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Identification of Symbiotic Arbuscular Mycorrhizal Fungi in Korean Ginseng Roots by 18S rDNA Sequence

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This study was carried out to perform the identification of arbuscular mycorrhizal fungi colonized in Korean Ginseng roots. Samples were soils and roots from various Ginseng cultivation sites of Chungcheong province in Korea. Those approaches include thorough investigation of morphological characteristics of spores harvested from Ginseng root associated soil and molecular biological identification of AM fungi in Ginseng roots. Based on the morphology of spores isolated from the soils, five distinguishable spore morpho–types AM fungi were found; *Glomus mosseae*, *Glomus intraradices*, *Gigaspora margarita*, *Acaulospora longular* and *Gigaspora gigantea*. Among these, *Glomus mosseae*, *Glomus intraradices* and *Acaulospora longular* were the most commonly encountered species. *Glomus* and *Acaulospora* were dominant with frequencies of 55% and 25%, respectively.

AM fungi colonization rates were progressed with the increase of cultivation period, relatively low infection rate in two year–old root, heavily infected in three year–old root, cube shape of intensive hyphal coil and distributed toward longitudinally in four year–old root, and eventually clear internal arbuscular hypae and vescicle were observed in the five year–old roots.

By sequence analysis of the small subunit of the nuclear ribosomal DNA (18S rDNA), it was disclosed that Ginseng roots were associated with AM fungi as follows; 4 *Glomus* and 1 *Acaulospora* genera, *Glomus sinuosum* (AJ133706), *Glomus fasciculatum* (Y17640), *Glomus intraradices* (AY635831), *Glomus mossae* (AJ699064) and *Acaulospora* spp. (AY394664). There were observed only one AM fungus, *Glomus sinuosum*, in one year–old Ginseng root, but three AM fungi, *Glomus fasciculatum*, *Glomus intraradices* and *Glomus sinuosum* in two year–old root. Three year–old root was associated with two AM fungi, *Glomus sinuosum* and one new AM fungus, *Acaulospora* spp. (AY394664). There were found three AM fungi, *Glomus sinuosum*, *Glomus fasciculatum* and *Glomus intraradices*, in five year–old root.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are ubiquitous in terrestrial ecosystem, forming symbiotic association with roots from the majority of plant species (Smith and Read, 1997a). In exchange for carbon from plant hosts, these fungi can facilitate plant uptake and transport of less mobile soil nutrients (such as phosphorus) (Bolan, 1991; Thingstrup *et al*., 2000; Jakobsen *et al*., 2001), enhance drought tolerance (Davies *et al*., 1993; Ruiz–Lozano *et al*., 2001; Kaya *et al*., 2003) and reduce pathogenic infections (Newsham *et al*., 1995; Abdalla and Abdel–Fattah, 2000). Although mycorrhizal fungi are traditionally believed to be non–host specific in their ability to infect plants, the benefit to each partner in any given interaction between AM fungus and host plant may depend on the particular species involved (Burrows and Pfleger, 2002a; Johnson *et al*., 2004).

It has been well documented that plant species is important as far as impacting individual fungal species or fungal assemblages (Bever *et al*., 1996; Eom *et al*., 1994; Eom *et al*., 2000; Lovelock *et al*., 2003). Since the root exudates of various plant species may affect the growth and colonization of specific mycorrhizal fungal species differently (Douds *et al*., 1996; Vierheilig *et al*., 1998), the co–existence of more than one plant species might alter mycorrhizal fungal growth, infection activity, spore production and species composition. Since higher plant diversity also likely enhances plant biomass (Tilman *et al*., 1996; Hector *et al*., 1999) resulting in a higher density of root material in the soil, coexistence of diverse plant species may better support mycorrhizal fungal growth and increase spore production. Mycorrhizal fungal species or fungal species assemblages have been shown to play an important role in the formation and maintenance of plant diversity and plant community structure (van der Heijden *et al*., 1998a, van der Heijden *et al*., 1998b and van der Heijden *et al*., 2003; Smith *et al*., 1999; Klironomos *et al*., 2000; O'Connor *et al*., 2002; Urcelay and Diaz, 2003). However, only a few studies have focused on whether and how plant coexistence or plant diversity influences the activities and species diversity of mycorrhizal fungi (Burrows and Pfleger, 2002a; Burrows and Pfleger, 2002b; Johnson *et al*., 2004). Burrows and Pfleger (2002a) reported that 30–150% more spores were found in the plots with 16

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coexisting plants than in the corresponding plots consisting of only a single species. Sporulation of mycorrhizal species with large spores also increased significantly with increasing plant species, while sporulation of smaller–spored species varied in response to host diversity. Despite this evidence, it is not clear how coexisting plant species affect the growth and functions of mycorrhizal fungi.

