Mycoscience (2011) 52:217–223
DOI 10.1007/s10267-010-0084-1

FULL PAPER

Arbuscular mycorrhizal fungi in roots of non-photosynthetic plants, *Sciaphila japonica* and *Sciaphila tosaensis* (Triuridaceae)

Masahide Yamato · Takahiro Yagame · Koji Iwase

Received: 8 June 2010 / Accepted: 20 October 2010 / Published online: 23 November 2010

Abstract The mycorrhizal fungi in the roots of achlorophyllous *Sciaphila japonica* and *S. tosaensis* (Triuridaceae) were identified by molecular methods. The habitats of *S. japonica* were in a tree plantation of Japanese cypress, *Chamaecyparis obtusa*, and bamboo forests, and those of *S. tosaensis* were in a camellia forest and a bamboo forest. In the root cortical cells of both plants, aseptate hyphal coils were observed, which suggested the *Paris*-type arbuscular mycorrhiza (AM). A phylogenetic analysis based on a partial sequence of an AM fungal nuclear small subunit ribosomal RNA gene showed that the fungal DNA sequences of *S. japonica* were separated into three closely related clades. Those of *S. tosaensis* were separated into two clades, which were also closely related to each other. The AM fungi of *S. japonica* and *S. tosaensis* were completely separated in the phylogenetic tree even among those found in the same habitat, which suggests the high specificities in the plant-fungal partnerships. All the detected AM fungi in these plants belonged to *Glomus*-group A. Even though the habitats are in quite common environments, both plant species are known as endangered species in Japan. Such a definite specificity in AM symbioses seems to restrict the distribution of the myco-heterotrophic plants.

Keywords *Glomus*-group A · Myco-heterotrophic plants · *Paris*-type AM · Specificity

Introduction

Arbuscular mycorrhiza (AM) is the symbiotic association between fungi in the phylum Glomeromycota (Schüßler et al. 2001) and various terrestrial plants. In general, AM fungi colonize chlorophyllous plants and provide them with mineral nutrients, especially phosphate, and in turn receive the photosynthates (Smith and Read 1997). Meanwhile, AM fungal colonization has been found in some achlorophyllous plants in Burmanniaceae, Corsiaceae, Gentianaceae, Thismiaceae, Triuridaceae (Yamato 2001; Bidartondo et al. 2002; Franke et al. 2006; Merckx and Bidartondo 2008), and achlorophyllous gametophytes of lycophytes in Lycopodiaceae and some ferns in Ophioglossaceae and Psilotaceae (Winther and Friedman 2007, 2008, 2009).

The Triuridaceae is a monocotyledonous family with 11 genera, in which all species are known as achlorophyllous plants (Maas-van de Kamer 1995). Because of the insufficient taxonomic characters with tiny flowers and reduced scale-like leaves, its phylogenetic position used to be controversial. Formerly, it was proposed to be classified in its own taxonomic group, order Triuridales (Cronquist 1988), superorder Triuridanae (Thorne 1992), and even in subclass Triurididae (Takhtajan 1997). However, recent molecular evidence based on sequences of a nuclear small subunit ribosomal RNA gene (nSSU rDNA) assigned it to Pandanales (Chase et al. 2000), which was allowed by the Angiosperm Phylogeny Group (2003). Due to the achlorophyllous feature, myco-heterotrophy (Leake 1994) has been supposed for the Triuridaceae species, and arbuscular

Electronic supplementary material The online version of this article (doi:10.1007/s10267-010-0084-1) contains supplementary material, which is available to authorized users.

M. Yamato (✉) · T. Yagame · K. Iwase
Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan
e-mail: m_yamato@muses.tottori-u.ac.jp

mycorrhizal (AM) fungi were identified in *Triuris hyalina* Miers (Imhof 1998) and *Sciaphila polygyna* Maas (Imhof 2003) from their morphological features, and in *S. tosaensis* Makino (Yamato 2001), *S. ledermannii* Engl. and *Kupea martinetugei* Cheek & S.A. Williams (Franke et al. 2006; Merckx and Bidartondo 2008) by molecular studies. In the molecular studies, it was found that the examined plants had symbiotic relations with specific groups of AM fungi in *Glomus*-group A as in other myco-heterotrophic AM plants (Bidartondo et al. 2002; Franke et al. 2006; Merckx and Bidartondo 2008).

