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Identification of Symbiotic Arbuscular Mycorrhizal Fungi in Korea by Morphological and DNA Sequencing Features of Their Spores

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In order to clarify the diversity of the arbuscular mycorrhizal fungi, 9 individual plant roots and soils were randomly chosen at 27 sites in the general cultivation fields in the Chungbuk– and Chungnam– provinces, middle parts of Korea. In terms of height growth of *Sorghum bicolor*, the soil in Cheongwon site (host plant *Fagopyrum esculentum*) resulted in the best growth, and the order of growth was *Platycodon grandiflorus, Miscanthus sinensis, Sesamum indicum*, and *Capsicum annuum*. It represents that AM fungi species are having host–specific differences. Concerned to AM fungi colonization of host plant, *Sorghum bicolor*, AM fungi colonization in the roots was an increasing colonization tendency with the increase of culture periods. At the initial culture stage, colonization upto 10 days was rather slow, and afterwards the rates were increased. This colonization increase has leveled off more than 40 days culture. In addition, both morphology–based and molecular approaches of the spores from various soils of Chungbuk– and Chungnam– provinces in Korea were applied to characterize the AM fungi. By following these approaches, in Chungbuk province, total 9 species of 6 genera, such as 3 species of *Glomus*, 1 species of *Paraglomus*, 1 species of *Gigaspora*, 2 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were identified. In Chungnam province, total 6 species of 5 genera, such as 2 species of *Gigaspora*, 1 species of *Glomus*, 1 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were confirmed.

INTRODUCTION

A mycorrrhiza is a symbiotic association between plant and fungus localized in root or root–like structure in which energy moves primarily from plant to fungus and inorganic resources from fungus to plant (Lewis, 1973). The term mycorrhiza (fungus root) was first used by Franke (1934) to describe the long–lived association between plant roots and fungal mycelium (Harley, 1969). Mycorrhiza may be one of the most important but least understood biological associations regulating community and ecosystem functioning (Harley, 1973). Most of the dominant plant species have mycorrhizae, and up to 96% of the root of the dominant species was mycorrhizal (Davidson and Christensen, 1977). These associations occur in most of the angiosperms, all gymnosperms, pteridophytes and some bryophytes (particularly liverworts). In addition, physiological interaction between plants and fungi begin very soon after contact has occurred. In some cases the changes induced in plants could be detrimental to the general physiology of the plant or even be fatal. These mutualistic associations may result in enhanced survival, nutrient acquisition, reproduction and growth for the component organisms (Smith and Read, 1997). The fungus invades plant root tissues and in general, gains a supply of carbon from the host. Representatives from all the taxonomic groups of fungi (Zygomycotina, Ascomycotina, Basidiomycotina, and Deuteromycotina) form mycorrhizal associations (Molina *et al*., 1997: Lugo and Cabello, 2002). Many of the mycorrhizal fungi are poor competitors in the soil environment, and some are obligate symbionts which are not capable of independent growth either in the natural environment or in vitro cultures. In general, the fungal partner maintains contact with the soil, sometimes forming an extensive mycelial network in the immediate vicinity which may have a very important role in the nutritional interdependence between the partners.

Mycorrhizas are classified (Harley and Smith, 1983) by the structural characteristics at maturity. At present, five kinds of mycorrhiza are known: ectomycorrhiza (EM) (mainly formed in roots of woody plants), arbuscular mycorrhiza (AM), ericoid mycorrhiza, orchid mycorrhiza, and ecto–endomycorrhiza (Peterson and Farquhar, 1994). Arbuscular mycorrhizal (AM) fungi are endomycorrhiza. The name 'vesicular–arbuscular' is derived from the structures formed within the host plant tissues by the invading fungus. After spore germination, hyphae branch to form a fan near the root surface and come in contact with the epidermal cells. And then, hyphae form appressoria and the fungus penetrates between the outermost layers of cells to grow within and

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between the cells of the root cortex (Eom *et al*., 1994; Koske and Gemma, 1989). In outer to middle cortical layers of root the fungus forms vesicles and within cells of the inner cortical layers, nearer to the central stele, the invading fungus forms arbuscules (Morton, 1986; Morton and Benny, 1990). These vesicular–arbuscular forms are the most common and widely occurring mycorrhizal associations. In fact, it has been suggested that up to 25,000 plant species have potential to form vesicular arbuscular mycorrhiza (Law and Lewis, 1983), with representatives largely from crop plants, herbs and tropical trees. These mycorrhizas are agriculturally important and have great economic significance.