Ginseng (*Panax ginseng* C.A. Meyer) has been cultivated for long period in Korea as one of the very important folks medicines. One major problem is root diseases encountered during long cultivation period. It was known that damping–off disease of Korean Ginseng is caused by mainly *Fusarium* sp., *Rhizoctonia solani*, and *Pythium* sp., root rots by *Cylindrocarpon destructans*, and root blight by *Phytophthora cactorum* (Oh *et al*., 1978; Yu *et al*., 1984; Darmon *et al*., 1991; Li. 1994; Shin *et al*., 1986). Consecutive planting of Ginseng in the same field may bring in serious root diseases and root–rot caused by *Fusarium* sp. (Li. 1994; Shin *et al*., 1986). Therefore Ginseng farmers have been paid their special attention to bed soil to avoid the introduction of any pathogens by means of completely exchanging top soil of the bed with new soil or sterilizing or fumigating the soil to kill any pathogens in the bed.

In recent years, mycorrhizae have been applied to environmental friendly agiculture to decrease the use of fertilizer and pesticide. It has been known that mycorrhizae may result in enhanced survival, nutrient acquisition, reproduction and growth for the component organisms (Smith and Read, 1997b; Molina, 1997: Lugo and Cabello, 2002). Ginseng is normally grown for 4 to 6 years in the same bed. Therefore in Ginseng cultivation, too much usuage of pesticide and fertilizer is strongly discouraged because of their slow degradation, possible accumulation in the soil and direct using Ginseng root by the people. Fortunately some researchers have been involved in mycorrhizal study related to Ginseng cultivation (Park *et al*., 1990; Han *et al*., 1996; Whitbread *et al*., 1996; McGonigle *et al*., 1999; Armstrong and Peterson, 2002; Woo *et al*., 2002; Lee *et al*., 2004; Auh, 2004). Lately Lee *et al*. (2004) precisely studied on the morphology of AM fungi infection in the ginseng roots related to the root age and soil texture. However, there are no enough informations on what kinds of mycorrhizal fungi exist in Ginseng roots, and how much affect those mycorrhizal fungi to the Ginseng growth because of very difficult identification of endomycorrhizal fungi not only in soil beds but also in roots.

Identification of biological species in the AM fungi was mostly investigated based on the morphological and developmental characteristics of fungal spores (Morton and Benny, 1990). More than 150 AMF species are described based on their spore morphology (Walker and Trappe, 1993), but spore morphotyping requires considerable experience (Clapp *et al*., 2001; Clapp *et al*., 2002) and spore counts may not reflect the in planta composition of AMF communities (Clapp *et al*., 1995; Merryweather and Fitter, 1998; Turnau *et al*., 2001), due to taxon–specific differences between sporulation and root colonization rates. Therefore, more adequate methods are needed to accurately identify the mycorrhizal fungi. The methodological advance in the study of mycorrhiza identification has been the application of PCR (Mullis and Falloona, 1987; Gardes *et al*., 1991; Henrion *et al*., 1992; Lanfranco *et al*., 1998). PCR–based molecular markers allowing differentiation between species or genotypes have become rapidly indispensable for ecological survey of the diversity of fungi (Lanfranco *et al*., 1998). The methodological advance in the study of mycorrhiza identification has been the application of PCR (Mullis and Falloona, 1987; Gardes *et al*., 1991; Henrion *et al*., 1992; Lanfranco *et al*., 1998). PCR–based molecular markers allowing differentiation between species or genotypes have become rapidly indispensable for ecological survey of the diversity of fungi (Lanfranco *et al*., 1998).

Progress in analyzing AMF diversity at species level in planta has recently been made by sequence analysis of the small subunit of the nuclear ribosomal DNA (18S rDNA) (Simon *et al*., 1992; Sanders *et al*., 1995; Helgason *et al*., 1998; Helgason *et al*., 1999, Kuhn *et al*., 2001; Vandenkoornhuyse *et al*., 2002). The rDNA has considerable utility to identify distantly related species or groups of related mycorrhizal fungi, and rDNA sequence data are accumulating very fast (Boscot *et al*., 2000). Thus, interspecies comparisons of the rDNA unit reveal a high degree of conservation in coding regions (18S, 5.8S, 25S and 5S genes) and considerable sequence differences in the internal transcribed spacer (ITS) and intergenic spacer (IGS)(Hijri *et al*., 1999) (Fig. 1). Eom *et al*. (2004) reported identification of AM fungi colonizing *Panax ginseng* in Korea using 18S rDNA Sequence. However there were some difficulties in AM fungi identification because of unselective amplification by primers and lack of accumulated sequencing data.

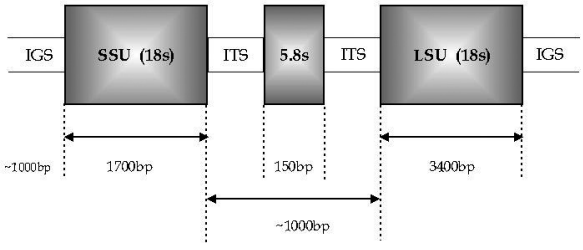


Fig. 1. Ribosomal DNA gene variability.
IGS; Intergenic spacer, ITS; Internal transcribed spacer, SSU; Small subunit, LSU; Large subunit.

