Dual colonization of Mucoromycotina and Glomeromycotina fungi in the basal liverwort, *Haplomitrium mnioides* (Haplomitriopsida)

Kohei Yamamoto[1], Masaki Shimamura[2], Yousuke Degawa[3], Akiyoshi Yamada[1,4,5,6]

[1] Department of Bioscience and Food Production Science, Interdisciplinary Graduate School of Science and Technology, Shinshu University, 8304 Minami-minowa, Nagano 399-4598, Japan

[2] Department of Biology, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan

[3] Sugadaira Research Station, Mountain Science Center, University of Tsukuba, 1278-294 Sugadaira, Ueda, Nagano 386-2204, Japan

[4] Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304, Minami-minowa, Nagano 399-4598, Japan

[5] Research Center for Fungal and Microbial Dynamism, Shinshu University, 8304 Minami-minowa, Nagano, 399-4598, Japan

[6] Division of Terrestrial Ecosystem, Institute of Mountain Science, Shinshu University, 8304 Minami-minowa, Nagano, 399-4598, Japan

Abstract

In general, Glomeromycotina was thought to be the earliest fungi forming mycorrhiza-like structure (MLS) in land plant evolution. In contrast, because the earliest divergent lineage of extant land plants, i.e. Haplomitriopsida liverworts, associates only with Mucoromycotina mycobionts, recent studies suggested that those fungi are novel candidate for the earliest mycobionts. Therefore, Mucoromycotina-Haplomitriopsida association currently attracts attention as an ancient mycorrhiza-like association. However, mycobionts were identified only in seven of 16 Haplomitriopsida species and the mycobionts diversity of this lineage is largely unclarified. To clarify the taxonomic composition of mycobionts in Haplomitriopsida, we observed MLSs in the rhizome of *Haplomitrium mnioides* (Haplomitriopsida), the Asian representative Haplomitriopsida species, and conducted molecular identification of mycobionts. It was recorded for the first time that Glomeromycotina and Mucoromycotina co-occur in Haplomitriopsida as mycobionts. Significantly, the arbuscule-like branching (ALB) of Glomeromycotina was newly described. As the Mucoromycotina fungi forming MLSs in *H. mnioides*, Endogonaceae and Densosporaceae were detected, in which size differences of hyphal swelling (HS) were found between the fungal families. This study provides a novel evidence in the MLS of Haplomitriopsida, i.e. the existence of Glomeromycotina association as well as the dominant Mucoromycotina association. In addition, since hyphal characteristics of the HS-type MLS were quite similar to those of fine endophytes (FE) of Endogonales in other bryophytes and vascular plants previously described, this MLS is suggested to be included in FE. These results suggest that Glomeromycotina and Mucoromycotina were acquired concurrently as the mycobionts by the earliest land plants evolved into arbuscular mycorrhizae and FE. Therefore, dual association of Haplomitriopsida, with Endogonales and Glomeromycotina will provide us novel insight how the earliest land plants adapted to terrestrial habitats with fungi.

Key words

Basal land plant · Endogonales · Fine endophyte · Glomeromycotina · Mucoromycotina · Mycorrhizal association

INTRODUCTION

It is widely accepted that the earliest land plant established its terrestrial habitat in association with Glomeromycotina fungi as a mycorrhiza-like association (Redecker et al., 2000; Simon et al. 1993; Smith and Read 2008). This hypothesis is strongly supported by arbuscule-like branching (ALB) of Glomeromycotina in the thalli of basal land plants, i.e. liverworts (at least 13 of 27 families in Marchantiopsida and Jungermanniopsida; Ligrone et al. 2007). In addition, bidirectional exchange of nutrient and carbon between Glomeromycotina and liverworts has been reported (Field et al., 2012).

In contrast, another mycorrhiza-like structure (MLS) has been reported in Haplomitriopsida liverworts, the extant earliest land plant and a sister lineage of the remaining liverworts (Marchantiopsida and Jungermanniopsida). In *Haplomitrium* (Haplomitriales), fungal colonization is restricted to the epidermis of the rhizome and the intracellular hyphae show hyphal swelling (HS) at the apex (Carafa et al. 2003). This HS was originally described as lump by Lilienfeld (1911) who observed MLS of *Haplomitrium hookeri* (Lyell ex Sm.) Nees. Similar MLS was then observed on *Treubia* (Haplomitriopsida) (Stahl 1949). These MLSs were observed in detail with transmission electron microscope and were recognized as a unique MLS type formed by Glomeromycotina. However, the hyphae observed in Haplomitriopsida are distinctly different from trunk hyphae (3–6 μm in diameter) and arbuscule (dichotomous branching, 0.8–1.5 μm in diameter) in Glomeromycotina (Smith and Read 2008), as they are fine, constant in width (0.8–1.5 μm in diameter), and irregularly branched (Bidartondo et al. 2011; Carafa et al. 2003; Duckett et al. 2006). In addition, the HS observed in *Haplomitrium* is thin-walled and therefore easily distinguishable from vesicles of Glomeromycotina (Smith and Read 2008). Interestingly, those distinctive mycobionts of Haplomitriopsida were exclusively identified as Mucoromycotina by molecular analyses (Bidartondo et al. 2011). Because basal clade of

land plants, i.e. Haplomitriopsida, associated only with Mucoromycotina, Bidartondo et al. (2011) created a novel hypothesis that the first land plant-fungal association was established by Mucoromycotina rather than Glomeromycotina. Recent phylogenetic studies suggest that Haplomitriopsida liverworts are associated with at least two distinct families of Endogonales in Mucoromycotina (i.e. Endogonaceae and Densosporaceae; Desirò et al. 2017). Endogonaceae and Densosporaceae are distinguished from one another by the morphology of their hyphae, with Endogonaceae forming thicker hyphae than Densosporaceae (Yamamoto et al. 2017b).

Several species of Marchantiopsida and Jungermanniopsida (Field et al. 2015c; Strullu-Derrien et al. 2014), and many hornworts (Desirò et al. 2013) are associated with both Mucoromycotina (Endogonaceae and Densosporaceae) and Glomeromycotina. Coexistence of diverse fungi associated with basal plants is suggested to mirror an ecological trait of ancient land plants from around four hundred million years ago (Field et al. 2015a). The existence of various fungal options for those plants might have facilitated subsequent plant diversification in ancient land (Field et al. 2015c). However, there is not enough information available to clarify the mycobiont of the earliest land plant. Although recent studies have emphasized that Mucoromycotina fungi, specific to Haplomitriopsida, are the most likely candidate, this hypothesis is open to question. In fact, mycobionts were identified only in seven (Bidartondo et al. 2011) of 16 Haplomitriopsida species accepted by Bakalin and Vilnet (2017) and Söderström et al. (2016). Furthermore, Glomeromycotina fungi were once reported as mycobionts of *Haplomitrium chilensis* R.M. Schust. (Ligrone et al. 2007). This finding is however based only on molecular identification, and whether Glomeromycotina has the ability to form ALB on Haplomitriopsida is not yet settled.

In this study, we conducted morphological characterization of MLSs in *Haplomitrium mnioides* (Lindb.) R.M. Schust. (Haplomitriopsida), the Asian representative

Haplomitriopsida species (Bartholomew-Began 1991; Shi and Zhu 2006), and molecular identification of its mycobionts for the first time. Here, we report the first evidence of Haplomitriopsida-Glomeromycotina associations. Additionally, we show morphological differences between the MLSs of Endogonaceae and Densosporaceae.

