FULL PAPER

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Morphology and colonization preference of arbuscular mycorrhizal fungi in *Clethra barbinervis*, *Cucumis sativus*, and *Lycopersicon esculentum*

Abstract *Clethra barbinervis* (Ericales), *Cucumis sativus*, and *Lycopersicon esculentum* were grown in soils collected from six different vegetation sites (cedar, cypress, larch, red pine, bamboo grass, and Italian ryegrass), and morphology and colonization preference of arbuscular mycorrhizal (AM) fungi were investigated by microscopic observation and PCR detection. *C. barbinervis* consistently formed *Paris*-type AM throughout the sites. *C. sativus* formed both *Arum*- and *Paris*-type AM with high occurrence of *Arum*-type AM. *L. esculentum* also formed both *Arum*- and *Paris*-type AM but with high occurrence of *Paris*-type AM. AM diversity within the same plant species was different among the sites. Detected AM diversity from AM spores in different site soils did not consistently reflect AM fungal diversity seen in test plants. Detected families were different, depending on test plants grown even in the same soil. AM fungi belonging to Glomaceae were consistently detected from roots of all test plants throughout the sites. Almost all the families were detected from roots of *C. barbinervis* and *L. esculentum*. On the other hand, only two or three families of AM fungi (Archaeosporaceae and/or Paraglomaceae and Glomaceae) but not two other families (Acaulosporaceae and Gigasporaceae) were detected from roots of *C. sativus*, indicating strong colonization preference of AM fungi to *C. sativus* among test plants. This study demonstrated that host plant species strongly influenced the colonization preference of AM fungi in the roots.

Key words AM fungal host preference · Arbuscular mycorrhizal (AM) fungi · *Clethra barbinervis* · Diversity · Glomales-specific primers

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Introduction

Arbuscular mycorrhizae (AM) are a symbiotic association between the roots of terrestrial plants and fungi of the order Glomales (Zygomycetes). Although new classification that AM fungi belong to Glomeromycota has been proposed (Schüßler et al. 2001), in this study we followed the conventional classification system. The order consists of 5 families: Glomaceae, Acaulosporaceae, Gigasporaceae, Archaeosporaceae, and Paraglomaceae. More than 150 species are known to form AM.

AM fungi are thought to have a wide host range because these 150 species have been found in more than 225 000 species of plants. Merryweather and Fitter (1998) found AM fungi belonging to 3 genera (*Glomus*, *Acaulospora*, and *Scutellospora*) in bluebell (*Hyacicthoides non-scripta*) grown in a field. On the other hand, *Sciaphila tosaensis* (Triuridaceae), an achlorophyllous plant, was found to be colonized by single species belonging to *Glomus* (Yamato 2001) indicating the host–AM fungal preference/specificity. Vandenkoornhuyse et al. (2002) reported that AM fungi colonizing *Trifolium repens* differed from that colonizing *Agrostis capillaris*, also indicating host–AM fungal preference.

Most studies showing the preference of AM fungi were based on percent of colonization, number of spores propagated, or growth performance by AM fungi. Sanders and Fitter (1992) reported that spore production of AM fungi responded differently according to the host species. Abbott and Robson (1991) found that spore numbers were poorly correlated with mycorrhiza formation. It is equally difficult to identify the fungal structures within plant roots at the genus level from morphological data. In recent studies, diversity of AM fungi colonizing plants in field was elucidated using molecular techniques (Daniell et al. 2001; Helgason et al. 1999; Husband et al. 2002). Redecker (2000) designed Glomales-specific primers that enabled monitoring AM fungi in a number of root samples. However, there are a few studies concerning colonization preference of AM fungi using molecular techniques.

Table 1. Characteristics of each sampling site

Dominant plant species	Soil pH	Soil property	Site
Cedar (*Cryptomeria japonica*)	4.3	Loam	Gifu
Cypress (*Chamaecyparis obtuse*)	4.5	Loam	Gifu
Larch (*Larix leptolepis*)	5.1	Loam	Nagano
Red pine (*Pinus densiflora*)	4.8	Sandy loam	Nagano
Bamboo grass (*Sasa senanensis*)	4.3	Loam	Nagano
Italian ryegrass (*Lolium multiflorum*)	6.7	Clay loam	Gifu

We have reported that *Clethra barbinervis* (Ericales), a naturally grown woody plant species from broad-leaved forests, forms *Paris*-type AM (Kubota et al. 2000). In this study, we investigated whether *Paris*-type AM is also formed in *C. barbinervis* grown in soils collected from different vegetation sites where *C. barbinervis* is absent. We also investigated the types of AM fungi colonizing to *C. barbinervis* using polymerase chain reaction (PCR) with Glomales-specific primers. *Cucumis sativus* and *Lycopersicon esculentum*, both well-known cultivated plants, were also used as comparison plants to clarify the host–AM fungal preference.