AM species is not easily manipulated in natural or laboratory settings. This is biotrophic obligate symbionts (Koske and Polson, 1984), and most of AM fungi either grow poorly or do not grow at all in culture, and also addition of spores or mycelia inoculums rarely results in colonization under non–sterile condition (Danielson andVisser, 1989; Horton and Bruns, 2001). Vegetative structures of these 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 AMF species are described based on their spore morphology (Walker and Trappe, 1993), but spore morphotyping requires considerable experience (Clapp *et al*., 2001; 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). However, biochemical and molecular approaches are being incorporated to define and relate taxa in the order Glomales (Simon *et al*., 1992; Graham *et al*., 1995; Schüßler *et al*., 2001; Madan *et al*., 2002), and to study the diversity of their populations in different soil–plant systems (Clapp *et al*., 1995; Helgason *et al*., 1998; Kowalchuk *et al*., 2002; Vandenkoornhuyse *et al*., 2002). Most of the recent molecular work has focused on the use of PCR techniques to analyze target sequences within the ribosomal gene cluster (Smith and Read, 1997; van Tuinen *et al*., 1998; Redecker *et al*., 2000; Schüßler *et al*., 2001). 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) (Sanders *et al*., 1995; Helgason *et al*., 1998, 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 spacers (ITS and intergeneric spacer (IGS)(Hijri *et al*., 1999) (Fig. 1.). Sequence data in the rDNA applied to question in the systematics, phylogeny and ecology of fungi.

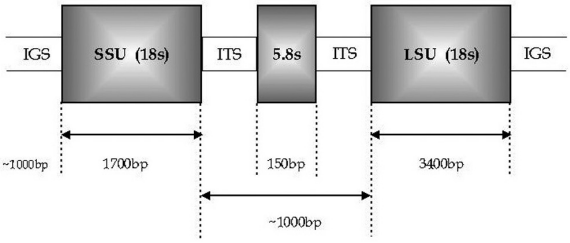

Fig. 1. Ribosomal DNA gene variability.
IGS; Intergenic spacer, ITS; Internal transcribed spacer, SSU; Small subunit, LSU; Large subunit.

The sequences of rDNA or its related informations made it easy to identify the AM fungal species (Egger, 1995; Bruns *et al*., 1993: Horton and Bruns, 2001). Much information about the sequences of rDNA with the specific name of species were accumulated in a gene bank of NCBI (http://www.ncbi.nlm.nih.gov), and also able to confirm the biological species with the specific primers. Several techniques of polymerase chain reaction (PCR)–randomly amplified polymorphism of DNA (RAPD) and PCR–restriction fragment length polymorphism (RFLP; Gardes and Bruns 1996; Pristsch *et al*., 1996; 1997) were developed for a last decade by using PCR with DNA polymerase™, including the analyzing the sequences of 18s rDNA by the universal primers (White *et al*., 1990; Gardes *et al*., 1991; Kikuchi *et al*., 2000).

This study was carried out to perform the identification of arbuscular mycorrhizal fungi which are contributed to the biomass production. Those approaches include thorough investigation of physiological and morphological characteristics and molecular biological techniques. Firstly various AM fungi in Chungbuk– and Chungnam– provinces are collected, and their identification was done by morphological and rDNA sequencing characteristics of spores of AM fungi by using designed the specific primers for AM fungi.

MATERIALS AND METHODS

Collection of plant roots and soils

Plant roots and soil samples were collected in general cultivation fields in the Chungbuk– (Table 1) and Chungnam–provinces (Table 2), middle parts of Korea. For the mycorrhizal survey 9 individual plant roots were randomly chosen at 27 sites in the two provinces. The samples were taken after removing the

Table 1. Collection of plants and its roots (Chungbuk Province)

Sample ID	Collection sites	Host plants
CB02009	Kyowon Univ.	*Fagopyrum esculentum*
CB02012	Sakyo village	*Platycodon grandiflorus*
CB01006	Kyowon Univ.	*Miscanthus sinensis*
CB02040	Songpyeung	*Sesamum indicum*
CB01007	Kyowon Univ.	*Capsicum annum*
CB02020	Boeun–Geosan	*Zea mays*
CB02015	Daesa–Mungyeong	*Capsicum annum*
CB03005	Kyowon Univ.	*Glycine max*
CB02001	Yongdong	*Capsicum annum*
CB02036	Danyang	*Glycine max*
CB02027	Chungju	*Fagopyrum esculentum*
CB02055	Mt. Wolak	*Allium fistulosum*
CB02043	Suanbo	*Glycine max*
CB02056	Mt. Wolak	*Glycine max*
CB02057	Mt. Wolak	*Capsicum annum*

Table 2. Collection of plants and its roots (Chungnam Province)

