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ORIGINAL PAPER

Specific arbuscular mycorrhizal fungi associated with non-photosynthetic *Petrosavia sakuraii* (Petrosaviaceae)

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Abstract Mycorrhizal fungi in roots of the achlorophyllous *Petrosavia sakuraii* (Petrosaviaceae) were identified by molecular methods. Habitats examined were plantations of the Japanese cypress *Chamaecyparis obtusa* in Honshu, an evergreen broad-leaved forest in Amami Island in Japan and a mixed deciduous and evergreen forest in China. Aseptate hyphal coils were observed in root cortical cells of *P. sakuraii*, suggesting *Paris*-type arbuscular mycorrhiza (AM). Furthermore, hyphal coils that had degenerated to amorphous clumps were found in various layers of the root cortex. Despite extensive sampling of *P. sakuraii* from various sites in Japan and China, most of the obtained AM fungal sequences of the nuclear small subunit ribosomal RNA gene were nearly identical and phylogenetic analysis revealed that they formed a single clade in the *Glomus* group A lineage. This suggests that the symbiotic relationship is highly specific. AM fungi of *P. sakuraii* were phylogenetically different from those previously detected in the roots of some mycoheterotrophic plants. In a habitat in *C. obtusa* plantation, approximately half of the AM fungi detected in roots of *C. obtusa* surrounding *P. sakuraii* belonged to the same clade as that of *P. sakuraii*. This indicates that particular AM fungi are selected by *P. sakuraii* from diverse indigenous AM fungi. The same AM fungi can colonize both plant species, and photosynthates of *C. obtusa* may be supplied to *P. sakuraii* through a shared AM fungal mycelial network. Although *C. obtusa* plantations are widely distributed throughout Japan, *P. petrosavia* is a rare plant species, probably because of its high specificity towards particular AM fungi.

Keywords Arbuscular mycorrhiza · *Chamaecyparis obtusa* · *Glomus* group A · Mycoheterotrophic plants · *Paris*-type AM

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Introduction

Non-photosynthetic plants that obtain carbon through symbiotic associations with fungi are termed mycoheterotrophic plants (Leake 1994). The application of molecular methods has led to the identification of the fungal partners of many mycoheterotrophic plant species. Furthermore, these studies have often demonstrated the existence of high specificity in the relationship with few fungal partners (Selosse and Cameron 2010). Among the mycoheterotrophic plants, morphological features suggesting arbuscular mycorrhiza (AM), a symbiotic association with fungi of the phylum Glomeromycota (Schüβler et al. 2001), have been shown in plants belonging to *Voyria* spp. in Gentianaceae (Imhof 1997, 1999a; Imhof and Weber 1997; Knöbel and Weber 1988); *Triuris hyalina* Miers and *Sciaphila polygyna* Maas in Triuridaceae (Imhof 1998, 2003); *Burmannia tenella* Benth. in Burmanniaceae (Imhof 1999b); *Arachnitis uniflora* Phil. in Corsiaceae (Domínguez et al. 2006) and *Epirixanthes* spp. in Polygalaceae (Imhof 2007) as well as

the achlorophyllous gametophytes of some ferns, such as *Psilotum nudum* (L.) Beauv. in Psilotaceae (Peterson et al. 1981) and *Lycopodium clavatum* L. in Lycopodiaceae (Schmid and Oberwinkler 1993). These mycorrhizas show features of the *Paris*-type AM (Smith and Smith 1997), characterized by intracellular hyphal coils. Molecular methods have been used to identify AM fungi in achlorophyllous plants, such as *Voyria* spp. and *Voyriella parviflora* (Miq.) Miq. in Gentianaceae; *A. uniflora* in Corsiaceae (Bidartondo et al. 2002); *Afrothismia* spp. in Thismiaceae; *Burmannia* spp. in Burmanniaceae; *Sciaphila ledermannii* Engl. and *Kupea martinetugei* Cheek & Williams in Triuridaceae; *Sebaea oligantha* Schinz in Gentianaceae (Franke et al. 2006); *Dictyostega orobanchoides* (Hook.) Miers in Burmanniaceae; *Voyria aphylla* (Jaquin) Persoon in Gentianaceae (Merckx et al. 2010) and *Sciaphila japonica* Makino and *Sciaphila tosaensis* Makino in Triuridaceae (Yamato et al. 2010). Furthermore, AM fungi in achlorophyllous gametophytes of some pteridophytes have been identified by molecular studies: *Botrychium* spp. in Ophioglossaceae (Winther and Friedman 2007); *Huperzia hypogaea* in Lycopodiaceae (Winther and Friedman 2008) and *P. nudum* in Psilotaceae (Winther and Friedman 2009). Almost all AM fungi of mycoheterotrophic plants that have been identified belong to *Glomus* group A as circumscribed by Schüβler et al. (2001), in which some specific relationships have been found. However, the degree of specificity varies among plants. For example, five species of *Afrothismia* collected in Southwest Cameroon showed highly specific relationships with different but phylogenetically related AM fungi (Merckx and Bidartondo 2008), whereas relationships with phylogenetically diverse AM fungi have been shown in some *Voyria* spp. (Bidartondo et al. 2002; Merckx et al. 2010).