Materials and methods

Sampling

Sampling sites with different dominant species were set in five forests (Masuda gun, Gifu Prefecture, Japan and Ina shi, Nagano Prefecture, Japan) and one grass field (Gifu shi, Gifu Prefecture, Japan) (Table 1) where *C. barbinervis* was absent. Four blocks of soil (40 cm × 40 cm × 5 cm) were collected from the base of dominant plant species, passed through 5-mm mesh, and completely mixed. These soils were used as AM fungal inoculum. To investigate AM spores in soil inoculum, field-collected soils were suspended in distilled water and sieved through a 500-μm screen. The soil suspension was gently transferred to a tube containing 60% sucrose solution and centrifuged for 3 min at 1600 g. Spores were collected, rinsed and, stored at −20°C until use for DNA extraction. A sample of AM spores was prepared from 5 g sieved soils with six replicates. Ten to 80 spores per 5 g soils were extracted.

Growth conditions

Clethra barbinervis seeds were collected in 1999 from plants in Gifu Prefecture, Japan. Commercial supplies were used for seeds of *Cucumis sativus* cv. Jibai and *Lycopersicon esculentum* cv. House Momotaro. Seeds were surface-sterilized with 70% ethanol for 1 min followed by 5% sodium hypochlorite for 10 min and rinsed three times in sterilized water. In preparation for use, surface-sterilized *C. barbinervis* seeds were sown on 0.7% water agar and incubated for 1 month in a growth chamber at 25°C with a light intensity of 120 μmol m^{-2} s^{-1} in a 14 h light:10 h dark daily

cycle. *C. sativus* and *L. esculentum* seeds were pregerminated on autoclaved Advantec no. 1 filter paper (Toyo Roshi, Tokyo, Japan) and kept moist with sterilized-distilled water for 2 days in an incubator at 25°C in the dark. Seedlings were transferred to pots 6 cm in diameter by 7.5 cm in length containing 50 ml sample soil overlaid with 100 ml of an autoclaved mixture of volcanic soil and sand (1:1 v/v, pH = 6.3). A 60-μm nylon mesh was placed between sample soil and autoclaved soil mixture to ease root sampling and improve sample conditions for following PCR. Seedlings were grown in a growth chamber at 25°C with a light intensity of 300 μmol m^{-2} s^{-1} in a 14 h light:10 h dark daily cycle. Water (10 ml) was supplied to each pot every other day; 10 ml 1000 times diluted 10-3-3 nutrient solution (HYPONEX Japan, Tokyo, Japan) was supplied every week after 2 weeks of growth.

Determination of root colonization

Roots of each plant were cut into 0.5-cm segments, and two sets of random subsamples of roots were taken by dispersing the entire root system in excess water. A set of subsample roots was freeze-dried and stored at −20°C until use for DNA extraction. Another set of sample roots was fixed in formyl-acetic alcohol (FAA), cleared in 10% KOH at 90°C for 1 h, and stained with 0.05% chlorazol black E solution made up in 80% lactic acid, glycerin, and distilled water in an 1:1:1 ratio by volume (Brundrett et al. 1984). After clearing and before staining, *C. barbinervis* roots were bleached for 15 min with diluted alkaline peroxide solution made as 30% hydrogen peroxide, 28% ammonium hydroxide solution, and distilled water in an 1:1:8 ratio by volume. Colonization was assessed according to McGonigle et al. (1990) at 200 × magnification to obtain the percentage of root length colonized by each of various types of fungal structure: hyphal coils, arbuscules, arbusculate coils, longitudinal hyphae, and vesicles. All photographs were obtained from thin mounts of squashed roots with an Olympus BX50F-3 camera (Olympus, Tokyo, Japan). Mycorrhizal colonization of roots was evaluated after 8 weeks of growth in pots. The experiment was repeated twice using four replicates per plant species.