Sciaphila japonica and *S. tosaensis* in Triuridaceae are found on understories in the western part of Japan (Kitamura et al. 2008). Both plants were sometimes found in the same habitats. In *S. tosaensis*, colonization of AM fungi was shown (Yamato 2001), but the fungal diversity has not been examined. In the present study, we have identified AM fungi in *S. japonica* and *S. tosaensis* in several habitats to reveal their symbiotic relations with AM fungi. Understanding of AM symbioses would be helpful to elucidate the ecological aspects on distribution of the myco-heterotrophic plants that are known as endangered species in Japan.

Materials and methods

Sampling

At the end of August 2009, the plant samples were collected at two habitats in Tokushima Pref. (T1, T2) and at two habitats in Kochi Pref. (K1, K2). The distances among the habitats are 22.6 km (T1–T2), 62.5 km (T1–K1), 56.4 km (T1–K2), 76.1 km (T2–K1), 69.0 km (T2–K2) and 7.5 km (K1–K2). Habitat T1 was in a plantation of Japanese cypress, *Chamaecyparis obtusa* Siebold & Zucc., in Naka-cho, Naka Gun, about 560 m above sea level, in which three individuals of *S. japonica* (T1H1, T1H2, T1H3) were collected. The three individuals were separately located 3–6 m apart in the habitat. Habitat T2 was in an evergreen broad-leaved forest with *Camellia japonica* L. and *Machilus thunbergii* Siebold & Zucc. in Minamicho, Kaifu Gun, about 140 m above sea level, where three individuals of *S. tosaensis* (T2U1, T2U2, T2U3), 1–2.5 m apart, were collected. Habitat K1 was in a forest of Japanese bamboo, *Phyllostachys bambusoides* Siebold & Zucc., in Kami City, about 250 m above sea level, and two individuals of *S. japonica* (K1H1, K1H2), 1.6 m apart, were collected. Habitat K2 was in a forest of naturalized Chinese bamboo, *P. pubescens* Mazel, in Kami City, about 100 m above sea level, where two individuals of *S. japonica* (K2H1, K2H2) and two individuals of *S. tosaensis* (K2U1, K2U2) were collected. The distances between K2H1 and K2H2, and K2U1 and K2U2 were 1.5 and 0.7 m, respectively. The closest individuals of the different species in the habitat, K2U1 and K2H2, were separated by 1.8 m. The collected samples were each kept cool in individual plastic bags until use within 2 days after sampling.

Light microscopy

The collected roots of *S. japonica* and *S. tosaensis* were cut into 1-cm fragments, and those with yellowish pigment, indicating fungal colonization (Yamato 2001), were selected. The root fragments were cleared with 10% KOH and stained with 0.1% Chlorazol black E (Brundrett et al. 1996). The stained root fragments were then flattened under a cover glass to observe the morphology of AM associations with an interference contrast microscope, Eclipse 80i (Nikon, Tokyo, Japan).

Molecular identification

In order to examine the phylogenetic relationship between the examined and other plant species in Pandanales, total DNA was extracted from the shoots of the two arbitrarily selected samples each of *S. japonica*, T1H1 and K1H2, and *S. tosaensis*, T2U2 and K2U2, using the DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Partial sequences of nSSU rDNA were amplified by polymerase chain reactions (PCR) using primers NS1 and NS8 (White et al. 1990) with TaKaRa Ex Taq^{TM} Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 2 µl of the extracted DNA solution, 0.75 U of Taq polymerase, 0.25 µM of each primer, 200 µM of each deoxynucleotide triphosphate and 3 µl of the supplied PCR buffer in 30 µl of the total amount. The PCR program performed on Program Temp Control System PC-818S (Astec, Fukuoka, Japan) was as follows: the initial denaturation step at 94°C for 2 min, followed by a step of 30 cycles at 94°C for 45 s, 55°C for 1 min, 72°C for 2 min and then the final elongation step at 72°C for 10 min.