Petrosavia sakuraii (Makino) J.J.Sm. ex Steenis is an achlorophyllous plant species distributed throughout Japan, Taiwan and south China. It is a small (10–20 cm tall), whitish plant that grows in the understorey of broad-leaved or coniferous forest (Takahashi et al. 1993). Furthermore, two other species of this genus, *Petrosavia stellaris* Becc. and *Petrosavia sinii* (K. Krause) Gagnep., are also achlorophyllous (Cameron et al. 2003; Chen and Tamura 2000; Ohba 1984). Molecular studies have shown that *Petrosavia* is sister to *Japonolirion* (Fuse and Tamura 2000; Soltis et al. 2000), and Cameron et al. (2003) proposed the family Petrosaviaceae, consisting of genera *Petrosavia* and *Japonolirion*, in the order Petrosaviales. The distinction of Petrosaviaceae from other monocots was confirmed by embryological studies that showed a glandular anther tapetum, simultaneous cytokinesis in microspore mother cells, anatropous and crassinucellate ovules in both genera (Tobe 2008; Tobe and Takahashi 2009). The chlorophyllous *Japonolirion* species is autotrophic, whereas *Petrosavia* species are considered to be mycoheterotrophic (Leake 1994; Wang and Qiu 2006). Watanabe (1944) observed hyphal coils in root cortical cells of *P. sakuraii*, while Stant (1970) observed similar structures in *P. stellaris*. The relationship of AM fungi with *Petrosavia* species has been indicated by the fungal structures. However, associated mycorrhizal fungi have never been identified in *Petrosavia* species.

In this study, we identified the mycorrhizal fungi of *P. sakuraii* collected from various habitats and investigated the morphological features to examine whether *P. sakuraii* is associated with AM fungi and whether specificity exists in the relationship. We also identified the mycorrhizal fungi in the roots of other plants found in the vicinity of *P. sakuraii* to examine whether the specificity in the relationship is based on the local availability of AM fungi.

Materials and methods

Plant materials

In mid-August 2007, five *P. sakuraii* plants (P1–P5) were collected from ‘Nagano A’ in Nagiso-machi, Nagano Prefecture, Japan, which is approximately 650 m above sea level. Plants in the flowering stage were collected from the understorey of a plantation of the Japanese cypress *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. This tree species is known to form *Arum*-type AM (Yamato and Iwasaki 2002). *P. sakuraii* root samples were collected with roots of *C. obtusa.* During root selection, the whitish roots of *P. sakuraii* were easily distinguishable from the brown roots of *C. obtusa*. The roots were thoroughly washed with tap water in a 250-μm stainless steel sieve to remove soil particles.

Samples of *P. sakuraii* were also collected from various habitats in an extensive survey of mycorrhizal fungi (Table 1). Four plants were collected from ‘Nagano A’ (Yukawa 09-47), described above, on 31 August 2009; five plants from ‘Nagano B’ (Yukawa 09-45), a conifer forest mainly comprising *C. obtusa* in Nagiso-machi, Kiso Gun, Nagano Prefecture, approximately 690 m above sea level, on 31 August 2009; four plants from ‘Gifu’ (Yukawa 08-31), a *C. obtusa* plantation in Ichinomiya-machi, Takayama, Gifu Prefecture, approximately 1,000 m above sea level, on 11 September 2008; three plants from ‘Amami’ (Yukawa 09-25), an evergreen broad-leaved forest comprising *Castanopsis sieboldii* (Makino) Hatus. ex T. Yamaz. and Mashiba, *Podocarpus macrophyllus* (Thunb.) Sweet and *Nageia nagi* (Thunb.) Kuntze in Amami Island in Kagoshima Prefecture, approximately 180 m above sea level, on 9 June 2009 and a single herbarium specimen ‘China’ (Takahashi 20907) from a mixed deciduous and

Table 1 Samples of *Petrosavia sakuraii* collected from various sampling sites in the extensive survey of arbuscular mycorrhizal fungi with their AM fungal DNA sequence types