Detection of AM fungi

DNA was extracted from AM spores collected from soil inoculum and from 200 mg freeze-dried root samples using
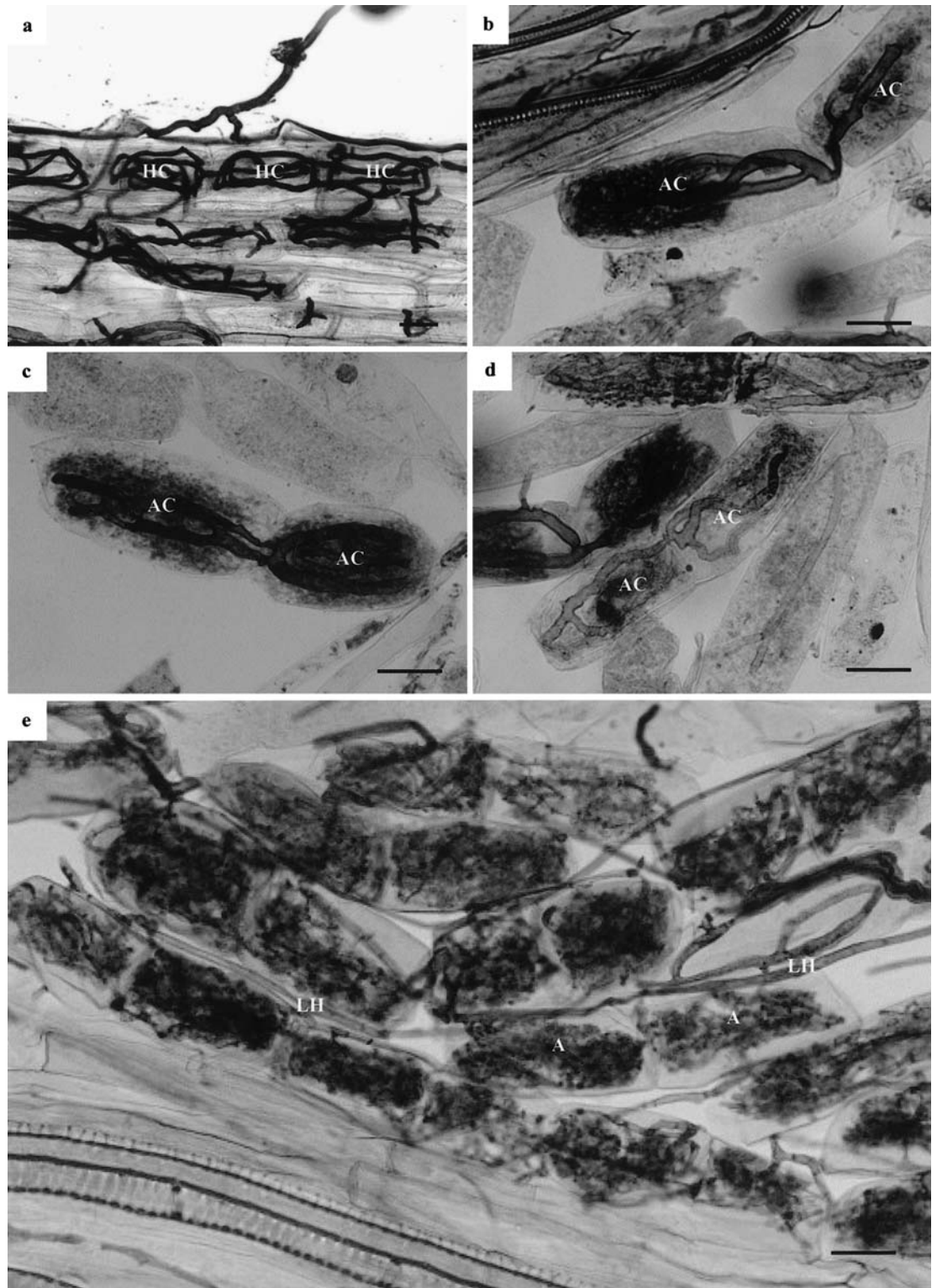
Fig. 1. Morphological characteristics of mycorrhizal association in *Clethra barbinervis* roots (**a–d**), in *Cucumis sativum* roots (**e**, **g**), and in *Lycopersicon esculentum* roots (**h**, **i**) grown in sample soils of cedar (**a**, **e**), cypress (**b**, **h**), larch (**c**), red pine (**g**, **i**), bamboo grass (**f**), and Italian ryegrass (**d**). **a** Hyphal coils (*HC*) extend from cell to cell. **b–d** Arbusculate coils (*AC*) formed from cell to cell. **e** Longitudinal hyphae (*LH*) extended intercellularly, and cells were fully occupied with arbuscules (*A*). **f** Arbuscules formed alongside longitudinal hyphae. **g** Hyphal and arbusculate coils. **h** Arbusculate and hyphal coils. **i** Arbusculate and hyphal coils extended from cell to cell. *Bars* 20 μm
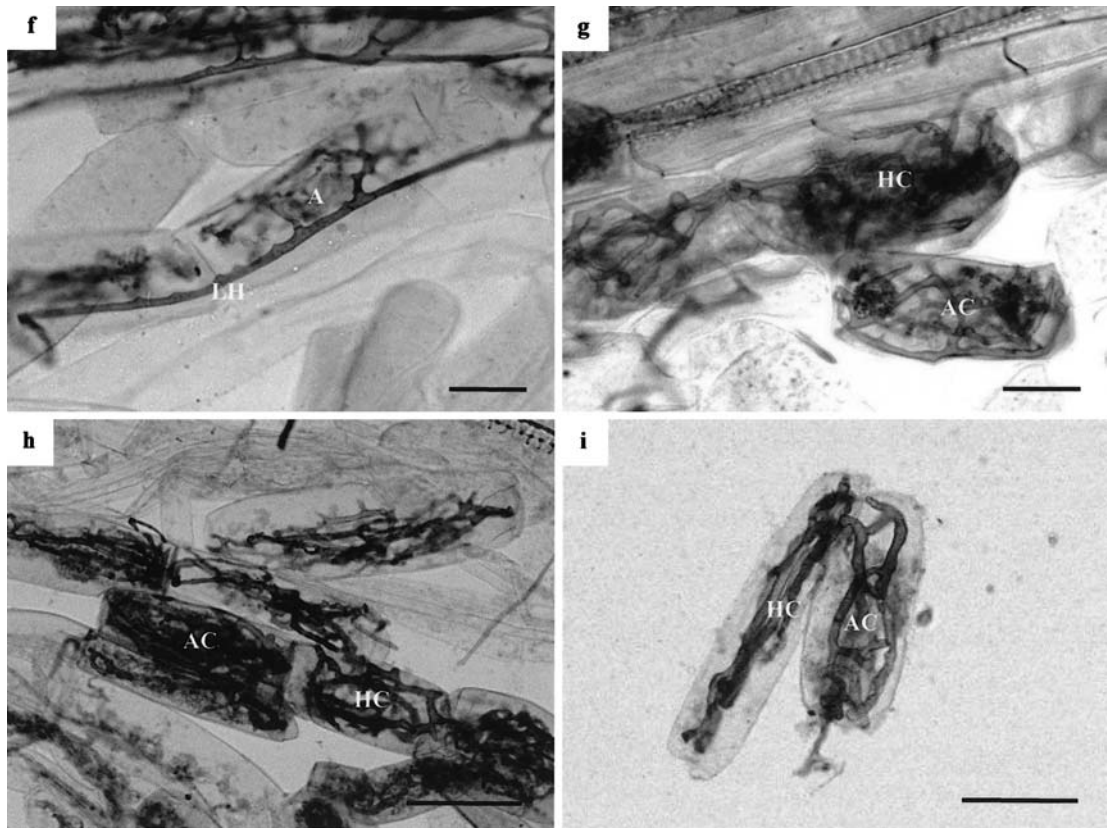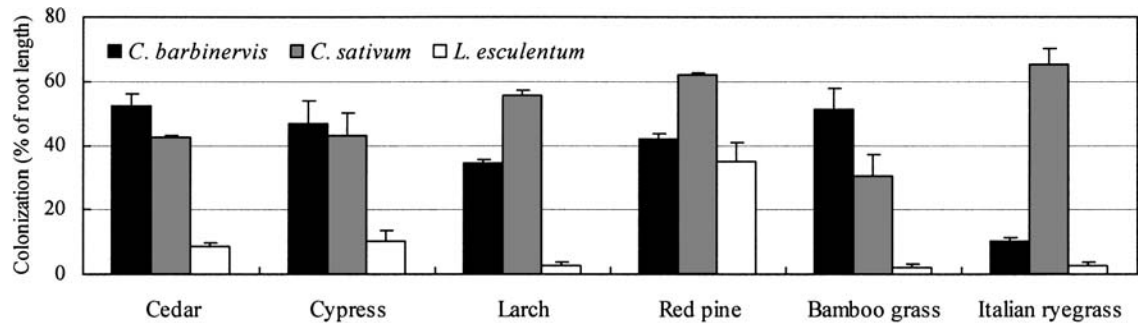
Fig. 1. *Continued*