In our preliminary study we found that native plant species in upland agroecosystem differed significantly in mycorrhizal species (Cho *et al*., 2006). Through both morphology–based and molecular biological approaches of the spores from various soils, total 9 species of 6 genera, such as 3 species of *Glomus*, 1 species of

Paraglomus, *Gigaspora*, *Acaulospora* and *Archaeospora*, respectively and 2 species of *Scutellospora* were identified at Chungbuk province, and in Chungnam province, total 6 species of 5 genera, such as 2 species of *Gigaspora*, 1 species of *Glomus*, *Scutellospora*, *Acaulospora* and *Archaeospora*, respectively were confirmed.

This study was carried out to perform the identification of arbuscular mycorrhizal fungi colonized in Korean Ginseng roots. Those approaches include thorough investigation of morphological characteristics of spores harvested from Ginseng root associated soil and molecular biological identification of AM fungi in Ginseng roots. Firstly various mycorrhizal fungi from the Ginseng root–associated soil beds in Chungcheong–province are collected, and their identification was done by morphological features. In addition rDNA sequencing characteristics of AM fungi in different aged Ginseng roots in terms of the root age were evaluated by using designed specific primers.

MATERIALS AND METHODS

Collection of root–associated soils and ginseng roots

The ginseng root–associated soil samples from each individual plant were collected, each sample consisting of five bulked sub–samples (200 cm^3 soil cores) randomly collected at 10 to 20 cm depth. Ginseng roots were collected in general crop fields in the Chungcheong province (Table 1), middle parts of Korea. For the mycorrhizal survey 8 individual Ginseng roots were randomly chosen at 5 sites in two provinces. Root samples for quantifying mycorrhizal colonization of different ages were collected and separated from the soil, washed and fixed in FAA (37% formaldehyde–glacial acetic acid–95% ethanol, 9:0.5:0.5, v/v/v).

Table 1. Collection sites of Ginseng roots

Sample ID	Collection sites	Note
G01	Munwei, Cheongwon, Chungbuk	1 yr root
G02	Kwangam, Jungpyung, Chungbuk	2 yr root
G03	Buyeon, Cheongwon, Chungbuk	3 yr root
G04	Buyeon, Cheongwon, Chungbuk	3 yr root
G05	Munduck, Cheongwon, Chungbuk	4 yr root
G06	Munduck, Cheongwon, Chungbuk	4 yr root
G07	Jungdo, Geumsan, Chungnam	4 yr root
G08	Jungdo, Geumsan, Chungnam	5 yr root

Isolations of spores from soils

The AM fungal spores were extracted from the rhizosphere soils by wet sieving and decanting followed by sucrose gradient centrifugation (Daniels and Skipper, 1982; Sieverding, 1991). After centrifugation, the supernatant was poured through a 50 μm mesh and quickly rinsed with tap water. Spores were grouped, under a dissecting microscope, according to their morphological characteristics and used to identify AM fungi and to initiate cultures of the different native AM fungal isolates.

Morphological characterization of spores by microscopical observation

After confirming under the light microscope the apparent purity of the cultures of the different AM morphotypes, spores were identified to genus and, when possible, to species level. Criteria for morphological spore characterization were mainly based on spore size and colour, wall structure and hyphal attachment (Walker, 1983; Morton and Benny, 1990; Schenk and Perez, 1990; Dodd and Rosendahl, 1996; INVAM, 1997). In order to assess the colonization, fine roots of host plant were stained with acid fuchsin (Kormaik and McGraw, 1982; Koske and Gemma, 1989). Colonization was determined by a grid intersect method (Giovannetti and Mosse, 1980) based on microscopical observation.

Molecular characterization (DNA sequencing)

Isolation of DNA

Total genomic DNA of the different AM fungal morphotypes was isolated from approximately 50 to 60 spores, which were placed in microcentrifuge tubes containing 40 μl milli Q–water and crushed with a miniature pestle. DNA extraction was done by DNeasy Plant Mini Kit (QIAGEN Science, USA) according to Technical Manual from QIAGEN (Gosselin *et al*., 1995; Wyss and Bonfante, 1993). The genomic DNA was repeatedly purified with the CTAB extracting buffer until a single band was obtained on gel. The total genomic DNA extracted above was amplified using the primers and conditions listed by Lee and Yoo (2000). The supernatant was frozen at −20 ˚C and used as template for PCR.

Polymerase chain reaction

Small subunit (SSU) rDNA gene sequences were PCR amplified from the purified total DNA by using the universal primer NS1–NS4 and the AM specific primer AML1–AML2 (Auh, 2004) (van Tuinen *et al*., 1998, Fig. 2). The latter primers were designed based on the small subunit of the nuclear ribosomal DNA (18S rDNA). The 1st PCR mixture consisted of 1 μl of universal primer NS1/NS4, 1 μl of DNA extract, 7 μl of nuclease free

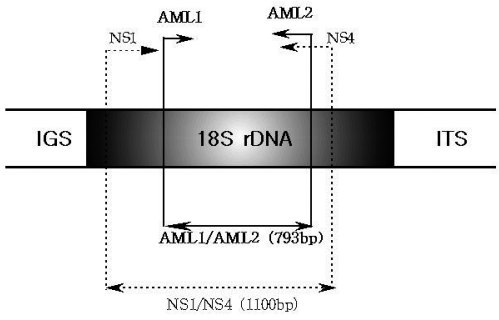


Fig. 2. 18S rDNA gene with annealing sites of primer pairs, NS1/NS4 and AML1/AML2 and their approximate DNA lengths (Lee, 2003).