Sampling site	Plant No.	AM fungal DNA sequence		
		No.	Type	Accession No.
Nagano A	NA1	Y98	A	AB601891
		Y100	A	
		Y101	A	
		Y112	A	
		Y125	A	
		Y126	A	
	NA2	Y102	A	
		Y103	A	
		Y104	A	
		Y105	A	
		Y113	A	
		Y122	A	
		Y123	A	
		Y124	A	
	NA3	Y114	A	
		Y115	A	
		Y116	A	
		Y117	A	
	NA4	Y119	A	
		Y120	A	
		Y131	A	
Nagano B	NB1	Y78	A	AB601892
		Y79	A	
		Y80	A	
		Y81	A	
	NB2	Y82	A	
		Y84	A	
		Y85	A	
	NB3	Y86	A	
		Y88	C	AB601893
		Y89	A	
	NB4	Y90	A	
		Y91	A	
		Y92	A	
		Y110	A	
	NB5	Y94	A	
		Y95	A	
		Y96	A	
		Y97	A	
		Y111	A	
		Y128	D	AB601894
		Y129	A	
		Y130	A	
Gifu	G1	M1212	A	AB601895
		M1213	A	
		M1214	A	
		M1215	A	
	G2	M1217	A	
		M1220	A	
	G3	M1221	A	
		M1223	A	
		M1224	A	
	G4	M1225	A	
		M1226	A	
Amami	A1	Y74	B	AB601897
		Y76	B	
		Y106	B	
		Y107	B	
		Y108	B	
		Y109	B	
	A2	Y134	A	AB601896
		Y135	B	
	A3	Y141	B	
		Y143	B	
China	C1	Y145	A	AB601898

evergreen forest in Longquan, Lishui District, China, on 19 September 1996. All of the voucher specimens are deposited in the Herbarium of National Museum of Nature and Science (TNS) except for Takahashi 20907 which is kept in the herbarium of Gifu University.

Light microscopy

Roots of *P. sakuraii* collected from Nagano A in 2007 were cut into 1-cm fragments, and those with yellowish pigmentation, indicating fungal colonization, were kept in 70% ethanol until use. The root fragments were cleared with 10% KOH and stained with 0.1% Chlorazol Black E (Brundrett et al. 1996). Roots from Gifu were also stained in the same way with 0.05% trypan blue. These stained fragments were squashed with a cover glass and the fungal morphology in root cortical cells was observed. To examine the fungal morphology related to root structure, root sections were observed as follows. Root fragments were cut into 2-mm long pieces, dehydrated in an ethanol series, transferred to propylene oxide and embedded in Spurr's low-viscosity resin (Spurr 1969). Semi-thin sections (0.8 μm) were cut with a diamond knife (Diatome, Bienne, Switzerland) on an ultramicrotome MT-7000 (RMC, Tucson, AZ, USA) and gently heat-fixed to glass microscope slides. The sections were stained with 0.5% toluidine blue O in 1% sodium borate, destained in tap water and then air-dried. The

morphology of AM associations in the stained roots was viewed under an interference contrast microscope (Eclipse 80i; Nikon, Tokyo, Japan).

Molecular identification of AM fungi

For the samples of *P. sakuraii* collected from Nagano A in 2007, approximately 150 mg of pigmented root fragments was randomly collected from each root sample (P1–P5). For the samples of *C. obtusa*, approximately 500 mg of fine roots was collected from three arbitrarily selected samples (C1–C3). The root samples of *C. obtusa* (C1, C2 and C3) were those collected with the roots of *P. sakuraii* (P1, P2 and P3), respectively. Total DNA was extracted from the root samples by the cetyltrimethylammonium bromide method (Weising et al. 1995). After additional purification using the Mag Extractor Plant Genome Kit (Toyobo, Osaka, Japan), DNA was dissolved in 50 μl Tris-ethylenediaminetetraacetic acid buffer. For samples of *P. sakuraii*, partial fungal nuclear small subunit ribosomal RNA gene (nSSU rDNA) sequences were amplified by polymerase chain reaction (PCR) using primers GEOA2 and GEO11 (Schwarzott and Schüβler 2001) and TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR 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 a total volume of 30 μl. The PCR programme, performed on a PC-818S Program Temp Control System (Astec, Fukuoka, Japan), used was as follows: initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 45 s, 57°C for 1 min, 72°C for 2 min and a final elongation step at 72°C for 10 min. For the three samples of *C. obtusa* and a single sample of *P. sakuraii* (P1), partial fungal SSU rDNA was amplified using the primers AML1 and AML2 (Lee et al. 2008), because the primers GEOA2 and GEO11 preferentially amplify plant DNA from the mycorrhizal roots of *C. obtusa*. The PCR mixture for the primers AML1 and AML2 was as described above and the PCR programme was as follows: initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final elongation step at 72°C for 5 min. The PCR products were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI, USA), and some clones were randomly chosen from each *P. sakuraii* sample. DNA inserts were sequenced by the dideoxy sequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA sequence data obtained were deposited in the DNA Data Bank of Japan (DDBJ) database with accession numbers AB546129 to AB546148 for *P. sakuraii* and AB546396 to AB546447 for *C. obtusa*. To estimate the evolutionary divergence between AM fungal DNA sequences of *P. sakuraii*, the number of base substitutions per site based on pair-wise analyses of the sequences (pairwise distances) was computed using the maximum composite likelihood method in MEGA 4 (Tamura et al. 2004, 2007).