Fig. 2. Colonization by arbuscular mycorrhizal fungi of plants grown in sample soils. Values are mean ± SE

Table 2. Summary of frequency of detection (%) based on the presence/absence of arbuscular mycorrhizal (AM) families in each test plant grown in soils from six sites (as shown in Fig. 3)

Plant	A/P	AC	GL	GI	Total
Clethra barbinervis	50.0 (3)	66.7 (4)	100.0 (6)	66.7 (4)	70.8 (17)
Cucumis sativum	50.0 (3)	0.0 (0)	100.0 (6)	0.0 (0)	37.5 (9)
Lycopersicon esculentum	83.3 (5)	33.3 (2)	100.0 (6)	66.7 (4)	70.8 (17)

Amplification with the primers were specific for Archaeosporaceae and/or Paraglomaceae (A/P), Acaulosporaceae (AC), Glomaceae (GL), and Gigasporaceae (GI), respectively
Values are number of sites from which each AM family was detected

the Isoplant DNA extraction kit (Nippon Gene, Toyama, Japan) and purified using the Geneclean Spin purification kit (BIO101 system; Qbiogene, Carlsbad, CA, USA). Nested PCR was conducted using AM fungi Glomales-specific primers (Redecker 2000). The first amplification with the universal primers NS5 and ITS4 (White et al. 1990) was performed as described by Redecker et al. (1997) with an annealing temperature of 51°C. Amplified products were diluted 1:10 and used as templates for the second PCR. The second PCR was conducted with various combinations of Glomales-specific primers (Redecker 2000), universal primers (White et al. 1990), and internal transcribed spacer (ITS)1F, which is specific for fungi (Gardes and Bruns 1993). Primer combinations were (i) ARCH1311, (ii) ACAU1660, (iii) GLOM1310 and LETC1670, each paired with ITS4, and (iv) a paring of ITS1F and GIGA5.8R. These combinations are specific for Archaeosporaceae and/or Paraglomaceae, Acaulosporaceae, Glomaceae, and Gigasporaceae, respectively. Annealing temperatures were 61°C for 5 cycles, then 60°C for 25 cycles, as described by Redecker (2000). PCR products were electrophoresed in 2% agarose gel, and amplification by each primer pair of the expected size of fragment was confirmed. The percent of detection was obtained by the following formula: (number of samples which amplification was obtained)/ (tested sample number, $n = 6$) $\times$ 100.

Results

Extent of colonization

Figure 1 shows representative morphologies observed in sample roots grown in soils collected from different vegetation sites. In *C. barbinervis*, hyphae extended by forming coils (hyphal coil) (Fig. 1a) or arbuscules (arbusculate coil) (Fig. 1b–d), which was *Paris*-type morphology. The *Paris*-type morphology was consistently observed in *C. barbinervis* grown in soils collected from throughout the sites. In *C. sativus*, hyphae mostly extended intercellularly (longitudinal hyphae) and formed arbuscules (Fig. 1e,f), which were *Arum*-type morphology, and sometimes arbusculate coils (Fig. 1g), which was *Paris*-type morphology. The *Arum*-type was more frequently seen than *Paris*-type in *C. sativus* grown in soils collected from different vegetation sites. Figure 1h,i shows hyphal and arbusculate coils formed in *L. esculentum*. Although both *Arum*- and *Paris*-type morphologies were seen in *L. esculentum*, the *Paris*-type morphology was more frequently observed than *Arum*-type throughout the sites.