MATERIALS AND METHODS

Preparation of *Haplomitrium mnioides* gametophytes

Haplomitrium mnioides were sampled at seven sites (Table S1) in the western and southern regions of Japan, where *H. mnioides* is mainly distributed (Shi and Zhu 2006). A single *H. mnioides* colony was collected at each site. As the MLS of *Haplomitrium* spp. actively develops throughout the seasons of sporophyte development and degenerates after spores release (Carafa et al. 2003), sampling of *H. mnioides* was tentatively conducted just before maturation of sporophytes in Kyoto (Table S1) in April 2012. Healthy MLSs were observed in the numerous epidermal cells of the sample. Following this, the other samples of *H. mnioides* were obtained a few months before sporophyte maturation. Samples were stored in a refrigerator at 5°C and used for analyses within 3 days. Each sample was washed with tap water and soil particles attached to rhizomes were removed under a dissection microscope (Zeiss Stemi 2000C). 15 or 30 rhizomes of each sample were used for morphological and molecular analyses (Table 1). Each washed rhizome was longitudinally sectioned into two using a razor under a dissection microscope, with one side used for morphological observation of the MLS, and the other used for molecular identification of mycobionts. Rhizome sections for molecular identification were immersed in 100 μL of TE buffer and stored at -65°C. Part of examined gametophyte of each sample was deposited as voucher specimen in Herbarium of Hiroshima University (HIRO) (Table S1).

DNA extraction, PCR amplification, and DNA sequencing

Total DNA was extracted from each rhizome (ca. 3 mm long) of *H. mnioides* according to Izumitsu et al. (2012) using a microwave with minor modifications. Before heating in a microwave, each rhizome fragment was crushed thoroughly using a homogenization pestle under a dissection microscope. The resultant supernatants were used as templates for PCR.

Partial sequences of the fungal 18S rDNA were amplified by PCR using a KAPA2G Robust Hotstart ReadyMix PCR Kit (KapaBiosystems, Wilmington, MA, USA), according to the manufacturer's instructions. In accordance with the protocol in Desirò et al. (2013), fungal universal primers, NS1 (White et al. 1990) and EF3 (Smit et al. 1999), were used for first PCR. Similar to Desirò et al. (2013), first PCR amplicons yielded insufficient DNA for sequencing, therefore, second PCR was conducted. The first PCR products were diluted 100-fold with sterilized distilled water and used as templates for second PCR. As Mucoromycotina and Glomeromycotina were most likely mycobionts of *H. mnioides* based on the preliminarily conducted morphological observations (data not shown), specific primer pairs for Mucoromycotina (EndAD1f and EndAD2r; Desirò et al. 2013) and Glomeromycotina (AML1 and AML2; Lee et al. 2008) were used separately for the second PCR of each sample. Both the first and second PCRs were performed using the ProFlex™ PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 2 min; 37 cycles of 95°C for 12 s, annealing at 55–58°C for 12 s, and 72°C for 12 s. If PCR products were difficult to sequence directly, they were cloned. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany).

DNA sequencing for the forward and reverse strands of PCR amplicons were performed using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies Inc., Carlsbad, CA, USA) with a reaction volume of 10 μL. The primers used for the second PCR described above were also used for the sequencing reactions. The sequencing reaction, purification of reaction products, and sequence analyses were performed as previously

described (Yamamoto et al. 2015). Chimera checking performed for newly generated sequences of mycobionts with UCHIME2 (Edgar 2016) and DECIPHER (Wright et al. 2012) resulted in detecting no chimeric sequences. Those sequences were deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp; LC417248–LC417301).

Phylogenetic analyses

Since the mycobionts of *H. mnioides* were inferred to belong to Endogonales and Glomeromycotina based on the results of NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), dataset-1 (Endogonales) and -2 (Glomeromycotina) were independently prepared for phylogenetic analyses. Dataset-1 included the following sequences: (1) putative Endogonales mycobionts of *H. mnioides*, (2) Mucoromycotina mycobionts of other Haplomitriopsida liverworts (Bidartondo et al. 2011; Field et al. 2015b), (3) sequences of Endogonales obtained from sporocarp specimens, and (4) outgroups, i.e. *Mortierella chlamydospora* (Chesters) Plaats-Niterink (Mortierellomycotina, Mucoromycota), *Calcarisporiella thermophile* (H.C. Evans) de Hoog and *Echinochlamydosporium variabile* X.Z. Jiang, H.Y. Yu, M.C. Xiang, X.Y. Liu & X.Z. Liu (Order *incertae sedis*, Mucoromycotina; Hirose et al. 2014). Dataset-2 included the following sequences: (1) putative Glomeromycotina mycobionts of *H. mnioides*, (2) Glomeromycotina mycobiont of *H. chilensis* (Ligrone et al. 2007), (3) sequences of known species and virtual taxa (Öpik et al. 2010) in the Glomeromycotina with high similarity to the mycobiont of *H. mnioides* and *H. chilensis* determined by blast search in a database of Glomeromycotina sequences (MaarjAM: http://maarjam.botany.ut.ee/; Öpik et al. 2010), (4) sequences of the two species in all orders of Glomeromycotina, i.e. Glomerales, Diversisporales, Archaeosporales, and Paraglomerales, and (5) outgroups (*M. chlamydospora* and *Endogone pisiformis* Link [Endogonales]). MUSCLE implemented in

MEGA ver. 6.06 (Tamura et al. 2013) was used for preliminary multiple alignments of nucleotide sequences and final alignments were adjusted manually. Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analyses. Finally, two datasets were prepared (dataset 1: 1605 bp; dataset 2: 777 bp) and deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S23178). Phylogenetic analyses of both datasets were conducted by maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. The general time reversible model of nucleotide substitution with a discrete gamma distribution was adapted for ML and BI. ML analyses were performed using raxmlGUI ver. 1.31 (Silvestro and Michalak 2012) as described in Yamamoto et al. (2017a). BI was performed using MrBayes 3.2.1 (Ronquist et al. 2012) as described in Yamamoto et al. (2015). Two runs with four chains of Markov chain Monte Carlo iteration were performed for 2 million generations. MP was performed using MEGA ver. 6.06 as described in Yamamoto et al. (2015). The tree bisection reconnection (TBR) model was adapted for MP.

Morphological observation of MLSs

Trypan blue staining was conducted for the rhizome samples from which sequences of mycobionts were obtained. The staining procedure followed the protocol of Koske and Gemma (1989), with minor modifications. Rhizomes were soaked in 2.5% KOH and heated at 105°C for 12 min, rinsed with tap water, and soaked in 1.5% HCl for 15 h at room temperature. Finally, rhizomes were stained with 0.05% trypan blue in lactoglycerol (100% lactic acid/100% glycerol/distilled water; 1:1:1, v/v/v) at 105°C for 1 h. Rhizomes were mounted with polyvinyl alcohol-lactoglycerol according to the procedure given in Koske and Tessier (1983). Microscopic observations were conducted using a differential interference contrast microscope (AXIO Imager A1, Carl Zeiss Inc.). In addition, the following structures

were measured for the abundance and size in each rhizome: number of host cells containing (1) HS and (2) ALB, (3) number of HS in a colonized host cell, (4) diameter of HS, and (5) number of putative intraradical spores of Glomeromycotina. Generally, surface of the HS is smooth in immature and winkled in mature MLS (Duckett et al. 2006; Field et al. 2015b). Measurements were conducted of mature HS and some rhizome samples were not measured due to immaturity or collapse of HS. The diameter of HS in samples from which Endogonaceae and Densosporaceae sequences were detected were compared statistically using KaleidaGraph ver. 4J (Hulinks Inc., Tokyo, Japan). Welch's *t*-test was adopted to determine significant difference ($P < 0.01$) between means.