The systematic affiliation of the DNA sequence data obtained was assessed using BLAST (Altschul et al. 1997). Representative fungal sequences that had high affinity to those from *P. sakuraii* were downloaded from the GenBank database. AM fungal nSSU rDNA sequences from other mycoheterotrophic plants, such as those of *A. uniflora* Phil.; *V. parviflora* (Miq.) Miq.; *Voyria corymbosa* Splitg (Bidartondo et al. 2002); *Afrothismia* spp.; *Burmannia hexaptera* Schltr.; *S. ledermannii* Engl. and *K. martinetugei* Cheek & SA Williams (Merckx and Bidartondo 2008) as well as mycoheterotrophic gametophytes of *Botrychium* spp. (Ophioglossaceae) and *H. hypogaea* B. (Lycopodiaceae; Winther and Friedman 2007, 2008), were also downloaded. For sequenced and downloaded data, multiple sequence alignment was performed using ClustalX 2.0.12 (Larkin et al. 2007). Neighbour-joining analyses (Saitou and Nei 1987) were performed for the aligned datasets using ClustalX with bootstrap analysis of 1,000 replications (Felsenstein 1985). The neighbour-joining tree was displayed using TreeView (Page 1996).

Extensive survey of mycorrhizal fungi

Roots of *P. sakuraii* were cut into 1-cm fragments and one fragment with yellowish pigmentation was used for DNA extraction. Total DNA was extracted from the root samples using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Partial AM fungal nSSU rDNA was amplified by PCR using the primers NS31 (Simon et al. 1992) and AM1 (Helgason et al. 1998) with TaKaRa Ex Taq (Takara Bio). The PCR mixture contained 1 μl of the extracted DNA solution, 0.25 U of Taq polymerase, 0.3 μM of each primer, 200 μM of each deoxynucleotide triphosphate and 1 μl of the supplied PCR buffer in a total volume of 10 μl. The PCR programme, performed using the GeneAmp PCR System 9700 (Applied Biosystems), was as follows: initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 7 min. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and directly sequenced by the dideoxy sequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3100 Genetic Analyzer (Applied Biosystems). The DNA sequence data obtained were deposited in the DDBJ database with accession numbers AB601891 to AB601898. The methods of data processing and analyses were as described above.

Results

Light microscopy

The roots of *P. sakuraii* collected from Nagano A were found to be always intermingled with the neighbouring roots of *C. obtusa*. Intracellular aseptate hyphal coils were found in the cortical cells, which developed vesicles (Fig. 1a) and eventually degenerated to amorphous clumps (Fig. 1b). In transverse (Fig. 2a) and longitudinal (Fig. 2b) sections of the roots, hyphal coils were observed in almost all cortical cells. Degeneration of hyphal coils occurred in various layers of the cortex. No hyphal coils were observed in the epidermis, exodermis, endodermis or stele. The same morphological features were also confirmed in a *P. sakuraii* sample from Gifu.

Molecular identification of AM fungi

Fungal partial nSSU rDNA from samples of *P. sakuraii* collected from Nagano A was amplified using primers GEOA2 and GEO11. The nested procedure described by Schwarzott and Schüβler (2001) was not necessary for these primer pairs, as demonstrated by Winther and Friedman (2007). After the PCR products had been cloned, four sequences of AM fungal partial SSU rDNA (1,766 or 1,767 bp) were obtained from each of the five *P. sakuraii* samples. Some fungal sequences were also obtained from Ascomycota or Basidiomycota, but these were excluded from further analyses because of the dominant AM fungal colonization observed by light microscopy. All amplified sequences (GEOA2–GEO11) from *P. sakuraii* were nearly identical. The pairwise distances among the sequences were low, ranging from 0 to 0.0053, with an overall average of 0.0023.