Percent of mycorrhizal colonization was varied among the plants grown with different sample soils (Fig. 2). High colonization was observed in *C. barbinervis* and *C. sativus* throughout the sites, except *C. barbinervis* grown in soil from Italian ryegrass. On the other hand, colonization in *L. esculentum* was low throughout the sites except from red pine. In *C. barbinervis*, colonization ranged from 10% to 52%. It was high, around 50%, in soils collected from sites

of cedar, cypress, and bamboo grass while it was as low as 10% in soil from Italian ryegrass. In *C. sativus*, colonization ranged from 30% to 66%. It was high, around 60%, in soils of larch, red pine, and Italian ryegrass. In *L. esculentum*, colonization was lowest among test plants. Highest colonization (35%) was observed in soil of red pine whereas colonization was less than 10% in soils of cedar, cypress, larch, bamboo, and Italian ryegrass.

Type of AM fungi colonizing plant roots

In *C. barbinervis*, AM fungi belonging to at least four families were detected from roots grown in soils from cypress and larch, and three families from cedar and Italian ryegrass showing high diversity of AM fungi (Fig. 3). In contrast, AM fungi belonging to only one or two families were detected from roots of *C. barbinervis* grown in soils from red pine and bamboo grass. In *L. esculentum*, AM fungi belonging to at least three families of Glomales were detected from roots grown in soils from cypress, larch, red pine, and bamboo grass. Archaeosporaceae and/or Paraglomaceae and Glomaceae were detected from roots of *L. esculentum* grown in soils from cedar and Italian ryegrass. In *C. sativus*, Archaeosporaceae and/or Paraglomaceae and Glomaceae were also detected, whereas only Glomaceae was detected from roots of *C. sativus* grown in soils from cedar, larch, and bamboo grass, showing poor diversity of AM fungi.

Percent of detection was varied depending on the combination of plant species and soils (see Fig. 3). In *C. barbinervis*, Archaeosporaceae/Paraglomaceae were detected with 100% from roots grown in soil from larch, and Glomaceae were detected with 100% from roots grown in soils from cedar and red pine. In *C. sativus*, Glomaceae was detected with 100% from roots grown in soils from cedar, cypress, and larch. In *L. esculentum*, Archaeosporaceae/ Paraglomaceae were detected with more than 80% from roots grown in soils from cedar, cypress, bamboo grass, and Italian ryegrass, Glomaceae was detected with more than 80% from roots grown in soils from cypress, larch, red pine, and Italian ryegrass, and Gigasporaceae was detected with 100% from roots grown in soils from cypress and red pine.

AM families detected from spores were different among soils and did not consistently reflect the AM families detected from test roots (see Fig. 3). Some AM families were only detected from plant roots and not from spores collected from soils, such as Archaeosporaceae and/or Paraglomaceae in soil of cedar, Acaulosporaceae in larch, red pine, and Italian ryegrass, Glomaceae in larch, and Gigasporaceae in larch and red pine.