RESULTS

Morphological characteristics of *Haplomitrium mnioides* in Japan

Gametophytes were composed of leafy shoots and underground creeping rhizomes which were covered with colorless mucilage (Fig. S1). The rhizomes consisted of main axes and short lateral branches (Fig. 1A). Pale parts were observed on the short branches of healthy green rhizomes (Fig. 1B, S1C, E, F), where epidermal and cortical cells contained fungi showing MLSs (Fig. 1D–R). Extraradical spores of Glomeromycotina (thick-walled, ca. 100 μm in diameter, with single subtending hypha) were rarely observed outside the surface of the rhizome with the connection of extraradical hyphae (Fig. 1C, S3).

Molecular identification of mycobionts of *Haplomitrium mnioides*

Forty-six sequences of Mucoromycotina (Endogonaceae and Densosporaceae; Fig. 2, Table 1) and eight sequences of Glomeromycotina (Fig. 3, Table 1) were obtained by direct sequencing with each specific primer pair. Of these, Glomeromycotina consisted of a *Glomus* sp. and three Glomeraceae with uncertain lower taxa in Glomerales, and four in the

sister clade of *Archaeospora-Geosiphon-Ambispora* clade in Archaeosporales.

Three morphotypes of the MLS and their phylogenetic relationships

Hyphae colonizing in the epidermal cells of rhizomes were stained purplish-blue by trypan blue (Fig. S2). Fungal intracellular colonization was patchily observed in the rhizomes with clusters of tens to hundreds of colonized epidermal cells. MLSs were largely grouped into two morphotypes based on their intracellular hyphal morphology: (1) HS-type and (2) ALB-type. Co-colonization of HS-type and ALB-type in single cell was never observed. HS and intracellular fine hyphae of HS-type (Fig. 1D–K) agreed with "lump" (Carafa et al. 2003; Duckett et al. 2006; Field et al. 2015b; Lilienfeld 1911) and hyphal coils, respectively, and were observed in the Endogonales-Haplomitriopsida associations (Bidartondo et al. 2011; Field et al. 2015b). ALB with trunk hyphae of ALB-type (Fig. 1L–P) closely resembled the MLS of Marchantiopsida- and Jungermanniopsida-Glomeromycotina associations (Ligrone et al. 2007). MLSs were present in all colonies of *H. mnioides* observed in this study, however the ratio of colonized rhizomes per examined rhizomes differed among samples: 93.3% in UW, 53.3% in IS, 40% in IN, 33.3% in KY, 23.3% in HI, 20 % in HA and 10% in NA (Table 1, S2). HS-type colonization was present in all colonies of *H. mnioides*, except HA which contained only ALB-type (Table 1). The ratio of host cells with HS (including degenerated and immature swellings) colonization per all colonized cells were 100% in NA, HI and UW, 79.8% in KY, 49.2% in IS, 15.7% in IN and 0% in HA (Table 1, S2). By contrast, ALB-type colonization was present only in four colonies of *H. mnioides* (Table 1). The ratio of host cells with ALB colonization per all colonized cells were 100 % in HA, 84.3% in IN, 50.8% in IS, 20.2% in KY, 0% in NA, HI and UW (Table 1, S2). In summary, the ratios of colonization by Mucoromycotina and Glomeromycotina were largely different between each *H. mnioides* colony. Co-colonization of HS-type (including

degenerated and immature swellings) and ALB-type was observed in single rhizome from Kyoto (KY-4, 5, 9 and 20), Ishigaki (IS-3, 4, 6 and 11) and Ino (IN-2, 4, 11 and 12) (Table 1, S2). Intraradical spores were also observed within the rhizomes containing ALB from Kyoto, Ino and Hachijo-jima Isl. Sequences of Mucoromycotina were always obtained from rhizomes containing HS-type colonization, and were not obtained from HA sample which contained only ALB-type (Table S2). In rhizome samples containing Mucoromycotina, the size of the HS was significantly different between Endogonaceae (large) and Densosporaceae (small) (Fig. 4, Table S2). In addition, ALB (and intraradical spores) presence and detection of Glomeromycotina sequences almost corresponded (Table 1, S2). Morphological differences in ALB between Glomerales and Archaeosporales were not clarified.

Morphological characteristics of MLS in each fungal taxon detected were described below:

Endogonaceae: HS-type MLSs were observed (Fig. 1D–G). Hyphae were 0.5–5.3 µm in diameter, often aseptate, rarely forming 1 secondary septa, randomly branching, lacking in dichotomous branching pattern (Fig. 1E). No hyphal colonization was observed from previously colonized cells to neighboring uncolonized cells. HS was spherical, terminal, 10–34 µm in diameter, 21 µm of mean (n = 258), stained deep purplish-bluish with trypan blue in the intracellular contents. Single colonized host cell involved 1–6 HSs, mean 1.8 (n = 105). This type was observed and molecularly identified in Kyoto (KY-6, 15 and 24), Ishigaki (IS-1, 3, 4, 6, 8, 10, 11 and 14), Higashi-hiroshima (HI-1, 7 and 11) and Uwajima (UW-1–10 and 12–15).

Densosporaceae: HS-type MLS was observed (Fig. 1H–K), but different from Endogonaceae in the following points. Hyphae were fine, 0.7–2.7 µm in diameter. (Fig. 1H). HS was 6–21 µm in diameter, 11 µm of mean (n = 530). Single colonized host cell involved

2–30 HSs, mean 8.3 (n = 59). This type was observed and molecularly identified in Kyoto (KY-1, 4, 9, 17 and 25), Nabari (NA-24, 28 and 30), Higashi-hiroshima (HI-8, 13, 14 and 16) and Ino (IN-2–4 and 10–12).

Glomeromycotina: ALB-type MLS was observed (Fig. 1L–P). ALB composed of trunk hyphae with 3.5–8.9 μm in diameter and treelike branching part. Hyphal colonization was frequently observed from previously colonized host cells to neighboring uncolonized cells (Fig. 1P). Intraradical spores [23–34 μm in diameter, 28.1 μm of mean (n = 11)] which are known to be formed inside roots by diverse lineages of Glomeromycotina, i.e. Paraglomerales, Archaeosporales, Diversisporales, and Glomerales (Morton and Redecker 2001), were sometimes formed in the rhizome (Fig. 1Q). Subtending hypha of those spores connected to hyphae colonizing in the epidermal cells of rhizomes. This type was observed and molecularly identified in Kyoto (KY-5, 9 and 20), Ishigaki (IS-11), Ino (IN-12) and Hachijo (HA-3, 5 and 8).