To examine AM fungal DNA, five fragments of pigmented root, 1 cm in length, were arbitrarily selected for each plant sample. Total DNA was extracted from the root samples as described above, and partial fungal nSSU rDNA was amplified by PCR using primers GEOA2 and GEO11 (Schwarzott and Schüßler 2001). We have confirmed that the primer pairs showed complete matches with some arbitrarily selected AM fungi in the four orders, Archaeosporales, Diversisporales, Glomerales and Paraglomerales (Table S1). The PCR reaction mixture was the same as that for the shoot sample except PCR primers. The PCR program was as follows: the initial denaturation step at 94°C for 2 min, followed by a step of 35 cycles at 94°C for 45 s,

57°C for 1 min, 72°C for 2 min, then the final elongation step at 72°C for 10 min.

The PCR products were cloned using pGEM-T Easy Vector System I (Promega, Tokyo, Japan), and four to five clones were arbitrarily chosen for each sample. The DNA inserts were sequenced by the dideoxysequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit in Genetic Analyser 3130 (Applied Biosystems, Tokyo, Japan).

For the phylogenetic analysis of the examined *Sciaphila* species, nSSU rDNA sequences of some plant families in Pandanales were downloaded from the GenBank database, while for that of AM fungi, the obtained DNA sequence data were subjected to BLAST searches (Altschul et al. 1997) to download some analogous DNA sequences. AM fungal nSSU rDNA sequences detected in other mycoheterotrophic plants (Bidartondo et al. 2002; Merckx and Bidartondo 2008) were also downloaded, i.e., *Arachnitis uniflora* Phil., *Voyriella parviflora* Miq., *Voyria corymbosa* Splitg. (Bidartondo et al. 2002), *Afrothismia* spp., *Burmannia hexaptera* Schltr., *Sciaphila ledermannii*, *Kupea martinetugei* (Merckx and Bidartondo 2008), and mycoheterotrophic gametophytes of *Botrychium* spp. (Ophioglossaceae) and *Huperzia hypogeae* (Lycopodiaceae) (Winther and Friedman 2007, 2008). Multiple sequence alignments were carried out for the sequenced and the downloaded data sets with Clustal X 2.0.12 (Larkin et al. 2007), and the neighbor-joining analyses (Saitou and Nei 1987) were performed for the aligned data sets by Clustal X with bootstrap analyses of 1,000 replications (Felsenstein 1985). The neighbor-joining trees were displayed using TreeView (Page 1996).

Results

Light microscopy

In root cortical cells of *S. japonica* and *S. tosaensis*, intracellular aseptate hyphal coils were observed with vesicles in some of them (Fig. 1). The morphological features suggested that the mycorrhizas are *Paris*-type AM. Degenerated amorphous fungal clumps were also observed in *S. japonica* (Fig. 1b) as well as in *S. tosaensis*. In some samples of *S. tosaensis*, a few aseptate hyphal coils consisting of bead-like hyphae were observed (Fig. 1d).

Phylogenetic relationship of the *Sciaphila* species

After cloning of the PCR products of partial plant nSSU rDNA (1,779 bp), at least three sequences were obtained from each sample to confirm the homogeneity and to select the majority sequences. The obtained DNA sequence data were deposited in the DDBJ database with accession numbers AB564589–AB564592. The two sequences in each *S. japonica* and *S. tosaensis* were completely identical, and the sequence identity between the species was 97.0% (1,725/1,779). From GenBank, eight plant species in