Table 2. Characteristics of primers used in this study and conditions of PCR (Lee, 2003)

Primer			PCR conditions
Name	Nucleotide sequences	Tm[a)]	
NS1/NS4 (30 cycles)			
NS1	5'–GTAGTCATATGCTTGTCTC–3'	37.7°C	95°C (3 min), 40°C (1 min), 72°C (1 min) – 1 cycle; 95°C (30 sec), 40°C (1 min), 72°C (1 min, 30 sec), 40°C –28
NS4	5'–TTCCGTCAATTCCTTTAAG–3'	48.3°C	cycles; 95°C (30 sec), 40°C (1 min), 72°C (10min) – 1 cycle
AML1/AML2 (30 cycles)			
AML1	5'–AACTTTCGATGGTAGGATAGA–3'	47.2°C	95°C (3 min), 47°C (1 min), 72°C (1 min) – 1 cycle;
AML2	5'–CCAAACACTTTGGTTTCC–3'	47.1°C	95°C (30 sec), 47°C (1 min), 72°C (1 min) – 28 cycles; 95°C (30 sec), 47°C (1 min), 72°C (10 min) – 1 cycle

[a)] Melting temperature was determined using 50 mM salts concentration

water, 10 μl of the PCR master mix (500 unit/ml taq DNA polymerase, 250 μmM dNTP, 1.5 mM $MgCl_2$, pH 8.5; Promega Co., USA) in a 20 μl reaction volume. PCR was performed in an automated thermal cycler (PTC–200 Peltier Thermal Cycler, MJ Research, INC. USA) and used to 2nd PCR–DNA sample. The 2nd PCR was done by using AM fungi specific primer AML1/AML2 (White *et al*., 1990; Lee, 2003). The PCR products were separated by electrophoresis in 1.0% agarose gels, stained with ethidium bromide, and visualized by UV trans–illumination. Table 2 represents characteristics of used primers and PCR condition.

Cloning and sequencing

PCR products were separated on an agarose gel, and the expected approximately 550–bp band was eluted by *AccuPrep*™ gel purification kit (Bioneer Co.). After purification, the sticky (–A) –ended PCR–product was cloned into the pGEM–T easy Vector™ (50 ng/$\mu\ell$; Promega, USA) and transformed into *Escherichia coli* (JM109; using as the competent cell). The positive transformed cells were screened by X–gal (white colonies were selected only) and extracted to insert–DNA included plasmid using *AccuPrep*™ plasmid extraction kit (Bioneer Co.). These plasmids were screened using the (NS1/NS4) primer amplification.

The amplication of insert DNA in plasmid was done using SP6 (5'–TATTTAGGTGACACTATAG–3') and T7 (5'–TAATACGACT CATATAGGG–3'). DNA sequences were done by automatic sequencer ABIPRISM™ (Perkin–Elmer, USA at Eugenetech Co., Korea). Sequencing was analyzed by both ways of reverse and forward sequences, and then re–calculated with matching procedures of forward and reverse readings. Reverse sequences were reverse complemented and aligned with forward sequences by GENDOC program. All sequences were submitted to a BLAST search using the GenBank database (http://www.ncbi.nlm.nih.gov, Gehrig *et al*., 1996; Tae, 2000; Tae *et al*., 2002; Thompson *et al*., 1994; Saitou and Nei, 1987; Altschul *et al*., 1990).

RESULTS AND DISCUSSION

Morphogical identification based on spores in ginseng root–associated soil

The arbuscular mycorrhizal (AM) fungi are currently all classified in the order Glomales (Morton, 1988). The term vesicular–arbuscular mycorrhiza (VAM) was originally applied to symbiotic association formed by all fungi in the Glomales, but because a major suborder lacks the ability to form vesicles in roots, AM is now the preferred acronym. The diagnostic feature of AM fungi is the development of a highly branched arbuscule within root cortical cells. The fungus initially grows between cortical cells, but soon penetrates the host cell wall and grows within the cell. As the fungus grows, the host cell membrane invaginates and envelops the fungus, creating a new compartment where material of high molecular complexity is deposited. The arbuscules are relatively short lived, less than 15 days, and are often difficult to see in field–collected samples. The order Glomales is further divided into families and genera according to the spore morphology.