Simultaneous colonizations of Mucoromycotina and Glomeromycotina were observed in several rhizomes, both based on morphology and phylogeny (Table S2, Fig. 2, 3): Endogonales and Glomerales were detected in the sample IS-11, Densosporaceae and Glomerales in sample KY-9 (Fig. 1R) and Densosporaceae and Archaeosporales in sample IN-12.

DISCUSSION

This study provides a novel evidence in the MLS of Haplomitriopsida, i.e. the existence of Glomeromycotina association. In this study, we failed to detect any morphological differences in ALB between Glomerales and Archaeosporales. Further samplings and observations are necessary. The ALB-type MLS on Haplomitriopsida is similar to the Marchantiopsida-Glomeromycotina symbiosis and arbuscular mycorrhizal (AM) symbioses

of vascular plants (Field et al. 2012; Humphreys et al. 2010; Smith and Read 2008). At present, all observed Haplomitriopsida species show MLS (*Apotreubia*: Inoue 1960; *Haplomitrium*: Bidartondo et al. 2011; Carafa et al. 2003; Grubb 1970; Hattori and Mizutani 1958; Lilienfeld 1911; Stahl 1949; *Treubia*: Bidartondo et al. 2011; Duckett et al. 2006; Goebel 1891). Hence, mycorrhiza-like associations are suggested to be essential for the growth of Haplomitriopsida in nature. It is also suggested that Haplomitriopsida does not depend on Mucoromycotina because analyzed colony of *H. mnioides* from Hachijo-jima Isl. lacked Mucoromycotina but showed MLS with Glomeromycotina (HA: Table 1). Several genes that rescue *Medicago truncatula* Gaertn. mutants for AM formation with *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler therefore essential for AM formation have been isolated from *Haplomitrium gibbsiae* (Steph.) R.M. Schust. (Wang et al. 2010). Furthermore, all subgenera constituting *Haplomitrium*, i.e. subg. *Calobryum* and subg. *Haplomitrium* (Stech and Frey 2004), include species that harbour Glomeromycotina mycobionts, i.e. *H. mnioides* and *H. chilensis*, respectively (this study; Ligrone et al. 2007). These suggest that both subgenera in *Haplomitrium* potentially have the ability to form AM-like association with Glomeromycotina. On the other hand, it was reported that 16 specimens from 5 species of *Haplomitrium*, including two *H. mnioides* from China, completely lacked Glomeromycotina mycobionts (Rimington et al. 2018). Thus, presence or absence of Glomeromycotina mycobionts in *Haplomitrium* is suggested to be largely dependent on species and locality of sampling. Recent study suggested that Mucoromycotina are more successful in competing with soil microbes for organic N resources than Glomeromycotina (Field et al. 2019). Thus, Mucoromycotina mycobionts might be dominant in the environment rich in organic matter. It should be also considered that phenology affects detection of Glomeromycotina in *H. mnioides* because active fungal-colonized cells are mainly present during its sporophyte development stage (see materials and methods). The

absence of Glomeromycotina in *Haplomitrium* reported by Rimington et al. (2018) is presumably more or less affected by those differences of sampling methods.

The present research determined that Endogonaceae and Densosporaceae form HS-type MLS on *H. mnioides*. In contrast, they form non-HS-type MLS on several other liverworts in Marchantiopsida and Jungermanniopsida and hornworts (Desirò et al. 2013; Field and Pressel 2018; Field et al. 2015c; Strullu-Derrien et al. 2014). The non-HS-type MLS was considered to be a kind of the fine endophyte (FE), i.e. AM-like structure of Endogonaceae and Densosporaceae (Desirò et al. 2017; Orchard et al. 2017a). Turnau et al. (1999) also illustrated FE as fine arbuscule and hyphae in the thalli of *Conocephalum conicum* (L.) Dumort. (Marchantiopsida) and *Pellia endiviifolia* (Dicks.) Dumort. (Jungermanniopsida). The current definition of FE includes vascular plants and non-vascular liverworts and hornworts as hosts (Walker et al. 2018).

Mycobionts of HS-type MLS on Haplomitriopsida and FE on other land plants had not been distinguished using molecular phylogeny, rather closely related (Bidartondo et al. 2011; Desirò et al. 2013, 2017). Hyphae of the HS-type MLS and FE shared common characteristics on hosts: (1) numerous hyphae developed at the intercellular space were thick-walled and multi-layered [HS-type: Duckett et al. (2006), Field et al. (2015b); FE: Gianinazzi-Pearson et al. (1981)], (2) two distinct hyphal wall layers were distinguishable by the PATAg test [HS-type: Fig. 10d in Duckett et al. (2006); FE: Gianinazzi-Pearson et al. (1981)], and (3) inter- and intracellular hyphae were up to 5 μm in diameter and the latter formed fine branch [HS-type: this study, Carafa et al. (2003), Duckett et al. (2006), Field et al. (2015b); FE: Błaszkowski (2012), Walker et al. (2018)]. In other words, hyphal characteristics of Endogonales-Haplomitriopsida-associated MLS coincide with those of FE. Hence, HS-type MLS is suggested to be a unique type of FE, specially formed on Haplomitriopsida. In addition, we were able to distinguish two HS-types in relation to

Endogonales families, finding that the hyphae were thicker and HS was larger in Endogonaceae than in Densosporaceae.

Mycobionts of *C. conicum* and *P. endiviifolia* were identified as ALB-type Glomeromycotina (Ligrone et al. 2007). However, those species growing along streams predominantly formed FE (Mucoromycotina) and only 10% of thalli formed ALB-type (Turnau et al. 1999). While colonization rates of Glomeromycotina have been shown to decrease under the flooded treatment as compared with dry conditions (Stevens et al. 2011), FE showed positive responses in colonization rates against waterlogging (Orchard et al. 2016). Furthermore, FE has been suggested to have a greater tolerance for physical disturbance than Glomeromycotina (Orchard et al. 2017b). Most species of *Haplomitrium*, including *H. mnioides*, frequently grow on easily eroding damp ground (Bartholomew-Began 1991). Therefore, it is suggested that Endogonales is superior to Glomeromycotina in forming MLS on *Haplomitrium* under such environments. In fact, the number of host cells colonized with Endogonales was much larger than Glomeromycotina in most of the *H. mnioides* samples, with the exception of two (Table S2). The cause of Glomeromycotina-dominance of sample from Hachijo-jima Isl is unclear, however weakly basic (pH=7.32: Oguchi and Sato 2003) ground water springing out just from our sampling site possibly caused an increase in soil pH. Some studies have shown that with increasing pH, colonization by FE decreases (Gucwa-Przepióra et al. 2013; Orchard et al. 2016; Postma et al. 2007; Wang et al. 1985). The surface area of the arbuscule per particular volume is considered to be larger than the HS, therefore the ALB-type MLS was likely structurally more efficient as a nutrient-exchange organ than HS-type. HS is suggested to function in nutrient exchange and storage (Field et al. 2015a, b) and is finally decomposed and absorbed by the host cell (Carafa et al. 2003; Duckett et al. 2006). As different developmental stages of HS are present in a single thallus (Carafa et al. 2003; Duckett et al. 2006; this study), HS

may occur repeatedly and develop area of MLS. Storage function of HS is possibly efficient to host plant for survive under difficulty of nutrient absorption from soil via extraradical hyphae. When the soil is disturbed, the extraradical mycelium is possibly cut and the supply of nutrients for host plant temporarily ceases. Therefore, frequent disturbance of the soil environment may confer advantage for Endogonales in the colonization on *Haplomitrium*.