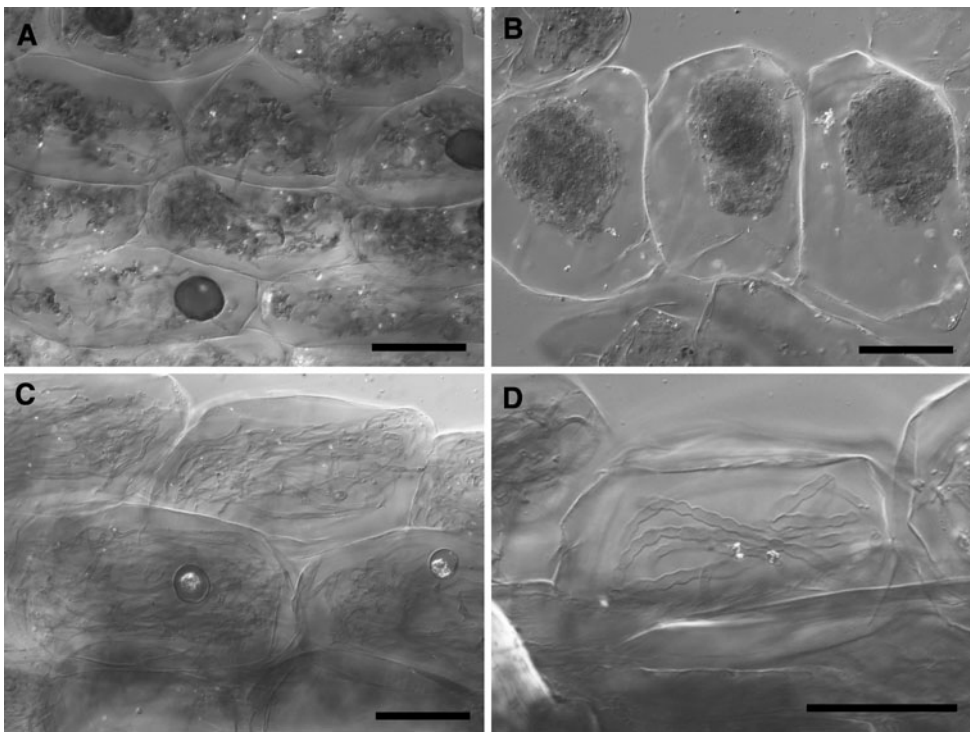

Fig. 1 Hyphal coils of mycorrhizal fungi colonizing cortical cells of *Sciaphila japonica* and *S. tosaensis*. **a** Aseptate hyphal coils with vesicles in *S. japonica*. **b** Degenerated hyphal coils in *S. japonica*. **c** Aseptate hyphal coils with vesicle in *S. tosaensis*. **d** A hyphal coil with bead-like structure in *S. tosaensis*. *Bars* 50 μm

Fig. 2 A neighbor-joining phylogenetic tree based on sequences of partial nuclear small subunit ribosomal RNA gene (nSSU rDNA) of plants in Pandanales. The tree is rooted to *Dioscorea elephantipes* (FJ215767) in Dioscoreales. The plant numbers were shown for *S. japonica* (T1H1 and K1H2) and *S. tosaensis* (T2U2 and K2U2). Bootstrap values are shown where they exceed 70% (1,000 replications). A scale is shown to infer the evolutionary distances. Accession numbers are given for all sequences

Pandanales including two species of *Sciaphila* and one *Kupea* in Triuridaceae were obtained, and the phylogenetic tree showed that *S. japonica* and *S. tosaensis* formed a monophyletic clade with other plants in Triuridaceae with a 99% bootstrap value (Fig. 2).

Molecular identification of AM fungi

Fungal partial nSSU rDNA was amplified by the primers GEOA2 and GEO11 in all examined samples. The nested procedure described by Schwarzott and Schüßler (2001) was not necessary for the primer pairs as demonstrated by Winther and Friedman (2007). After cloning of the PCR products, four or five sequences of AM fungal partial SSU rDNA (1,760–1,768 bp) were obtained from each sample of *S. japonica* and *S. tosaensis*. In total, 31 DNA sequences were obtained from 7 individuals of *S. japonica* and 21 sequences from 5 individuals of *S. tosaensis*. The obtained DNA sequence data were deposited in the DDBJ database with accession numbers AB555654–AB555684 for those of *S. japonica* and AB556916–AB556936 for those of *S. tosaensis*. The sequence identity among those obtained from *S. japonica* was 92–100% and that of *S. tosaensis* was 94–100%. Some fungal sequences in Ascomycota or Basidiomycota were also obtained from *S. japonica*, because of the low specificity of the primers towards AM fungi. They were excluded from the further analysis because of their dismembering into different fungal taxa and the dominance of AM fungi in the microscopy. The blast homologies of the sequences are shown in Table S2.