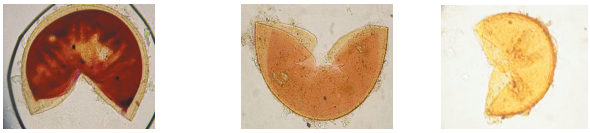
Vegetative structures of AM fungi (*i.e.* mycorrhizae and mycelium in the soil) occur largely under ground, and are difficult to be tracked and identified. Identification of biological species in the AM fungi was mostly investigated based on the morphological and developmental characteristics of fungal spores (Morton and Benny, 1990). More than 150 AM fungi are described based on their spore morphology (Walker and Trappe, 1993), but spore morphotyping requires considerable experiences (Clapp *et al*., 2001), and spore counts may not reflect the in planta composition of AMF communities (Clapp *et al*., 1995; Merryweather and Fitter, 1998; Turnau *et al*., 2001), due to taxon–specific differences between sporulation and root colonization rates. Therefore, more adequate methods are needed to accurately identify the mycorrhizal fungi.

Morphology–based approach of the spores from ginseng root–associated soils was applied to characterize the AM fungi spores. This is the first step to analyze the diversity of AM fungi in the Ginseng fields of Chungcheong province, Korea. By following this

approach, only five distinguishable spore morpho–types, three genera of AM fungi, were found. As shown in Fig. 3, five morphological species, *Glomus mosseae*, *Glomus intraradices*, *Gigaspora margarita*, *Acaulospora longular* and *Gigaspora gigantea* were recorded as spores in the soil samples. Among these, *Glomus mosseae*, *Glomus intraradices* and *Acaulospora longular* were the most commonly encountered species

Three genera of AM fungi were identified, among which *Glomus* and *Acaulospora* were dominant with frequencies of 55% and 25%, respectively. The frequency of *Gigaspora* genera was low. *Glomus mosseae* has globose or irregular shape of spore, 80–240μm in diameter, consisted to thin outer and thick inner walls, and funnel–type mycelia at spore base attached. Spores of *Glomus intraradices* were globose, obovate, to irregular in shape, 40–120μm in diameter, hyaline to pale yellow in color. They are formed singly and freely in soil or in colonized roots. *Gigaspora margarita* Becker & Hall has globose and obovate spores with 2 or 3 layers and 30μm in thickness, 240–350μm in diameter, milky to pale yellow in color and strong dark purple by Melzer's reagent. *Gigaspora gigantea* has dark pinkish red in colored spores, globose or obovate shape with 10μm thin wall and 40μm bulb–shaped suspensor attached, and 200–350μm in diameter, pale yellow green in color and pale pinky colored by Melzer's reagent. Spores of *Acaulospora longula* have globose or obovate shape with 5 layers wall and 100–200μm in diameter, milky to pale yellow in color and red colored inner wall by Melzer's reagent.

Glomus mosseae *Glomus intraradices*

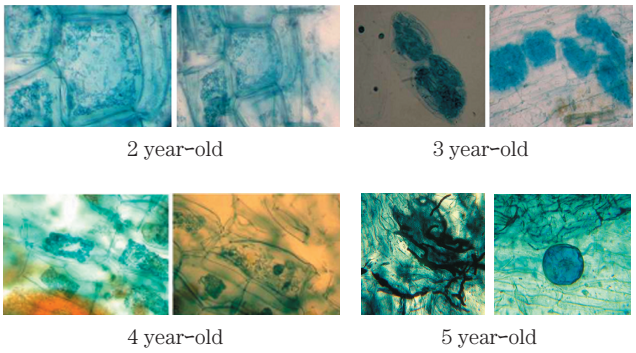


Acaulospora longular *Gigaspora margarita* *Gigaspora gigantea*

Fig. 3. Identified spores in the Ginseng rhizosphere soils.

AM fungi colonization

AM fungi colonization in the Ginseng roots were observed by Microscope as in Fig. 4. Two and three year–old roots showed infected by AM fungi, particularly heavily infection in 3 year–old root of Buyeon, Cheongwon, Chungbuk. There were no typical vesicle and only abundant arbuscules in the cortex. Especially the colonization of 4 year old root showed very complicate distribution of AM fungi arbusucles. Typical vesicle (V) and arbuscule (A) were observed in the five year–old roots.



2 year-old 3 year-old

4 year-old 5 year-old

Fig. 4. Microscopic features of intracellular cells in the cortical layers of Ginseng roots. View of arbuscular hyphae (A, left) and vesicle (V, right) in hairy root of 5 year–old Ginseng. Sites: 2 year–old: Kwangam, Jungpyung, Chungbuk; 3 year–old: Buyeon, Cheongwon, Chungbuk; 4 year–old: Munduck, Cheongwon, Chungbuk; 5 year–old: Jungdo, Geumsan, Chungnam.

AM fungi colonization in the roots was measured by a grid intersect method (Giovannetti and Mosse, 1980). There was some increasing colonization tendency with the increase of culture periods. The colonization rates were progressed with the increase of cultivation period, relatively low infection rate in the two year–old root, heavily infected in the three year–old root, cube shape of intensive hyphal coil and distributed toward longitudinally in four year–old root, and eventually showed clear internal arbuscular hypae and vescicle.