Sample ID	Collection sites	Host plants
CN02057	Taean, CN	*Capsicum annum*
CN02028	Taean, CN	*Capsicum anuum*
CN02032	March hill, CN	*Fagopyrum esculentum*
CN03025	Buyeo, CN	*Glycine max*
CN02048	Mt. Chilgap, CN	*Glycine max*
CN03001	March hill, CN	*Zea mays*
CN03029	Gongju, CN	*Artemisia princeps v. orientalis*
CN03022	Seochun, CN	*Ipomoea batatas*
CN03005	Daechun, CN	*Zea mays*
CN03002	Boryung, CN	*Artemisia princeps v. orientalis*
JB02020	Jangsu, JB	*Glycine max*
JN02018	Mt. Jiri, JN	*Sorghum bicolor*

top litter layer (5–10 cm) and digging out an appropriate amount of soil close to the roots of the host plant from a depth of 10–15 cm. The samples were collected in triplicate. Soil particles attached to fine feeder roots were removed by generous shaking. The soil samples brought to the laboratory were stored at 4–8 ˚C to sustain the viability of arbuscular mycorrhizal spores.

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 (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

In order to assess the colonization, fine roots of host plant were stained with acid fuchsin (Kormaik and McGraw, 1982). Colonization was determined by a grid intersect method (Giovannetti and Mosse, 1980). Colonized areas were classified by 5 different colonization rates, 0–20% = 1, 20–40% = 2, 40–60% = 3, 60–80% = 4 and 80–100% = 5 based on microscopical observation. After confirming under the light microscope the apparent purity of the cultures of the different AM morphotypes, these were identified to genus and, when possible, to species level. Criteria for morphologi-cal 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).

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 form 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

SSU rRNA 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 the DNA extract, 7 μl of nuclease free 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 (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 3 represents characteristics of used primers and PCR condition.

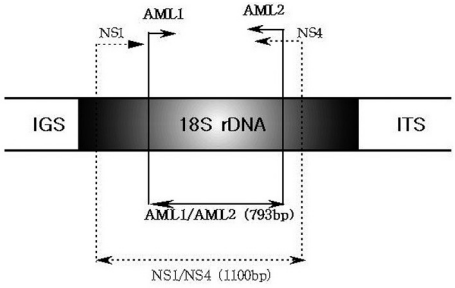

Fig. 2. 18S ribosomal DNA gene with annealing sites of primer pairs, NS1/NS4 and AML1/AML2 and their approximate DNA lengths (Lee, 2003).

Table 3. 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 cycles;
NS4	5'–TTCCGTCAATTCCTTTAAG–3'	48.3°C	95°C (30 sec), 40°C (1 min), 72°C (10 min) –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; 95°C (30 sec), 47°C (1 min), 72°C (1 min) –28 cycles; 95°C (30 sec),
AML2	5'–CCAAACACTTTGGTTTCC–3'	47.1°C	95°C (30 sec), 47°C (1 min), 72°C (10 min) –1 cycle

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

Sequence analysis

The amplication of insert DNA in plasmid was done by automatic sequencer ABIPRISMTM (Perkin–Elmer, USA at Eugenetech Co., Korea). DNA sequences were 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

Growth of Sorghum bicolor on various Chungbuk– and Chungnam– provinces soils

The Growth of *Sorghum bicolor* on various soils collected from Chungbuk– and Chungnam– provinces, weights of seedling, total weights, numbers of seedlings, and their average heights were measured as shown in Table 4. The high growth variability among sampling sites were shown from the data among host plants and sampling sites. Total weights were the highest from the soil growing *Glycine max* in Cheongwon site, and the next soil site growing pepper. In terms of weight growth

Table 4. Growth of *Sorghum bicolor* on various collected soils

Sites	Weight/1 seedling g	Total weight G	# of seedlings	Host plant	Height cm
CB02009	0.167	3.33	20	*Fagopyrum esculentum*	32.2
CB02012	0.210	1.89	9	*Platycodon grandiflorus*	28.5
CB01006	0.315	3.468	11	*Miscanthus sinensis*	26.4
CB02040	0.323	1.291	4	*Sesamum indicum*	25
CB01007	0.501	3.504	7	*Capsicum annuum*	24.7
CN03005	0.163	2.122	13	*Zea mays*	23.4
CB02015	0.114	1.367	12	*Capsicum annuum*	22.6
CN02048	0.366	2.193	6	*Glycine max*	22.2
CN02028	0.363	2.54	7	*Capsicum annuum*	22
CB02036	0.142	2.137	15	*Glycine max*	21.2
CB02027	0.106	1.061	10	*Fagopyrum esculentum*	21
CB02055	0.165	1.82	11	*Allium fistulosum*	20.7
CB02043	0.102	1.93	19	*Glycine max*	20.7
CB02056	0.182	2.372	13	*Glycine max*	20.5
CB02057	0.152	1.819	12	*Capsicum annuum*	20.5
CN02057	0.345	2.414	7	*Capsicum annuum*	20.4
CB02001	0.284	1.418	5	*Capsicum annuum*	20.2
CN02032	0.178	1.954	11	*Fagopyrum esculentum*	20.2
CN03025	0.257	2.314	9	*Glycine max*	18.9
CB03005	1.532	1.532	1	*Glycine max*	18.7
CN03001	0.161	1.611	10	*Zea mays*	17.4
CN03029	0.221	1.767	8	*Artemisia princeps v. orientalis*	17.3
CN03022	0.167	1.002	6	*Ipomoea batatas*	17.2
CB02020	0.259	2.072	8	*Zea mays*	17
CN03002	0.076	1.221	16	*Artemisia princeps v. orientalis*	17
JB02020	0.226	2.484	11	*Glycine max*	16.7
JN02018	0.346	1.039	3	*Sorghum bicolor*	16.5