Molecular dating analysis estimated that the ages of the most recent common ancestors (MRCA) of Endogonales as 420 million years ago (Mya), and the age of MRCA of Glomeromycotina as 426 Mya (Chang et al. 2018). In other words, Endogonales originated in the mid-late Silurian, contemporary to the origin of Glomeromycotina. This suggests that the establishment of the Endogonales-plant association may have co-occurred around the same time as the AM association, both during the initial colonization of land by plants (Chang et al. 2018). Based on this analysis and the present finding, we provide a hypothesis of how early land plants adapted from the waterside to drier habitats. At the primary stage of plant terrestrialization, ancestral land plants grew on the waterside in association with Endogonales and Glomeromycotina in parallel. With the expansion of habitats and invasion to new environments, host-fungus combinations diverged to suit newly acquired habitats by developing a tolerance against drier conditions. As a result, early extant land plant linages of liverwort, such as *H. mnioides* have been forced into limited habitats, including under shaded forest condition and waterlogged sites near brooks and streams, by association with primarily HS-type Endogonales (Endogonaceae and Densosporaceae) and secondarily Glomeromycotina. Further studies of dual colonization of Mucoromycotina and Glomeromycotina in *H. mnioides* will give us additional insight into the various selection mechanisms of mycobionts in basal land plants.

Acknowledgments

We are grateful to Prof. Mitsuru Moriguchi, Dr. Takamichi Orihara, Ms. Yumiko Oba, Mr. Yoshiteru Kawabata, and Dr. Kanami Yoshino for supporting for our sampling. We are also grateful to Prof. Tomio Yamaguchi for depositing of *H. mnioides* specimens. We thank the technical staff in the Division of Instrumental Analysis, Research Center for Human and Environmental Sciences, Shinshu University for DNA sequencing. This study was supported in part by KAKENHI (No. JP14J09199, No. JP25291084 and JP15H01751) from the Japan Society for the Promotion of Science (JSPS).

References

Bakalin V, Vilnet A (2017) How many species are in *Apotreubia* S. Hatt. & Mizut. (Marchantiophyta)? Nova Hedwig 104:473–482. doi: 10.1127/nova_hedwigia/2016/0386

Bartholomew-Began SE (1991) A morphogenetic re-evaluation of *Haplomitrium* Nees (Hepatophyta). Bryophyt Bibl 41:1–297

Bidartondo MI, Read DJ, Trappe JM, Merckx V, Ligrone R, Duckett JG (2011) The dawn of symbiosis between plants and fungi. Biol Lett 7:574–577. doi: 10.1098/rsbl.2010.1203

Błaszkowski J (2012) *Glomeromycota*. W Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland

Carafa A, Duckett JG, Ligrone R (2003) Subterranean gametophytic axes in the primitive liverwort *Haplomitrium* harbour a unique type of endophytic association with aseptate fungi. New Phytol 160:185–197. doi: http://10.1046/j.1469-8137.2003.00849.x

Chang Y, Desirò A, Na H, Sandor L, Lipzen A, Clum A, Barry K, Grigoriev IV, Martin FM, Stajich JE, Smith ME, Bonito G, Spatafora JW (2019) Phylogenomics of Endogonaceae and evolution of mycorrhizas within Mucoromycota. New Phytol 222:511–525. doi: 10.1111/nph.15613

Desirò A, Duckett JG, Pressel S, Villarreal JC, Bidartondo MI (2013) Fungal symbioses in hornworts: a chequered history. Proc R Soc B: Biol Sci. doi: 10.1098/rspb.2013.0207

Desirò A, Rimington WR, Jacob A, Pol NV, Smith ME, Trappe JM, Bidartondo MI, Bonito G (2017) Multigene phylogeny of Endogonales, an early diverging lineage of fungi associated with plants. IMA Fungus 8:245–257. doi: http://10.5598/imafungus.2017.08.02.03

Duckett JG, Carafa A, Ligrone R (2006) A highly differentiated glomeromycotean association with the mucilage-secreting, primitive antipodean liverwort *Treubia* (Treubiaceae): clues to the origins of mycorrhizas. Am J Bot 93:797–813. doi: http://10.3732/ajb.93.6.797

Edgar RC (2016) UCHIME2: improved chimera prediction for amplicon sequencing. bioRxiv. doi: 10.1101/074252

Field KJ, Bidartondo MI, Rimington WR, Hoysted GA, Beerling D, Cameron DD, Duckett JG, Leake JR, Pressel S (2019) Functional complementarity of ancient plant-fungal mutualisms: contrasting nitrogen, phosphorus and carbon exchanges between Mucoromycotina and Glomeromycotina fungal symbionts of liverworts. New Phytol 223:908–921. doi: 10.1111/nph.15819

Field KJ, Cameron DD, Leake JR, Tille S, Bidartondo MI, Beerling DJ (2012) Contrasting arbuscular mycorrhizal responses of vascular and non-vascular plants to a simulated Palaeozoic CO_2 decline. Nat Commun 3:835. doi: 10.1038/ncomms1831

Field KJ, Pressel S (2018) Unity in diversity: structural and functional insights into the ancient partnerships between plants and fungi. New Phytol 220:996–1011. doi: 10.1111/nph.15158

Field KJ, Pressel S, Duckett JG, Rimington WR, Bidartondo MI (2015a) Symbiotic options for the conquest of land. Trends Ecol Evol 30:477–486. doi: 10.1016/j.tree.2015.05.007

Field KJ, Rimington WR, Bidartondo MI, Allinson KE, Beerling DJ, Cameron DD, Duckett JG, Leake JR, Pressel S (2015b) First evidence of mutualism between ancient plant lineages (Haplomitriopsida liverworts) and Mucoromycotina fungi and its response to simulated Palaeozoic changes in atmospheric CO_2. New Phytol 205:743–756. doi: 10.1111/nph.13024

Field KJ, Rimington WR, Bidartondo MI, Allinson KE, Beerling DJ, Cameron DD, Duckett JG, Leake JR, Pressel S. (2015c) Functional analysis of liverworts in dual symbiosis with Glomeromycota and Mucoromycotina fungi under a simulated Palaeozoic CO_2 decline. ISME J 10:1514–1526. doi: 10.1038/ismej.2015.204

Gianinazzi-Pearson V, Morandi D, Dexheimer J, Gianinazzi S (1981) Ultrastructural and ultracytochemical features of a *Glomus tenuis* mycorrhiza. New Phytol 88:633–639. doi: 10.1111/j.1469-8137.1981.tb01739.x

Goebel K (1891) Morphologische und biologische studien. IV. Über javanische Lebermoose. 1 *Treubia*. Ann Jard Bot Buitenzorg 9:1–11

Grubb PJ (1970) Observations on the structure and biology of *Haplomitrium* and *Takakia*, hepatics with roots. New Phytol 69:303–326. doi: 10.1111/j.1469-8137.1970.tb02430.x