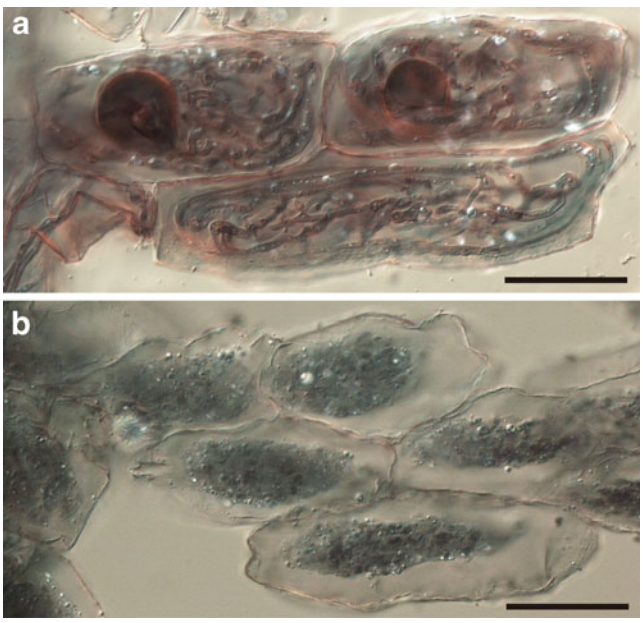

Fig. 1 Hyphal coils of arbuscular mycorrhizal fungi colonizing root cortical cells of *P. sakuraii* **a** Aseptate hyphal coils with vesicles. **b** Degenerating hyphal coils. *Bars* = 50 μm

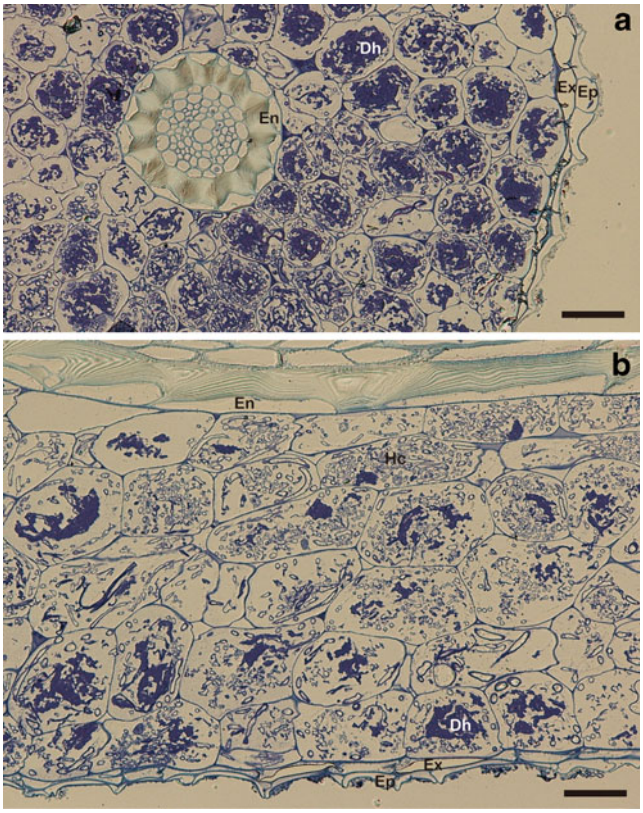

Fig. 2 Root sections of *P. sakuraii* showing hyphal coils of arbuscular mycorrhizal fungi in cortical cells **a** Transverse section. **b** Longitudinal section. *Hy* hyphal coil, *Dh* degenerating hyphal coils, *Ep* epidermis, *Ex* exodermis, *En* endodermis. *Bars* = 50 μm

Phylogenetic analysis of the region common to all sequences (517–528 bp) revealed that AM of *P. sakuraii* formed a single clade belonging to *Glomus* group A, which is most closely related to *Glomus* sp. W3347 (AJ301857) and uncultured *Glomus* (EF447222; Fig. 3). They differed from AM fungi previously detected in other mycoheterotrophic plants and none of the GenBank database entries matched the AM fungal clade associated with *P. sakuraii*.

For samples of *C. obtusa*, the primers GEOA2 and GEO11 preferentially amplified plant DNA; therefore, primers AML1 and AML2 were used to amplify the AM fungal partial nSSU rDNA. A total of 52 sequences of AM partial nSSU rDNA (793–800 bp) were obtained from three samples of *C. obtusa*. The primer pairs AML1 and AML2 were also applied to the sample of *P. sakuraii* from which DNA sequences in the same AM fungal group as those obtained by GEOA2 and GEO11 were exclusively obtained (data not shown). For the partial nSSU rDNA sequences (AML1 and AML2), another phylogenetic tree was obtained (Fig. 4). In this analysis, 20

