WK1 and KR1) had more connections than others (Fig. 7).

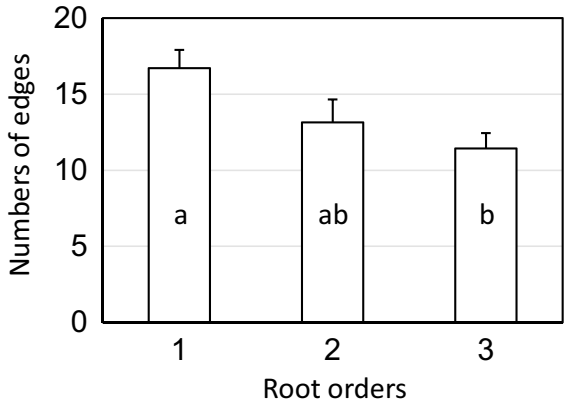

Fig. 7 Number of edges shared by arbuscular mycorrhizal fungal taxa in each root order detected among seven *Cryptomeria japonica* forests. Data are shown as means ($n=7$) ± SE. Different letters indicate significant differences in edge numbers among order roots by Steel–Dwass non-parametric multiple comparison tests ($P<0.05$)

Discussion

AM community structure in fine-root systems

In this study, our hypothesis that lower-order roots have higher AM fungal taxa than higher-order roots and AM fungal communities are varied among different root orders was partly supported. Within the fine-root systems of *C. japonica*, first-order roots harbored significantly more AM taxa than third-order roots (Fig. 5, Table S4). Moreover, the taxa detected in the third-order roots were also found in second- and first-order roots (Fig. 6). However, there was no significant NMDS clustering of AM fungal communities among either root orders or study sites (Fig. S4a). Since we examined *C. japonica* roots collected at same seasons in limited geographical areas, the influence of climate and plant identity can be excluded and microhabitat factors such as root orders and soil environment were possible to affect colonization and community patterns of AM fungi. Under this condition, the communities in locally planted *C. japonica* forests may be established by random colonization by the fungi. This was also inferred partly from the network pattern of AM taxa: first-order roots were significantly more connected with other fine roots than were third-order roots irrespective of the sites (Figs. 7, S5). However, the clustering patterns of the fungi in roots of each order revealed that those in second- and third-order roots were ordinated with some soil conditions, such as soil pH and N concentration (Figs. S4c, d). In addition, unique Glomeraceae selected as indicator taxa were found at either higher (HK and MR) or lower (WK and KR) pH sites (Tables 1 and S4). Soil pH has been suggested to be one of the environmental factors affecting the assemblage patterns of soil organisms (Fierer and Jackson 2006; Dumbrell et al. 2010; Kitagami et al. 2020), occasionally forming nested assemblage patterns of AM fungi (Kawahara et al. 2016). Woody plants can live longer than annual plants and are thus required to adapt to changes in the surrounding growth environment. In this respect, soil acidity in other *C. japonica* forests has been found to shift towards either a higher or lower pH depending on soil buffer capacity, that is responsible for calcium concentration (Tanikawa et al. 2014), leading to bulk soil pH changes. Moreover, the exudation of H^+ during root growth decreases the pH in the rhizosphere (Kuzyakov and Razavi 2019). Thus, higher-order roots should persist structurally longer in the field than first-order roots under different time scales of acidity changes, i.e. rhizospheric and bulk soil pHs. Thus, community shifts and loss of AM diversity within fine root systems may be affected by acidity conditions of AM fungal habitats.

In this study, the AM community in temperate coniferous forests was dominated by members of the Glomeraceae. This is in line with the findings of previous global-, continental-, and regional-scale studies (Davison et al. 2015; Põlme et al. 2016; Rodríguez-Echeverría et al. 2017). Moreover, the prevalence of Glomeraceae fungi was suggested in *Chamaecyparis obtusa* and *Cunninghamia lanceolata* plantation forests, which belong to the same Cupressaceae family as *C. japonica* (Lu et al. 2019; Miyake et al. 2020). In addition, the most dominant and second-most dominant AM fungi, VT84 and VT166, respectively, in our study have also been detected as common dominant taxa in forest ecosystems (Miyake et al. 2020). These taxa may therefore have a high affinity for woody species, possibly coniferous trees. Linking the community structure of AM fungi with their functional significance at the species or strain level would provide insights into tree growth and ecosystem functioning in forest ecosystems (Koch et al. 2017; Mathieu et al. 2018; Powell and Rillig 2018).