Gucwa-Przepióra E, Błaszkowski J, Kurtyka R, Małkowski Ł, Małkowski E (2013) Arbuscular mycorrhiza of *Deschampsia cespitosa* (Poaceae) at different soil depths in highly metal-contaminated site in southern Poland. Acta Soc Bot Pol 82:251–258. doi: 10.5586/asbp.2013.033

Hattori S, Mizutani M (1958) What is *Takakia lepidozioides*? J Hattori Bot Lab 38:115–121

Hirose D, Degawa Y, Yamamoto K, Yamada A (2014) *Sphaerocreas pubescens* is a member of the Mucoromycotina closely related to fungi associated with liverworts and hornworts. Mycoscience 55:221–226. doi: 10.1016/j.myc.2013.09.002

Humphreys CP, Franks PJ, Rees M, Bidartondo MI, Leake JR, Beerling DJ (2010)

Mutualistic mycorrhiza-like symbiosis in the most ancient group of land plants. Nat Commun 1:103. doi: 10.1038/ncomms1105

Inoue H (1960) Studies in *Treubia nana* (Hepaticae) with special reference to the antheridial development. Bot Mag Tokyo 73:225–230. doi: 10.15281/jplantres1887.73.225

Izumitsu K, Hatoh K, Sumita T, Kitade Y, Morita A, Gafur A, Ohta A, Kawai M, Yamanaka T, Neda H, Ota Y, Tanaka C (2012) Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR. Mycoscience 53:396–401. doi: 10.1007/S10267-012-0182-3

Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 92:486–488. doi: 10.1016/S0953-7562(89)80195-9

Koske RE, Tessier B (1983) A convenient, permanent slide mounting medium. Mycol Soc Am Newslett 34:59

Lee J, Lee S, Young JP (2008) Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 65:339–349. doi: 10.1111/j.1574-6941.2008.00531.x

Ligrone R, Carafa A, Lumini E, Bianciotto V, Bonfante P, Duckett JG (2007) Glomeromycotean associations in liverworts: a molecular, cellular, and taxonomic analysis. Am J Bot 94:1756–1777. doi: http://10.3732/ajb.94.11.1756

Lilienfeld F (1911) Beiträge zur kenntnis der art *Haplomitrium hookeri* Nees. Bull Acad Sci Crac Sér B 1911:315–339

Morton JB, Redecker D (2001) Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia 93:181–195. doi: 10.2307/3761615

Oguchi S, Sato K (2003) Springing out mechanisms to groundwater at the upper reaches of

rivers of Mt. Higashiyama, Hachijo-jima Island. Proc Inst Nat Sci Nihon Univ 38:233–242.

Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier U, Zobel M (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol 188:223–241. doi: 10.1111/j.1469-8137.2010.03334.x

Orchard S, Hilton S, Bending GD, Dickie IA, Standish RJ, Gleeson DB, Jeffery RP, Powell JR, Walker C, Bass D, Monk J, Simonin A, Ryan MH (2017a) Fine endophytes (*Glomus tenue*) are related to Mucoromycotina, not Glomeromycota. New Phytol 213:481–486. doi: 10.1111/nph.14268

Orchard S, Standish RJ, Dickie IA, Renton M, Walker C, Moot D, Ryan MH (2017b) Fine root endophytes under scrutiny: a review of the literature on arbuscule-producing fungi recently suggested to belong to the Mucoromycotina. Mycorrhiza 27:619–638. doi: http://10.1007/s00572-017-0782-z

Orchard S, Standish RJ, Nicol D, Gupta VVSR, Ryan MH (2016) The response of fine root endophyte (*Glomus tenue*) to waterlogging is dependent on host plant species and soil type. Plant Soil 403:305–315. doi: 10.1007/s11104-016-2804-6

Postma JWM, Olsson PA, Falkengren-Grerup U (2007) Root colonisation by arbuscular mycorrhizal, fine endophytic and dark septate fungi across a pH gradient in acid beech forests. Soil Biol Biochem 39:400–408. doi: 10.1016/j.soilbio.2006.08.007

Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921. doi: 10.1126/science.289.5486.1920

Rimington WR, Pressel S, Duckett JG, Field KJ, Read DJ, Bidartondo MI (2018) Ancient plants with ancient fungi: liverworts associate with early-diverging arbuscular mycorrhizal fungi. Proc R Soc B: Biol Sci. doi: 10.1098/rspb.2018.1600

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542. doi: 10.1093/sysbio/sys029

Shi XQ, Zhu RL. 2006. A range extension for *Haplomitrium mnioides* (Lindb.) R.M.Schust. Trop Bryol 27:87–90. doi: 10.11646/bde.27.1.11

Silvestro D, Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. Org Divers Evol 12:335–337. doi: 10.1007/s13127-011-0056-0

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69. doi: 10.1038/363067a0

Smit E, Leeflang P, Glandorf B, Van Elsas JD, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Environ Microbiol 65:2614–2621

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, Cambridge, UK

Söderström L, Hagborg A, von Konrat M, Bartholomew-Began S, Bell D, Briscoe L, Brown E, Cargill DC, Costa DP, Crandall-Stotler BJ, Cooper ED, Dauphin G, Engel JJ, Feldberg K, Glenny D, Gradstein SR, He X, Heinrichs J, Hentschel J, Ilkiu-Borges AL, Katagiri T, Konstantinova NA, Larraín J, Long DG, Nebel M, Pócs T, Puche F, Reiner-Drehwald E, Renner MA, Sass-Gyarmati A, Schäfer-Verwimp A, Moragues JG, Stotler RE, Sukkharak P, Thiers BM, Uribe J, Váňa J, Villarreal JC, Wigginton M, Zhang L, Zhu RL (2016) World checklist of hornworts and liverworts. PhytoKeys 27:1–828. doi: 10.3897/phytokeys.59.6261

Stahl M (1949) Die Mycorrhiza der Lebermoose mit besonderer Berucksichtigung der thallosen formen. Planta 37:103–148. doi: 10.1007/BF01929705

Stech M, Frey W (2004) Molecular circumscription and relationships of selected Gondwanan species of *Haplomitrium* (Calobryales, Haplomitriopsida, Hepaticophytina). Studies in austral temperate rain forest bryophytes 24. Nova Hedwig 78: 57–70. doi: 10.1127/0029-5035/2004/0078-0057

Stevens KJ, Wall CB, Janssen JA (2011) Effects of arbuscular mycorrhizal fungi on seedling growth and development of two wetland plants, *Bidens frondosa* L., and *Eclipta prostrata* (L.) L., grown under three levels of water availability. Mycorrhiza 21:279–288. doi: 10.1007/s00572-010-0334-2

Strullu-Derrien C, Kenrick P, Pressel S, Duckett JG, Rioult J-P, Strullu D-G (2014) Fungal associations in *Horneophyton ligneri* from the Rhynie Chert (c. 407 million year old) closely resemble those in extant lower land plants: novel insights into ancestral plant–fungus symbioses. New Phytol 203:964–979. doi: 10.1111/nph.12805

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi: 10.1093/molbev/mst197

Turnau K, Ronikier M, Unrug J (1999) Role of mycorrhizal links between plants in establishment of liverworts thalli in natural habitat. Acta Soc Bot Pol 68:63–68. doi: 10.5586/asbp.1999.011