Table 2 shows the summary of frequency of AM fungal families detected from test plants grown in soils from six sites. Glomaceae was consistently detected (100%) among test plants. From *C. barbinervis* and *L. esculentum*, all the families were detected (50%–100% and 33%–100%, respectively). From *C. sativus*, Archaeosporaceae and/or Paraglomaceae and Glomaceae were frequently detected (50% and 100%, respectively) whereas Acaulosporaceae
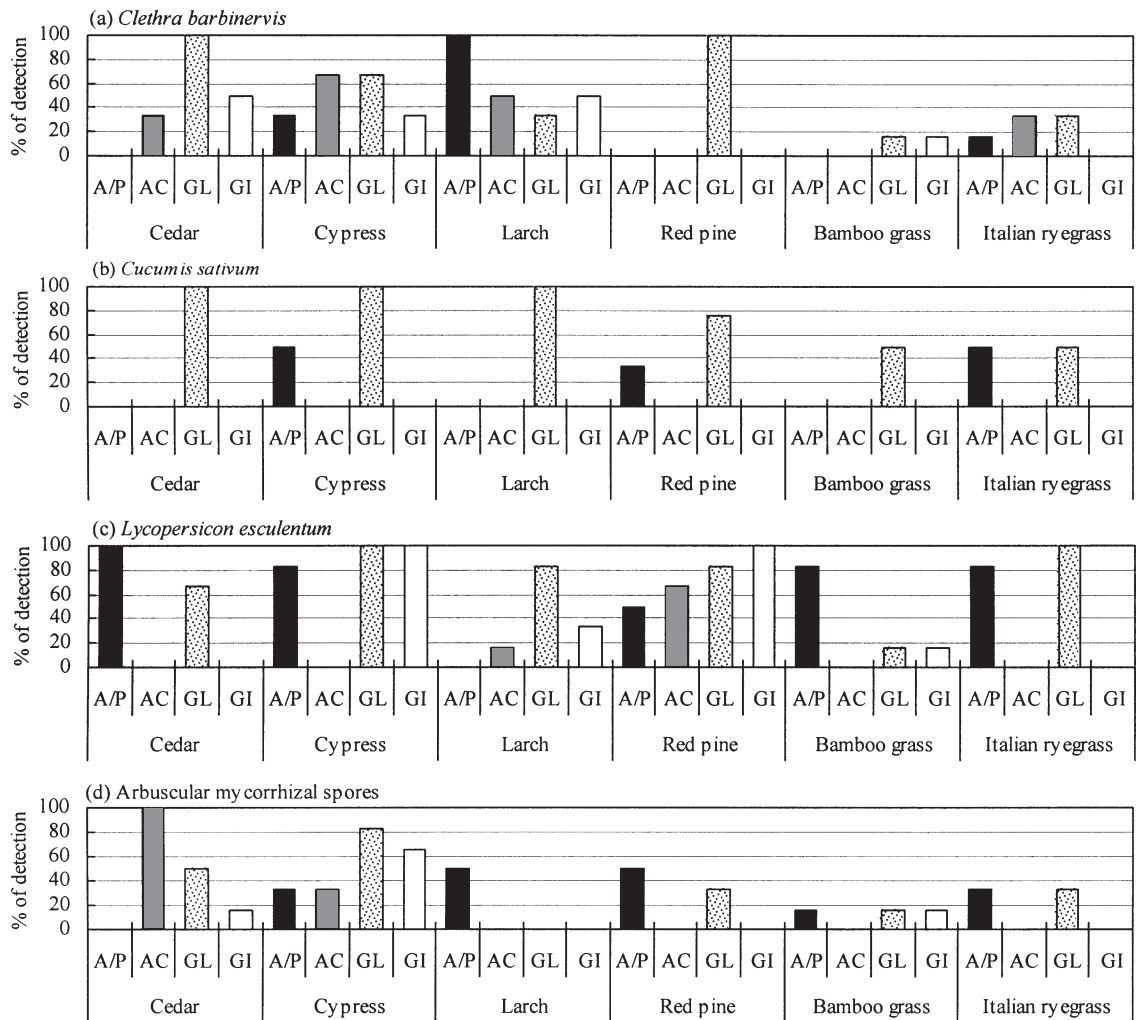
Fig. 3. Percent of detection with Glomales-specific primers from roots grown in sample soils and arbuscular mycorrhizal spores in sample soils. Amplification was with primers specific for Archaeosporaceae and/or Paraglomaceae (*A/P*), Acaulosporaceae (*AC*), Glomaceae (*GL*), and Gigasporaceae (*GI*), respectively. Values were obtained by the formula: (number of samples which amplification was obtained)/ (tested sample number, $n = 6$) × 100

and Gigasporaceae were not detected at all. Total frequency of detection of each test plant was high (71%) both in *C. barbinervis* and *L. esculentum*, while it was low (38%) in *C. sativus*.

Discussion

A canopy species, *C. barbinervis* belonging to Ericales is one of the trees comprising native forests in Japan and has been reported to form AM of *Paris*-type morphology (Kubota et al. 2000). In this study, we confirmed that *C. barbinervis* grown in soils collected from different vegetation sites consistently formed only *Paris*-type morphology. On the other hand, *C. sativus* formed both *Arum*- and *Paris*-type AM with high occurrence of *Arum*-type AM. *L. esculentum* also formed both *Arum*- and *Paris*-type AM but with high occurrence of *Paris*-type AM. The result obtained

here corresponds with the findings of Cavagnaro et al. (2001), who demonstrated that both morphologies were formed in *L. esculentum* using separate pots that contain different AM fungal species. Application of the molecular method in addition to morphological observation allowed better understanding of fungi involved in each test plant. *C. barbinervis*, grown in soils collected from different vegetation sites, was found to be colonized by AM fungi belonging to at least four or five families: Archaeosporaceae and/or Paraglomaceae, Acaulosporaceae, Glomaceae, and Gigasporaceae. Morphologically although only *Paris*-type was seen in *C. barbinervis*, several AM fungal families were detected from the roots. Similar results that several AM fungal families detected from *C. barbinervis* grown in soils collected from below *C. barbinervis* tree showed only *Paris*-type AM were also obtained (data unpublished). *L. esculentum* forming both *Arum*- and *Paris*-type AM was also found to be colonized by AM fungi belonging to at least four or five families.