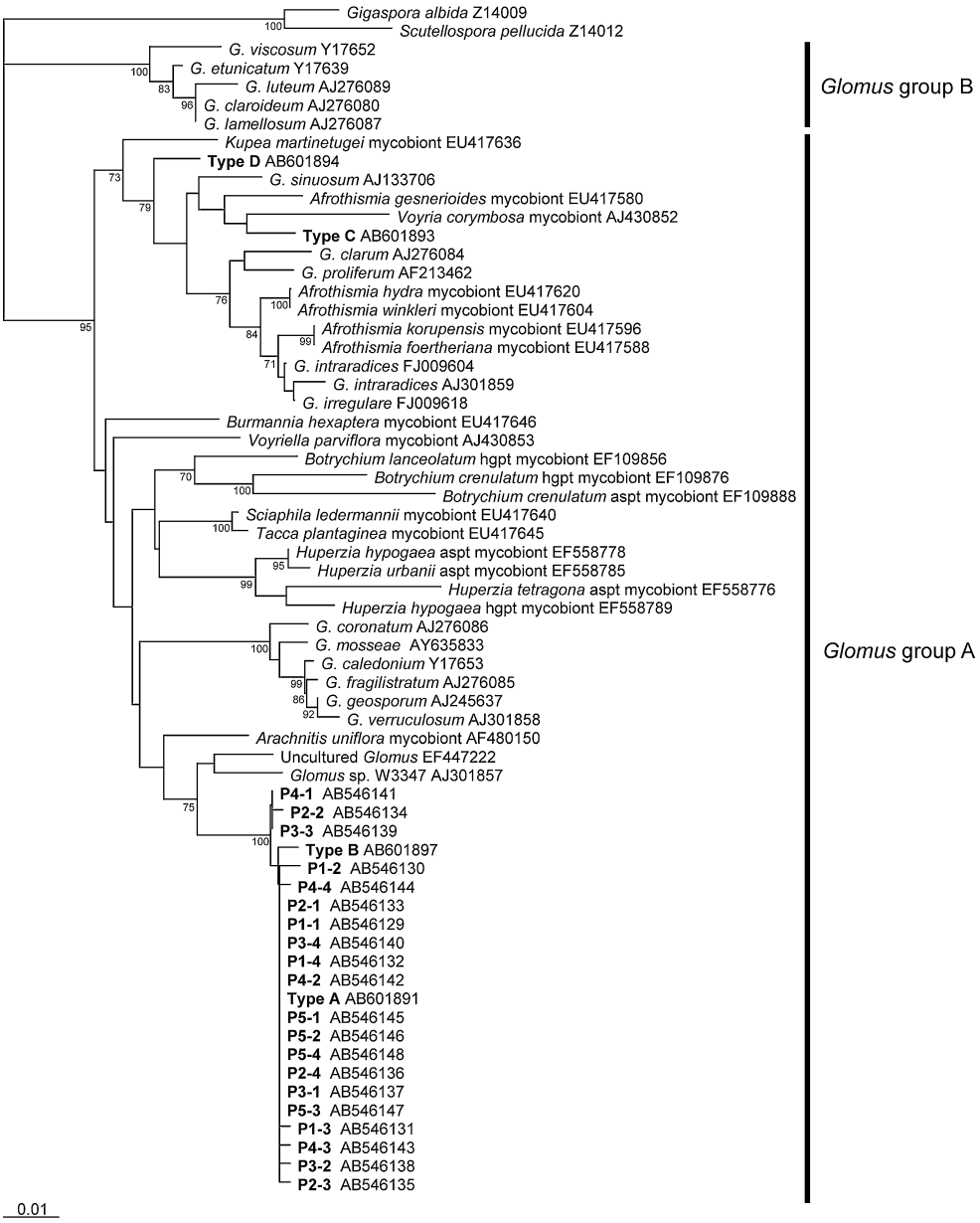

Fig. 3 Neighbour-joining phylogenetic tree based on partial nSSU rDNA sequences of arbuscular mycorrhizal fungi in Glomerales obtained from *P. sakuraii* and the GenBank database. The tree is rooted to *Gigaspora albida* (Z14009) and *Scutellospora pellucida* (Z14012) in Diversisporales, Glomeromycota. The sequence numbers relate to plant numbers of *P. sakuraii* (P1–P5) and the clone numbers. The sequence types obtained from the extensive survey (*Types A*, *B*, *C* and *D*) are shown in Table 1. Bootstrap values are shown where they exceed 70% (1,000 replications). The scale is shown so that evolutionary distances can be inferred. Accession numbers are given for all sequences. *hgpt* heterotrophic gametophyte; *aspt* autotrophic sporophyte

DNA sequences of AM fungi in *P. sakuraii* amplified by GEOA2 and GEO11 were also included. The phylogenetic tree showed that all AM fungi detected in *C. obtusa* belonged to *Glomus* group A and approximately 44% (23/52) shared a clade with those detected in *P. sakuraii*. In the clade of *P. sakuraii* symbionts, sequences from both *P. sakuraii* and *C. obtusa* were intermixed. Among them, eight sequences from *P. sakuraii* and 10 from *C. obtusa* were identical. AM fungi belonging to this clade were detected in all *C. obtusa* samples examined (C1–C3).