Molecular characterization (DNA sequencing)

The molecular biological identification of AM fungi has been a complicated task in the past because of the considerable degree of variation detected among the copies of rDNA within single spores(Calvente *et al.*, 2004). However, recent reports indicated that SSU rRNA sequence analysis is a suitable tool to inter–phylogenetic relationship among AM fungi since the expected sequence differences could be more easily linked to a taxonomic entity than the possibly multi–allelic ITS sequences (Schwarzott and Schüßler, 2001).

PCR primers were used to confirm AM fungi in Ginseng roots. Small subunit (SSU) rDNA gene sequences were PCR amplified from the purified total DNA by using the universal primer NS1–NS4 and the AM specific primer AML1–AML2 (Auh, 2004) (van Tuinen *et al.*, 1998). Finally, 8 cloned DNA fragments were selected, and displayed in the agarose gel as shown in Fig. 5.

After purification, the sticky (–A) –ended PCR–product was cloned and transformed into *E. coli*. The positive transformed cells were extracted to insert–DNA included plasmid using *AccuPrep*™ plasmid extraction kit (Bioneer Co.). These plasmids were screened using the primer (NS1/NS4) amplification. The cloned DNA fragments were selected and re–amplified to the plasmids as shown in Fig. 6. Then, selected one clone from each group and purified plasmids were

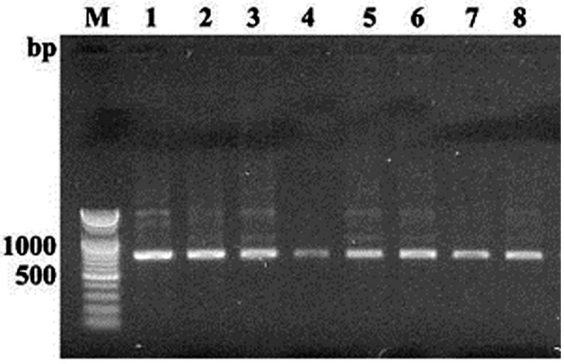


Fig. 5. PCR products of 18s rDNA fragment of AM fungi in Ginseng roots. Lane M, molecular size marker (100–bp ladder), 1: G01 (1 yr–old), 2: G02 (2 yr–old), 3: G03 (3 yr–old), 4: G03 (3 yr–old), 5: G04 (4 yr–old), 6: G04 (4 yr–old), 7: G04 (4 yr–old), 8: G05(5 yr–old).

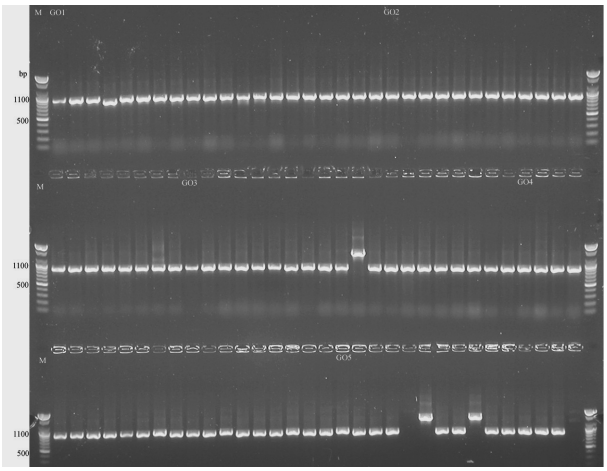


Fig. 6. The confirmation of the plasmid (pGEM–T Easy Vector™) inserted with the fragment of 18s rDNA's amplified with the specific primers. Lane M, molecular weight marker (100–bp ladder); Lanes were corresponded to the lanes of Fig. 5.

automatically sequenced on an ABIPRISM™377 (Perkin–Elmer, USA). The standard primer SP6 and T7 were used for sequencing of insert–DNA regions (Helgason *et al*., 2002).

Based on the above collaborated molecular techniques, the AM fungi in the Ginseng roots were genetically characterized. As shown in Table 3, the accession number for the new sequences, as deposited in the EMBL database are as follows: 4 *Glomus* and 1 *Acaulospora* genera, *Glomus sinuosum* (AJ133706), *Glomus fasciculatum* (Y17640), *Glomus intraradices* (AY635831), *Glomus mossae* (AJ699064) and *Acaulospora* spp. (AY394664).

There was observed only one AM fungus, *Glomus sinuosum*, from one year–old Ginseng root, but two year–old root had three AM fungi, *Glomus fasciculatum*, *Glomus intraradices* and *Glomus sinuosum*. Three year–old root had two AM fungi, *Glomus sinuosum* and one new AM fungus, *Acaulospora* spp. (AY394664). There were found three AM fungi in 5 year–old root, *Glomus sinuosum*, *Glomus fasciculatum* and *Glomus intraradices*. Comparisons of the obtained sequences with those present in the database revealed that they showed more than 95–99% identity to the SSU rDNA gene of different AM fungal isolates.

The sequences of DNA band of AM fungi in Ginseng root were aligned with GENDOC program as shown in Fig. 7. After making the consensus sequences using GENDOC program, Tree View and CLUSTAL X (Thompson *et al*., 1994) was used for multiple alignment and neighbor–joining phylogenetic dendrogram (Saitou and Nei, 1987) as shown in Fig. 8 using rDNA sequences of AM fungi selected from Gene Bank of NCBI (Gehrig *et al*., 1996; Tae *et al*., 2002; Eom *et al*., 2004).