The phylogenetic analysis for the common region among the sequences (1,216–1,228 bp) showed that the AM fungi of *S. japonica* and *S. tosaensis* belong to *Glomus*-group A as well as those of other myco-heterotrophic plants (Fig. 3). The phylogenetic tree showed that the obtained AM fungal nSSU rDNA sequences of *S. japonica* were separated into three clades with 82, 98 and 99% bootstrap values, which were closely related to one another. An AM fungal sequence of myco-heterotrophic *Voyria corymbosa* (AJ430852) was closely related to one of them, *S. japonica* symbionts clade-3. It was also shown that almost all sequences of *S. tosaensis* were separated into two clades with 94 and 99% bootstrap values, which were closely related to each other. The AM fungal sequences detected in myco-heterotrophic *S. ledermannii* (Triuridaceae), a liverwort *Marchantia foliacea* and photosynthetic *Tacca plataginea* were also included in one of them, *S. tosaensis* symbionts clade-1. Furthermore, the AM fungal sequences of myco-heterotrophic *Arachnitis uniflora* were nearly related to both clades as well as those of *Botrychium* spp. and *Huperzia* spp.

Discussion

The morphological features of mycorrhizal fungi observed in root cortical cells of *S. japonica* and *S. tosaensis* were the intracellular spread of hyphal coils with some vesicles and conceivably their degenerated structure, amorphous clumps, which were similar to those of *S. tosaensis* reported previously (Yamato 2001) as well as those found in other myco-heterotrophic plants (Imhof 1999, 2003, 2007). Almost all the observed hyphal coils are aseptate, indicating the dominance of AM fungi. A few aseptate hyphal coils with bead-like structure were observed in some *S. tosaensis* samples. The bead-like hyphae were also observed in *S. tosaensis* collected in Fukui Pref., Japan (Yamato 2001). Imhof (2003) also observed the bead-like