In contrast, *C. sativus* mostly forming *Arum*-type AM was found to be colonized by AM fungi belonging to fewer than two or three families: Archaeosporaceae and/or Paraglomaceae and Glomaceae. Such cooccurrence of different AM families colonizing single plant species has also been seen in previous studies (Clapp et al. 1995; Brundrett et al. 1999; Helgason et al. 1999; Daniell et al. 2001; Husband et al. 2002; Gollotte et al. 2003). Helgason et al. (1999) and Vandenkoornhuyse et al. (2002) reported that both bluebell and *Agrostis capillaries* and *Trifolium repens*, naturally growing in woodland and grassland, respectively, were colonized by three families of AM fungi: Acaulosporaceae, Glomaceae, and Gigasporaceae.

Detected AM diversity from AM spores in different site soils did not consistently reflect AM fungal diversity seen in test plants. The poor correlation of spore numbers with mycorrhiza formation was pointed out by previous researchers (Abbott and Robson 1991; Clapp et al. 1995; Morton et al. 1995; Merryweather and Fitter 1998). Although *C. barbinervis* and *L. esculentum* showed higher AM diversity comparing to *C. sativus*, the AM diversity within the same plant species depended on soils in which test plants were grown. In case of *C. barbinervis* grown in soil from red pine, only one family of AM fungi belonging to Glomaceae was detected from roots, and in soil from bamboo grass, only two families, Glomaceae and Gigasporaceae, were detected. *C. sativus* consistently showed lower AM diversity (fewer than two or three families), and in soils from cedar, larch, and bamboo grass, only Glomaceae was detected. These variations of AM diversity within single plant species could be attributed to various AM species with different inoculum levels existing in the collected soils. AM fungal competition, antagonism, or suppression effect via plants could also be considered as the factors causing different AM diversity seen in each test plant grown in soils from different vegetation sites. Pearson et al. (1993) reported that high *Scutellospora calospora* treatment in clover (*Trifolium subterraneum*) reduced colonization by *Glomus* sp.

Even using the same soil, colonized families of AM fungi were different depending on the test plants. For example, in soil of cedar, Archaeosporaceae and/or Paraglomaceae were detected with high frequency from roots of *L. esculentum*, but it was not found at all from roots of *C. barbinervis* and *C. sativus*. Brundrett et al. (1999) emphasized that differences between soils were more important than the influence of host plants or extremes in soil fertility for colonized species of AM fungi. However, this study showed that host plant species strongly influenced the species of AM fungi or AM diversity in the roots.

In the present study, AM fungi belonging to Glomaceae were the most dominant family detected among the test plants. Brundrett et al. (1999) investigated the speed of colonization by different AM fungi and showed that *Glomus* species colonized more rapidly in first 12 weeks than *Scutellospora* species did. We investigated colonization by AM fungi at 8 weeks, and that timing might have resulted in the dominance of Glomaceae among the test plants. From *C. sativus*, Acaulosporaceae and Gigasporaceae were not detected at all, suggesting that the plant has strong colonization preference for Archaeosporaceae/Paraglomaceae and Glomaceae. On the other hand, *C. barbinervis* and *L. esculentum* might not have such colonization preference by AM fungi as seen in *C. sativum*.

Although colonizing AM species differ depending on host species, host plants seem to have the following two types: (1) host determines the morphology, and (2) host reflects the AM fungal character itself. In the case of *C. barbinervis*, AM morphology is controlled by host regardless of colonizing AM fungi. *L. esculentum* could be the latter type, as Cavagnaro et al. (2001) demonstrated. *C. sativus* may seem to be the former type because of the high occurrence of *Arum*-type morphology; however, it would rather be the latter type because it has strong preference to Archaeosporaceae and/or Paraglomaceae and Glomaceae. Further investigation is necessary to elucidate the relations in frequency of AM fungi colonizing and the morphologies formed in plants. Host–fungal preference should be also studied to clarify the functional interaction between plants and AM fungi.

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