AM morphological types on roots of different orders

We examined AM colonization rates in first-, second- and third-order roots, and the rates generally decreased from the first to third order roots, irrespective of AM morphological types (Fig. 2). Thus, this tendency supported our hypothesis, and the lower root orders showed more association with AM fungi. Formation of different AM morphological types is suggested to be influenced by the type of host plant, the associated fungi, and the surrounding environment (Dickson et al. 2007). Our study clearly showed that both Arum and Paris types were simultaneously detectable on the lower-order roots, and the latter type predominated at all study sites (Fig. 2). However, the AM types on *C. japonica* have been reported as being of Paris type (Yamato and Iwasaki 2002) or of both types (Fujimaki et al. 2001). Natural soils contain various species of AM fungal inocula, and the identity of the AM species can affect the morphological type. In fact, different AM fungal species have been demonstrated to form either Paris or Arum types, and Cavagnaro et al. (2001) demonstrated intraspecific variations in AM morphology by inoculating *Solanum lycopersicum* with different species in the genera *Glomus*, *Gigaspora*, and *Scutellopora*. Accordingly, the colonization morphology depends on the combination of host plant and fungal species and can differ within individual plants and within AM taxa (Bedini et al. 2000; Dickson 2004). Thus, experimental inoculations of *C. japonica* with known AM fungal species should be conducted to understand morphological variations in future.

The colonization rate of Paris types at HK site showed a different trend from the other sites in that the rate of second-order roots was higher than that of first-order roots. This pattern is known from studies of broad-leaved trees, in which higher AM colonization rates have been detected in roots of higher orders than the first-order (Guo et al. 2008; Eissenstat et al. 2015). In another study of *C. japonica* forests, intraspecific variations in the fine-root architecture, e.g. the diameter and specific root length were observed (Wada et al. 2019). Among them, the same study site as ours, i.e. HK site, showed significantly finer root diameter and larger specific root length than other sites. Plant species with interspecific traits such as thinner and longer roots may forage for nutrients via their own growth rather than by depending on association with AM fungal hyphae (Eissenstat et al. 2015; Chen et al. 2016, 2018). In support of the concept of plastic modification of fine-root systems, the second-order roots at HK may have functioned similar to first-order roots and may have been exposed occasionally to infection by AM fungi.

The detection of both AM morphological types may imply the presence of phenotypic plasticity reflecting functional diversification within lower-order roots. Frequent occurrences of the Paris type, which agrees with the common pattern of woody plants, can likely be attributed to the slower growth of the plants relative to that of fast-growing annual plants (Brundrett and Kendrick 1990; Dickson et al. 2007). On the other hand, the Arum type tended to occur more frequently in first-order roots than in the roots of other orders, although the relative colonization rate of this type was limited, at no more than about 5% (Fig. 2). In terms of root anatomical structure, first-order roots of *C. japonica* formed exclusively primary roots with certain protoxylem groups, such as monarch, diarch, or triarch, but higher order roots were secondary roots with triarch or tetrarch structures without monarch (Hishi et al. 2017). Thus, the AM morphology can be linked to, and altered with the anatomical differences of root orders within fine-root systems.

For nutrient acquisition, first-order roots can have higher capacity than other order roots (McCormack et al. 2015). This was indirectly supported in the study, and root nitrogen concentration and C/N tended to be higher or lower in the first order roots than others, respectively (Fig. 3b). Based on AM morphological differences, the Arum type can have higher surface areas per volume than the Paris type (Dickson and Kolesik 1999). This difference may explain the variations in the pattern of nutrient exchanges. In this respect, a recent study suggested that *Paris quadrifolia* formed a Paris type AM, using substantial amounts of fungal-derived carbons for its growth, unlike *Arum maculatum*, which formed an Arum type and was likely a full autotroph (Giesemann et al. 2020). However, Paris type formation was experimentally shown to promote the growth of host plant, *Smilax aspera* (Bedini et al. 2000). Thus, although AM morphology can be a signal for functional variations, the phenotypic plasticity of the morphology on individual roots of different orders should

be evaluated in terms of nutrition and growth of host trees under experimental conditions.

Conclusion

We clarified the morphological and community traits of AM fungi associated with *C. japonica* (Cupressaceae) from first to third order roots inferring structural and assemblage variations within fine-root systems in temperate coniferous forests. Both the Arum type and the Paris type generally occurred more commonly on first-order roots than on second- or third-order roots, although colonization rates with the Paris type were much higher than rates with the Arum type. Moreover, in the lower-order roots, colonization rates were higher as well as more AM fungal taxa, mainly species of the Glomeraceae, were found. AM fungal taxa detected on higher-order roots were a subset of those on lower-order roots, possibly because of plant-fungal interactions during the course of root growth, as well as due to environmental filtering towards certain taxa. Future studies integrating root orders, root lifespan, and AM fungal assemblage within distal fine-root systems can lead to a deeper understanding of resource acquisition and nutrient flows in the below ground region of forest ecosystems.

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Author Contributions Y.M. planned and designed the study; all authors conducted fieldwork; K.K., T.T. and Y.K. performed laboratory measurements (soil chemistry, microscopy, and DNA extraction and sequencing); Y.M. and Y.K. performed the data analyses; Y.M. wrote the first draft of the manuscript; and all authors discussed and contributed to the final version of paper.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data accessibility DNA sequences: Genbank accessions LC550571-LC551791; DDBJ DRA: DRA011250.

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