Survey of mycorrhizal fungi

In total, 65 sequences were obtained from 17 plants collected from Nagano A, Nagano B, Gifu, Amami and China (Table 1). Fifty-four of these sequences (Type A) were identical. Nine sequences obtained from the Amami samples (Type B) differed only in two bases from Type A. Types A and B were the same as or almost identical to the AM fungal sequences obtained from *P. sakuraii* in Nagano A using the primers GEOA2 and GEO11 (Fig. 3). Two other AM fungal sequences (Types C and D) were also obtained from the Nagano B population.

Discussion

The morphological features of the mycorrhizal fungi of *P. sakuraii*, i.e. the intracellular spread of hyphal coils with some vesicles and their degeneration to amorphous clumps,

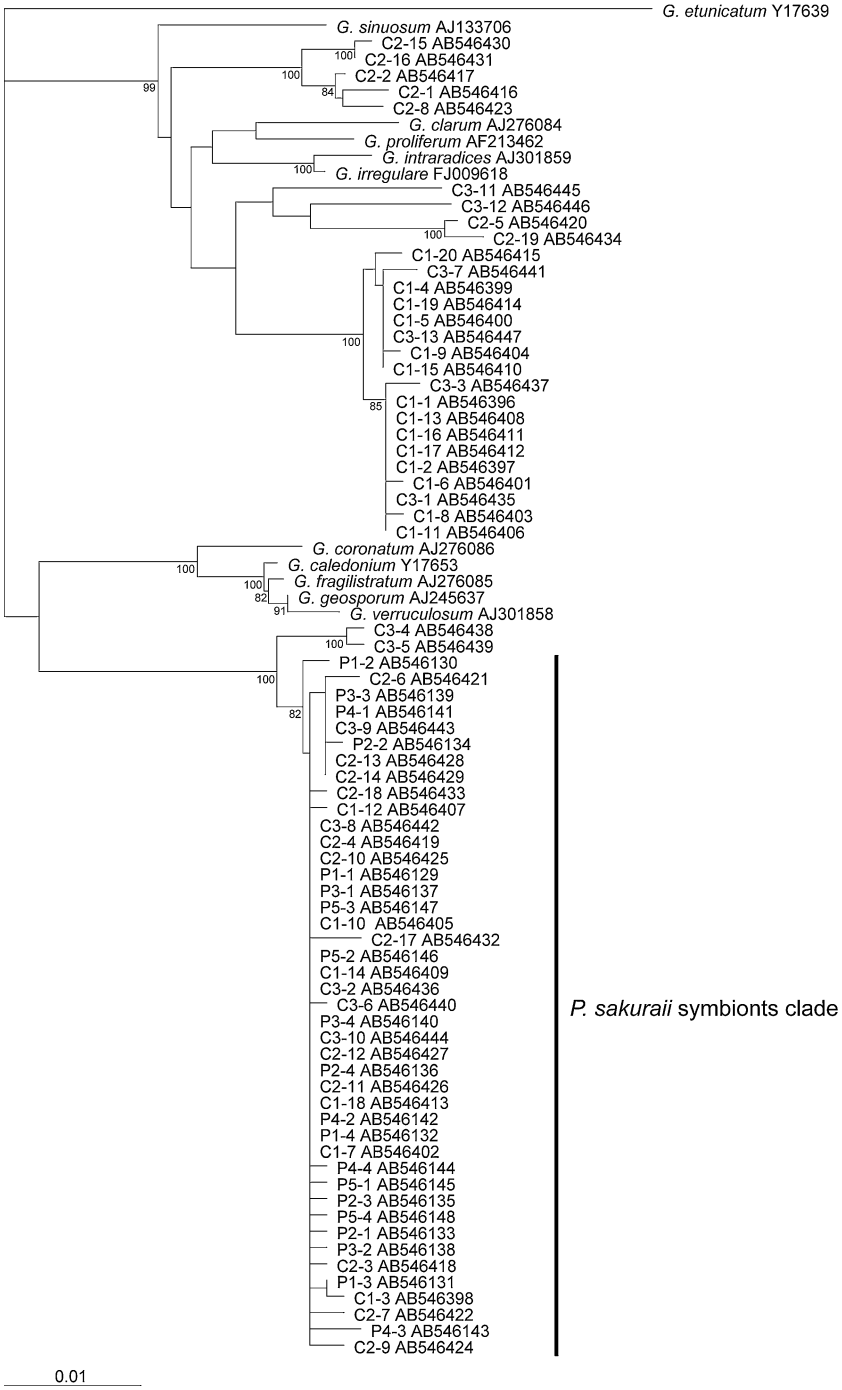

Fig. 4 Neighbour-joining phylogenetic tree based on AML1–AML2 nSSU rDNA sequences of arbuscular mycorrhizal fungi in *Glomus* group A obtained from *P. sakuraii*, *C. obtusa* and the GenBank database. The tree is rooted to *Glomus etunicatum* (Y17639) in *Glomus* group B. The sequence numbers relate to plant numbers of *P. sakuraii* (P1–P5) or *C. obtusa* (C1–C3) and the clone numbers. Bootstrap values are shown where they exceed 70% (1,000 replications). The scale is shown so that evolutionary distances can be inferred. Accession numbers are given for all sequences

were similar to those previously found in other mycoheterotrophic plants (Imhof 1999a, 2003, 2007; Yamato 2001). Almost all hyphal coils observed were aseptate, indicating the dominance of AM fungi. Watanabe (1944) illustrated mycorrhizal fungi in the roots of *P. sakuraii* and reported that the fungal mycelia passed through the two outermost root cell layers, the epidermis and exodermis, and formed hyphal coils in all layers of cortical cells. He also observed the degeneration of hyphal coils. In the present study, we confirmed the all morphological features indicating *Paris*-type AM, and AM fungal identity was shown by molecular methods.

In the phylogenetic analysis of AM fungal nSSU rDNA sequences, AM fungi of *P. sakuraii* formed a clade in the *Glomus* group A lineage, separate from most AM fungi previously detected in mycoheterotrophic plants. AM fungi were related to *Glomus* sp. W3347, which was different from *Glomus* groups Aa and Ab according the phylogenetic