These identification results concerned to the AM fungi in Ginseng roots emphasize the importance of

Table 3. BLAST search on NCBI with analyzed sequences for Identification of AMF in *Panax ginseng* roots

Years	Host Plant	Fungal Species	Accession no.	Identities
1	*P. ginseng*	*Glomus sinuosum*	AJ133706	765/790 (96%)
2	*P. ginseng*	*Glomus fasciculatum*	Y17640	789/792 (99%)
		Glomus intraradices	AY635831	789/792 (99%)
		Glomus sinuosum	AJ133706	767/790 (97%)
3	*P. ginseng*	*Acaulospora spp.*	AY394664	782/789 (99%)
		Glomus sinuosum	AJ133706	770/790 (97%)
4	*P. ginseng*	*Glomus sinuosum*	AJ133706	769/791 (97%)
		Acaulospora spp.	AY394664	782/795 (98%)
		Glomus mossae	AJ699064	699/727 (96%)
5	*P. ginseng*	*Glomus sinuosum*	AJ133706	766/789 (97%)
		Glomus fasciculatum	Y17640	790/792 (99%)
		Glomus intraradices	AY635831	684/714 (95%)

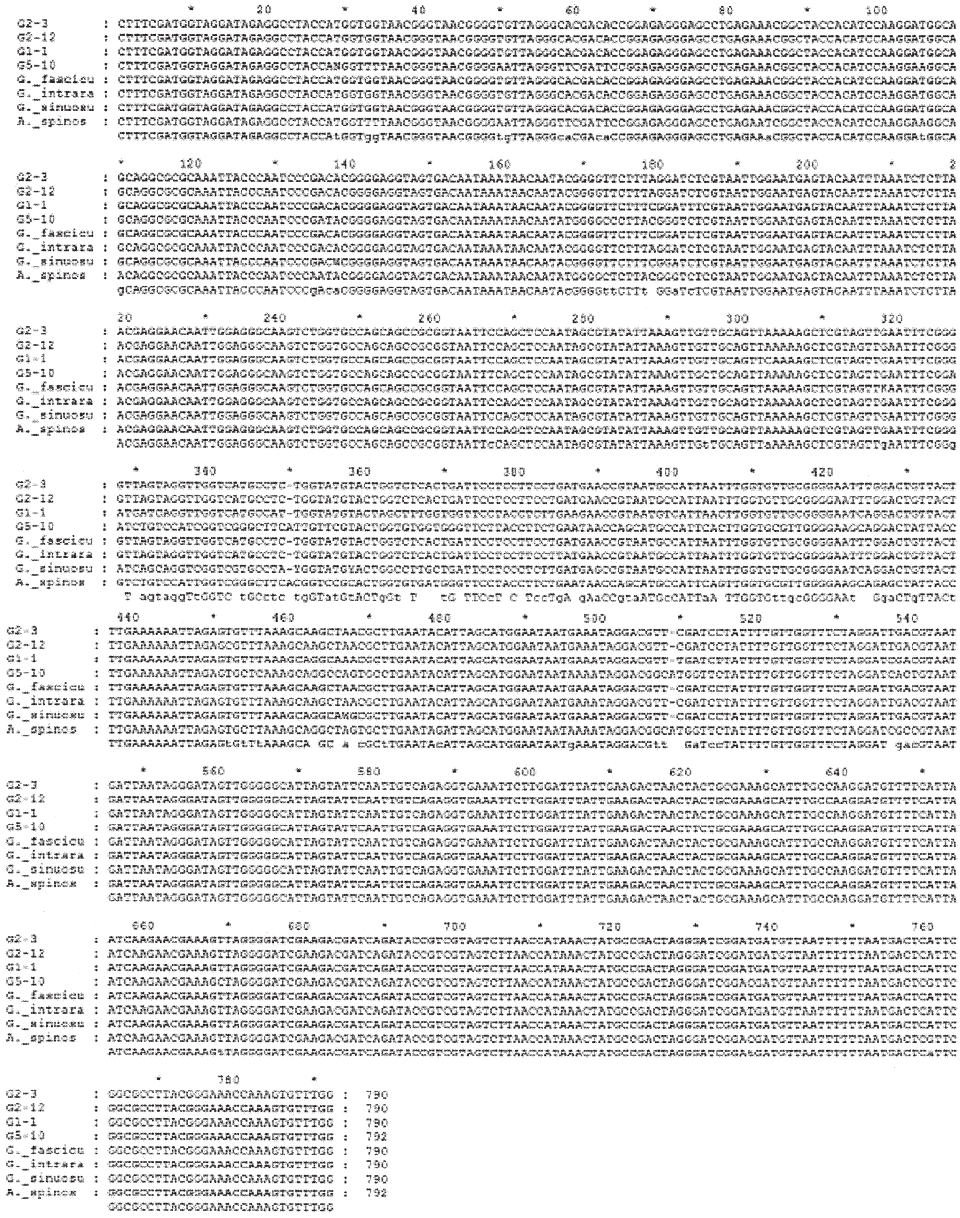


Fig. 7. Sequence alignment of PCR products amplified with AM fungi specific primers.