Walker C, Gollotte A, Redecker D (2018) A new genus, *Planticonsortium* (Mucoromycotina), and new combination (*P. tenue*), for the fine root endophyte, *Glomus tenue* (basionym *Rhizophagus tenuis*). Mycorrhiza 28:213–219. doi: 10.1007/s00572-017-0815-7

Wang GM, Stribley DP, Tinker PB (1985) Soil pH and vesicular-arbuscular mycorrhizas. In: Fitter AH (ed) Ecological Interactions in Soil: Plants, Microbes and Animals. Blackwell

Scientific, Oxford, UK, pp 219–224

Wang B, Yeun LH, Zue J-Y, Liu Y, Ané J-M, Qiu Y-L (2010) Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. New Phytol 186:514–525. doi: 10.1111/j.1469-8137.2009.03137.x

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a Guide to Methods and Applications. Academic Press, New York, USA, pp 315–322

Wright ES, Yilmaz LS, Noguera DR (2012) DECIPHER, a search-based approach to chimera identification for 16S rRNA Sequences. Appl Environ Microbiol 78:717–725. doi: 10.1128/AEM.06516-11

Yamamoto K, Degawa Y, Hirose D, Fukuda M, Yamada A (2015) Morphology and phylogeny of four *Endogone* species and *Sphaerocreas pubescens* collected in Japan. Mycol Prog 14:86. doi: 10.1007/s11557-015-1111-6

Yamamoto K, Degawa Y, Takashima Y, Fukuda M, Yamada A. (2017a) *Endogone corticioides* sp. nov. from subalpine conifer forests in Japan and China, and its multi-locus phylogeny. Mycoscience 58:23–29. doi: 10.1016/j.myc.2016.08.001

Yamamoto K, Endo N, Degawa Y, Fukuda M, Yamada A (2017b) First detection of *Endogone* ectomycorrhizas in natural oak forests. Mycorrhiza 27:295–301. doi: 10.1007/s00572-016-0740-1

Table 1 – Number of rhizome fragments indicating fungal colonizations of Mucoromycotina and Glomeromycotina in each sample.

Sample name	Number of examined rhizome fragment	Number of rhizome fragments observed mycorrhiza-like structure			Number of rhizome fragments detected following taxa by the sequencing*						
		Hyphal-swelling type		Arbuscule-like branching type	Mucoromycotina		Glomeromycotina		Co-detections of:		
		Large	Small		E	D	G	A	E-G	D-G	D-A
KY	30	3	5	4	3	5	1	2	0	1	0
NA	30	0	3	0	0	3	0	0	0	0	0
IS	15	8	0	4	8	0	1	0	1	0	0
HI	30	3	4	0	3	4	0	0	0	0	0
UW	15	14	0	0	14	0	0	0	0	0	0
IN	15	0	2	4	0	6	0	1	0	0	1
HA	15	0	0	3	0	0	2	1	0	0	0

*E, Endogonaceae; D, Densosporaceae; G, Glomerales; A, Archaeosporales.

Figure legends

Fig. 1 Gametophyte of *Haplomitrium mnioides* and its mycorrhiza-like structures (MLSs). (A) Gametophyte showing epigeous leafy shoots and subterranean rhizome, collected from Kyoto. (B) Rhizome of gametophyte collected from Uwajima. Arrows indicate epidermal cells that show pale color due to fungal colonization. (C) Extraradical spore of Glomeromycotina on the rhizome collected from Ino. (D–Q) MLSs identified in mycobionts both by morphology and molecular phylogeny. (D–G) Endogonaceae (D: KY-15; E: IS-14; F: HI-11; G: UW-1). Mature (D, F, G) and young (E) stages are indicated. (H–K) Densosporaceae (H: KY-1; I: KY-17; J: HI-13; K: IN-2). Mature (H, I, K) and young (J) stages are indicated. (H) Hyphae and swellings leaked out from colonized host cell. (L–Q) Glomeromycotina (L: KY-9; M: KY-20; N: IS-11; O: IN-12; P: HA-5; Q: HA-3). Arbuscular MLS (L–P) and intraradical spore (Q). (P) Hyphae extending from colonized cell penetrated host cell wall (arrow) and colonized the adjoining cell. (R) Dual colonization of Densosporaceae and Glomeromycotina (KY-9). Different types of MLSs are present close together on a rhizome. S, hyphal swelling of Endogonaceae and Densosporaceae; A, arbuscule-like structure of Glomeromycotina, T; trunk hyphae of Glomeromycotina. Bars: (A) 3 mm; (B) 1 mm; (C) 100 μm; (D–R) 20 μm.

Fig. 2 Maximum likelihood (ML) phylogeny of Endogonaceae, Densosporaceae, and Mucoromycotina mycobionts of Haplomitriopsida based on 18S rDNA sequences (dataset 1). Notably, all Mucoromycotina mycobionts of Japanese *H. mnioides* (red; sample name is indicated) and other Haplomitriopsida liverworts (blue: sequenced in Bidartondo et al. (2011) and Field et al. (2015b)) belong to Endogonaceae or Densosporaceae. *Mortierella chlamydospora* (AF157143), *Echinochlamydosporium variabile* (EU688964), and *Calcarisporiella thermophila* (AB597205) were used as outgroup taxa. Bootstrap (BS)

values > 50% from ML (left) and maximum parsimony (MP) (center), and Bayesian posterior probability (PP) > 0.5 (right) are shown near the nodes. Branches supported by MLBS/MPBS > 70% and PP > 0.95% (black) and MLBS/MPBS > 70% (gray) are depicted as thick lines. AUS, Australia; JPN, Japan; MEX, Mexico; MYS, Malaysia; NZL, New Zealand; SCO, Scotland.

Fig. 3 ML phylogeny of Glomeromycotina (Glomerales, Diversisporales, Archaeosporales, and Paraglomerales) and Glomeromycotina mycobionts of Haplomitriopsida based on 18S rDNA sequences (dataset 2). Glomeromycotina mycobionts of Japanese *H. mnioides* (red; sample name is indicated) and *Haplomitrium chilensis* (blue: sequenced in Ligrone et al. (2007)) belong to Glomerales or Archaeosporales. *Endogone pisiformis* (LC107347) and *M. chlamydospora* (AF157143) were used as outgroup taxa. BS values > 50% from ML (left) and MP (center), and PP > 0.5 (right) are shown near the nodes. Branches supported by MLBS/MPBS > 70% and PP > 0.95% (black) and MLBS/MPBS > 70% (gray) are depicted as thick lines. Sequence name with the initials VTX stand for virtual taxa from MaarjAM. CHL, Chile.

Fig. 4 Boxplot showing the diameter of hyphal swellings (HS) in Endogonaceae- (n = 258) and Densosporaceae-identified (n = 530) rhizome fragments. Asterisk shows significance difference between two families at $P < 0.01$ by the Welch's t-test.