analysis (Schüβler 2010). This suggests that the *P. sakuraii* symbionts do not belong to *Glomus* group Aa or Ab but to the other group in *Glomus* group A. The sequences obtained were similar, suggesting strong specificity in the relationship. In the survey of AM fungi from Nagano A, Nagano B, Gifu, Amami and China, almost all AM fungi in *P. sakuraii* detected by primers NS31 and AM1 were categorized as Types A and B. Types A and B sequences were identical to some of those obtained in Nagano A using primers GEOA2 and GEO11. In Nagano A, Nagano B and Gifu, *C. obtusa* was the dominant canopy tree, whereas in Amami and China, the habitats were evergreen broad-leaved forests without *C. obtusa* trees. The detection of the identical AM fungal sequence in various types of vegetation indicates a strong and specific relationship between *P. sakuraii* and AM fungi. It was also found that 44% of the AM fungal DNA sequences from the roots of *C. obtusa* formed a clade with mycobionts from *P. sakuraii* roots. This suggests that the same AM fungi colonize both plant species. Considering the obligate biotrophic state of AM fungi (Bonfante and Perotto 1995) and the achlorophyllous nature of *P. sakuraii*, transfer of photosynthates from *C. obtusa* to *P. sakuraii* through a shared AM mycelial network may be probable. The detection of some other AM fungi in the roots of *C. obtusa* indicates that particular AM fungi are selected among diverse AM fungi.

Majority of the photosynthetic plant species capable of forming AM can be colonized by almost any AM fungal isolate under artificially controlled conditions (Smith and Read 2008). In natural ecosystems, however, there is ecological specificity between photosynthetic plants and AM fungi, probably caused by fungal preferences for certain host plants or environmental features (Sanders 2002). The relationship between *P. sakuraii* and AM fungi may be under genetic regulation because the same group of AM fungi were detected in *P. sakuraii* from various environments and AM fungi were selected from a diverse AM fungal community. Such specific relationships with AM fungi have also been found in other mycoheterotrophic plants (Bidartondo et al. 2002; Franke et al. 2006; Merckx and Bidartondo 2008; Merckx et al. 2010; Yamato et al. 2010).

Most of the known habitats of *P. sakuraii* are in plantations of *C. obtusa*. This tree species is important for Japanese timber production, and plantations cover 2,595,000 ha of Japan (http://www.rinya.maff.go.jp/j/keikaku/genkyou/pdf/hinoki_7.pdf). Thus, the sites examined represent a common vegetation type in Japan. In contrast, *P. sakuraii* is a rare plant species that is categorized as critically endangered on the Red List (Ministry of the Environment 2007). Though the diversity and distribution of AM fungi in *C. obtusa* plantations remain unresolved, high specificity in mycorrhizal symbiosis may restrict the distribution of *P. sakuraii*.

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