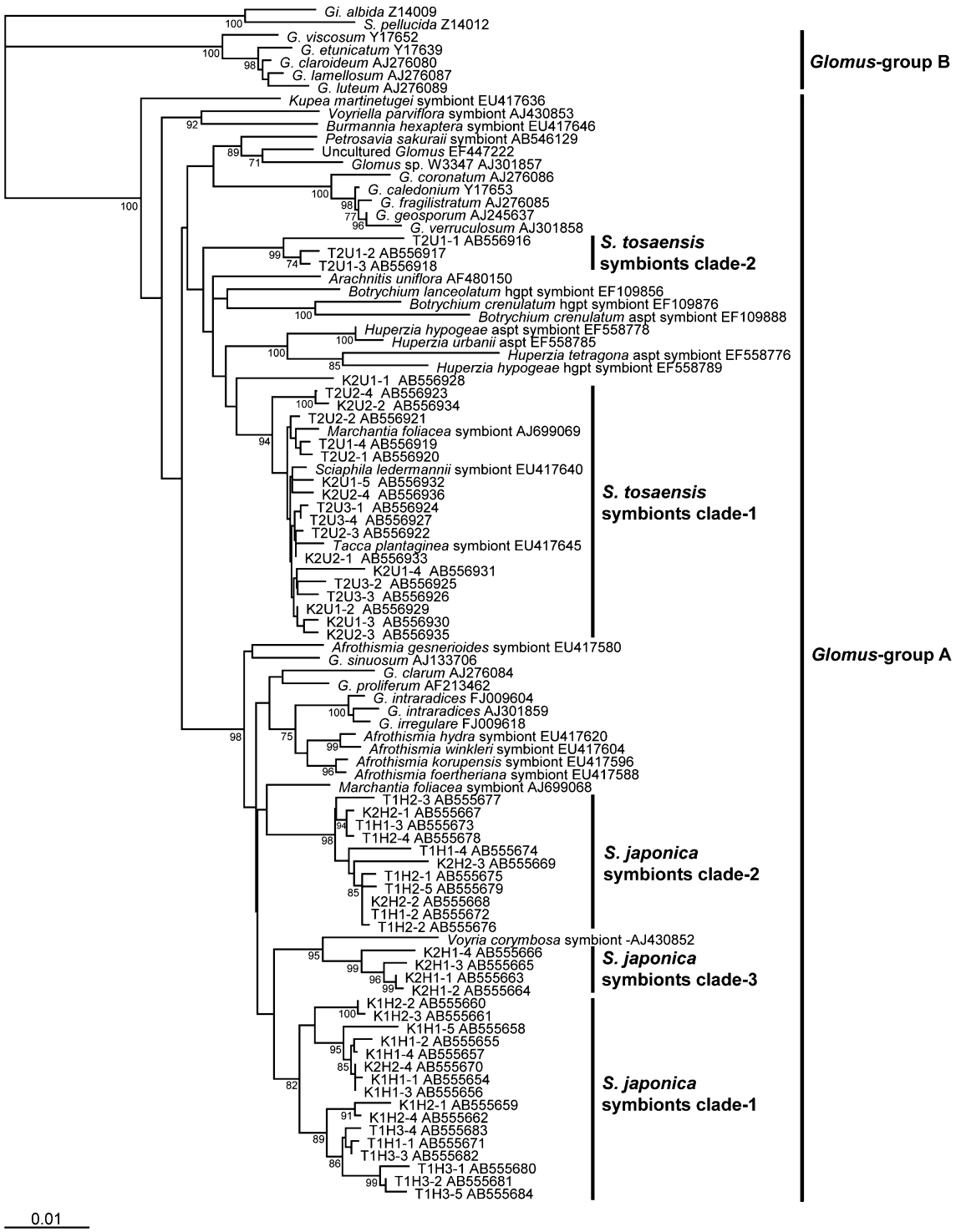

Fig. 3 A neighbor-joining phylogenetic tree based on sequences of a partial nuclear small subunit ribosomal RNA gene (nSSU rDNA) of arbuscular mycorrhizal (AM) fungi in Glomelares obtained from *Sciaphila japonica*, *S. tosaensis* and the GenBank database. The tree is rooted to *Gigaspora albida* (Z14009) and *Scutellospora pellusida* (Z14012) in Gigasporaceae, Diversisporales in Glomeromycota. The *sequence numbers* relate to plant numbers of *S. japonica* (T1H1, T1H2, T1H3, K1H1, K1H2, K2H1 and K2H2) and *S. tosaensis* (T2U1, T2U2, T2U3, K2U1 and K2U2), and the clone numbers. Bootstrap values are shown where they exceed 70% (1,000 replications). A scale is shown to infer the evolutionary distances. Accession numbers are given for all sequences. *hgpt* Heterotrophic gametophyte, *aspt* autotrophic sporophyte

hyphae in *S. polygyna* Maas, in which direct transitions from beaded to non-beaded hyphae were confirmed, suggesting that the same AM fungus could form two different hyphal morphologies in *Sciaphila* spp. The degeneration of hyphal coils into amorphous clumps was also observed in other myco-heterotrophic AM plants (Yamato 2001; Imhof 2003, 2007), and this feature is very similar to that of orchid mycorrhizas. For the degeneration, the carbon transfer from the fungi to myco-heterotrophic plants through the digestion of hyphal coils has been suggested (Leake and Cameron 2010).

The phylogenetic analysis of the plant nSSU rDNA in Pandanales confirmed that *S. japonica* and *S. tosaensis* are included in Triuridaceae. To reveal the phylogenetic relationship within Triuridaceae, however, more DNA sequences of other plant species have to be included.