Fig 1

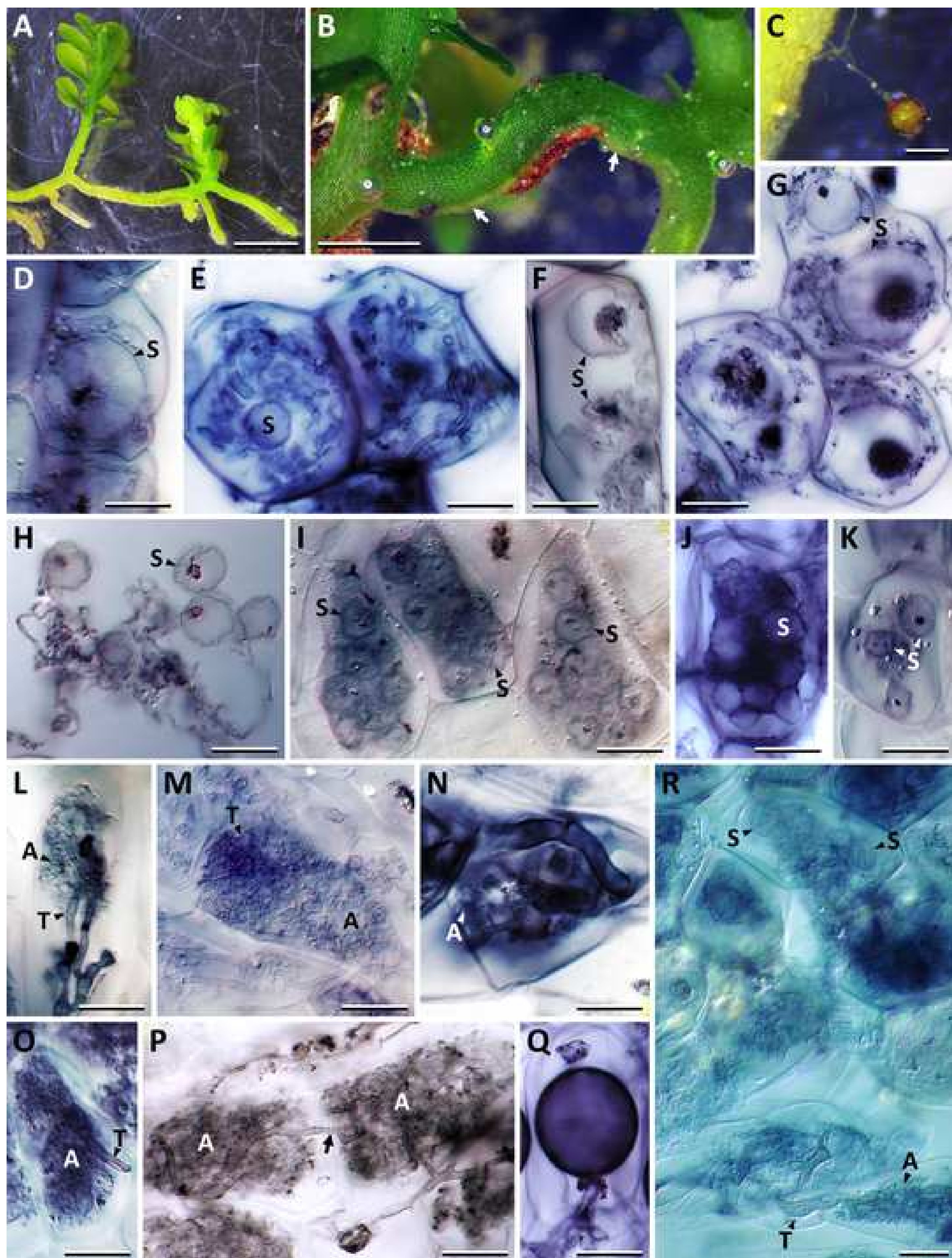

Fig 2

Fig 3

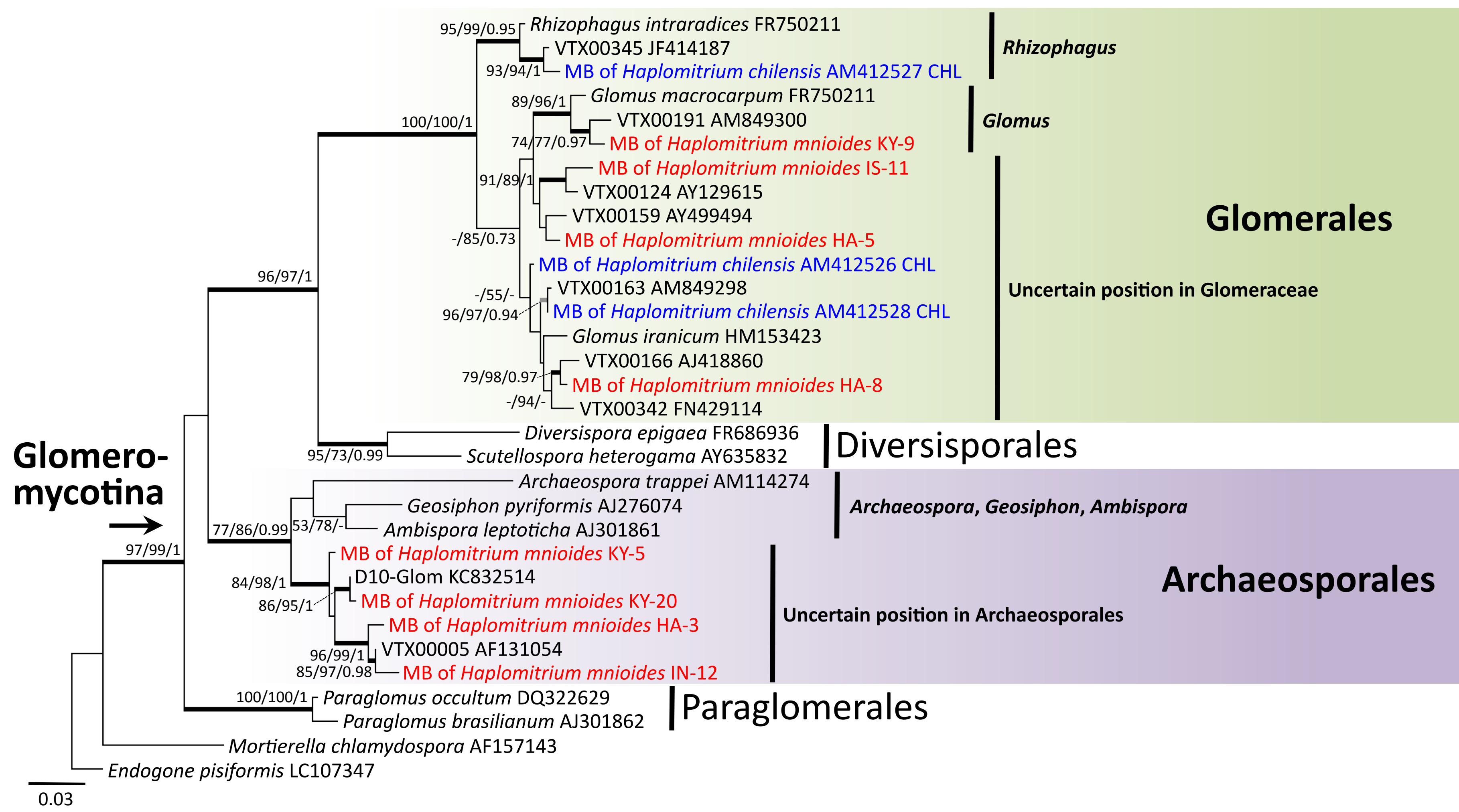

Fig 4