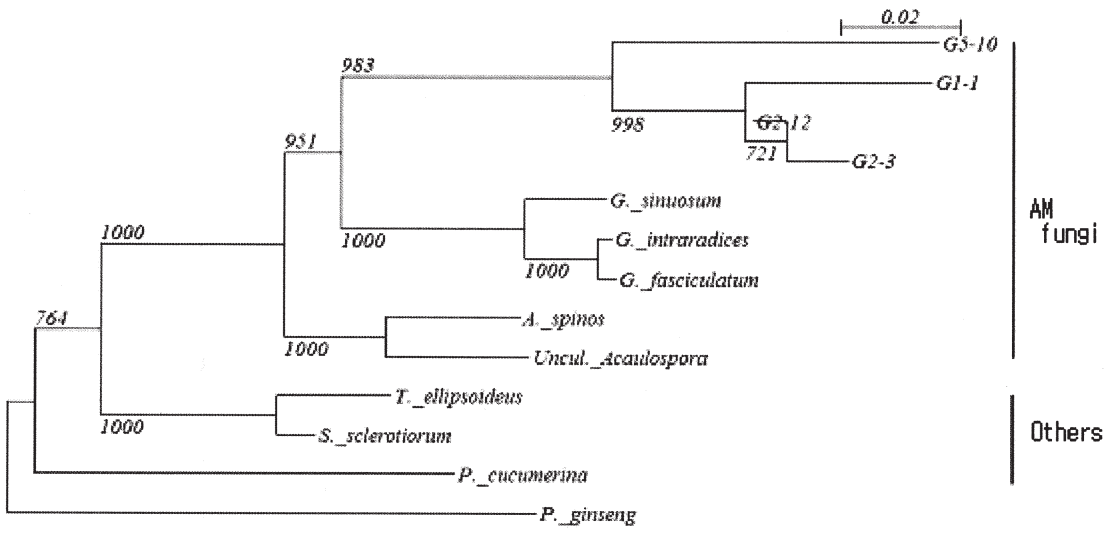


Fig. 8. Neighbour–joining phylogenetic relationship among AM fungi colonizing in Ginseng root based on 18s rDNA sequences.

exploiting the natural diversity of AM fungi not only in Ginseng root–associated soils but also in Ginseng roots, particulary in Chungcheong province, Korea, and would be a starting point to formulate particular AM fungi inoculants to be applied for the Ginseng production with optimized quality.

CONCLUSIONS

This study was carried out to perform the collection of AM fungi in soils and roots from various Ginseng cultivation sites of Chungcheong province in Korea and to identify the AM fungi which are associated to symbiotic relation with Ginseng. Those approaches include thorough investigation of morphological characteristics and molecular biological techniques.

Based on morphology–based approach of the spores from ginseng root–associated soils, five distinguishable spore morpho–types, three genera of AM fungi, were found; *Glomus mosseae*, *Glomus intraradices*, *Gigaspora margarita*, *Acaulospora longular* and *Gigaspora gigantea*. Among these, *Glomus mosseae*, *Glomus intraradices* and *Acaulospora longular* were the most commonly encountered species. *Glomus* and *Acaulospora* were dominant with frequencies of 55% and 25%, respectively. The frequency of *Gigaspora* genera was low.

Two and three year–old roots showed infected by AM fungi, particularly heavily infection in 3 year–old root. The colonization of 4 year old root showed very complicate distribution of AM fungi arbusucles. Typical vesicle (V) and arbuscule (A) were observed in the five year–old roots. AM fungi colonization rates were progressed with the increase of cultivation period, relatively low infection rate in the two year–old root, heavily infected in the three year–old root, cube shape of intensive hyphal coil and distributed toward longitudinally in four year–old root, and eventually showed clear internal arbuscular hypae and vescicle.

By sequence analysis of the small subunit of the nuclear ribosomal DNA (18S rDNA), the AM fungi in the Ginseng roots were identified as follows; 4 *Glomus* and 1 *Acaulospora* genera, *Glomus sinuosum* (AJ133706), *Glomus fasciculatum* (Y17640), *Glomus intraradices* (AY635831), *Glomus mossae* (AJ699064) and *Acaulospora* spp. (AY394664). There was observed only one AM fungus, *Glomus sinuosum*, from one year–old Ginseng root, but two year–old root had three AM fungi, *Glomus fasciculatum*, *Glomus intraradices* and *Glomus sinuosum*. Three year–old root had two AM fungi, *Glomus sinuosum* and one new AM fungus, *Acaulospora* spp. (AY394664). There were found three AM fungi in 5 year–old root, *Glomus sinuosum*, *Glomus fasciculatum* and *Glomus intraradices*. These identification results concerned to the AM fungi in Ginseng roots would be a starting point to formulate particular AM fungi inoculants to be applied for the Ginseng cultivation with optimized quality.

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