In the phylogenetic analysis of AM fungal nSSU rDNA sequences from *S. japonica* and *S. tosaensis*, specificity with certain groups of AM fungi in *Glomus*-group A lineage was shown across the varied habitats. The symbionts of *S. japonica* and *S. tosaensis* were, however, completely separated into the different clades in the phylogenetic tree even among those collected in the same habitat, K2. Merckx and Bidartondo (2008) showed that the phylogenetic relationships of myco-heterotrophic *Afrothismia* species were closely mirrored by those of their closely related AM fungal associates, which suggested a codiversification of *Afrothismia* plants and *Glomus* fungi. Meanwhile, the obvious separation of the AM fungi of *S. japonica* and *S. tosaensis* into different clades was found in the phylogenetic tree in the present study. The plants themselves were also somewhat distantly related; thus, codiversification of plant and fungi was not confirmed in the relations. The DNA sequences of the AM fungi of *S. tosaensis* obtained in this study were compared to an AM fungal sequence of *S. tosaensis* in Fukui Pref. by Yamato (2001) in the region (143–146 bp in length) between the primers VANS1 (Simon et al. 1992) and VAGLO (Simon et al. 1993). The sequence identity between that of Fukui and those of this study was 94–97%, whereas among those of this study it was 96–100%. This suggested that more extensive study on mycorrhizal fungi of *S. tosaensis* may detect some other AM fungi nearly related to those of this study.

High specificities toward AM fungi have been also recognized in other myco-heterotrophic plants in Corsiaceae, Burmanniaceae, Gentianaceae, Thismiaceae and Triuridaceae (Bidartondo et al. 2002; Franke et al. 2006; Merckx and Bidartondo 2008). In *S. tosaensis* symbionts clade-1, a mycorrhizal fungus of *Sciaphila ledermannii* (Merckx and Bidartondo 2008) was included as well as those of a liverwort *Marchantia foliacea* Mitt. (Russell and Bulman 2005) and a perennial dicotyledonous plant *Tacca plantaginea* (Hance) Drenth in Taccaceae (Merckx and Bidartondo 2008). The inclusion of *S. ledermannii* symbiont in the same clade suggested the preference of this fungal group for *Sciaphila* species.

The examined habitats of *S. japonica* were in a cypress tree plantation (T1) or in bamboo forests (K1 and K2), and those of *S. tosaensis* were in a camellia forest (T2) or in a bamboo forest (K2). In the previous study on *S. tosaensis* by Yamato (2001), the examined habitat was also in a camellia forest. These results suggested that the symbiosis of *S. tosaensis* may have some preference for camellia trees. Almost all the plants forming canopies in the habitats are known to form AM (Mejstrik 1974; Yamato 2002; Muthukumar and Udaiyan 2006). Considering the non-saprobic feature of AM fungi and non-photosynthetic ability of *Sciaphila* species, it was evidently suggested that some photosynthates of the canopy plants would be supplied to *Sciaphila* species through the hyphal connections of AM fungi. It is therefore conceivable that the association with the particular groups of AM fungi would be indispensable for the growth of these plants. Both currently examined plant species are categorized as endangered species in the Red Data Book of the Ministry of the Environment, Japan, although the habitat environments—cypress tree plantation, bamboo forest and camellia forest—are quite common in Japan. It was supposed that such a definite specificity of AM symbioses might restrict the distribution of these myco-heterotrophic plants.

Acknowledgments We thank Dr. Tsutomu Teramine in Kochi Gakuen College, Mr. Hiroshi Fukuhara in The Kochi Prefectural Makino Botanical Garden, and Yasuo Katayama, Takeki Tabuchi and Hisanori Takeuchi, plant fanciers in Tokushima and Kochi Pref., for their kind help in the plant sampling. This study was supported by the Global COE Program "Advanced utilization of fungus/mushroom resources for sustainable society in harmony with nature" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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