of *Sorghum bicolor*, host plants were *Glycine max*, *Capsicum annuum*, *Sorghum bicolor*, *Sesamum indicum*, *Miscanthus sinensis*, and *Fagopyrum esculentum*. On the other hand, in terms of height growth of *Sorghum bicolor*, the soil in Cheongwon site (host plant *Fagopyrum esculentum*) was the best, and the order of hight growth *Platycodon grandiflorus*, *Miscanthus sinensis*, *Sesamum indicum*, and *Capsicum annuum*. It refers that AM fungi species are having host–specific differences. Several authors (Lopez–Sanchez and Honrubia, 1992; Michel–Rosales and Valdes, 1996; Helgason *et al*., 1999; 2002) reported that there was no correlation between the colonization of adjacent plants. Together with the fact that some AM fungi species show host–specific differences (Bever, 2002), the differences in pH are also known to have an impact on spore communities (Porter *et al*., 1987; Bever, 2002; Michel–Rosales and Valdes, 1996) but not on colonization. This result supports the potentiality of AM inoculation for many crops in Korea of current agronomic interest.

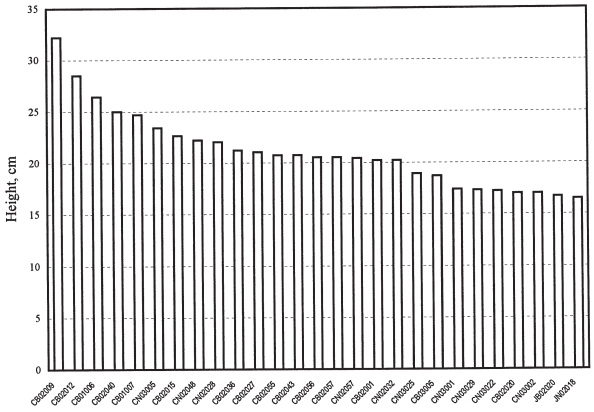

Fig. 3. Growth of *Sorghum bicolor* on various collected soils.

AM fungi colonization

AM fungi colonization of host plant, *Sorghum bicolor*, was measured as shown in Fig. 4. There was some increasing colonization tendency with the increase of culture periods. At the initial culture stage, colonization upto 10 days was rather slow, and afterwards the rates were getting so fast. This colonization increase has leveled off more than 40 days culture. In addition, AM fungi colonization of host plant, *Sorghum bicolor*, varied widely, both within samples taken at different sites and between individuals.

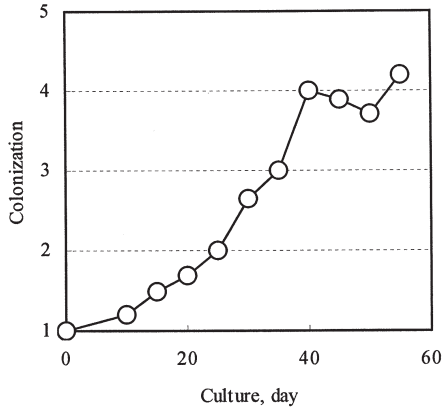

Fig. 4. Colonization index of infected *Sorghum bicolor* roots.

AM fungi colonization in the roots were observed by Microscope as in Fig. 5. The first site, 2nd, 3rd and 5th sites are from Cheongwon, Chungbuk province, and 1st, 2nd and 26th (Jangsu, Chonbuk) sites showed typical vesicle in a root cell, but no in 3rd and 5th sites, and only aseptate hyphal coils in root tissue cells were observed. Moreover 3rd and 21st sites showed typical arbuscules. Gongju site (22nd) in Chungnam–province was shown very specific clusters of AM fungi spores.

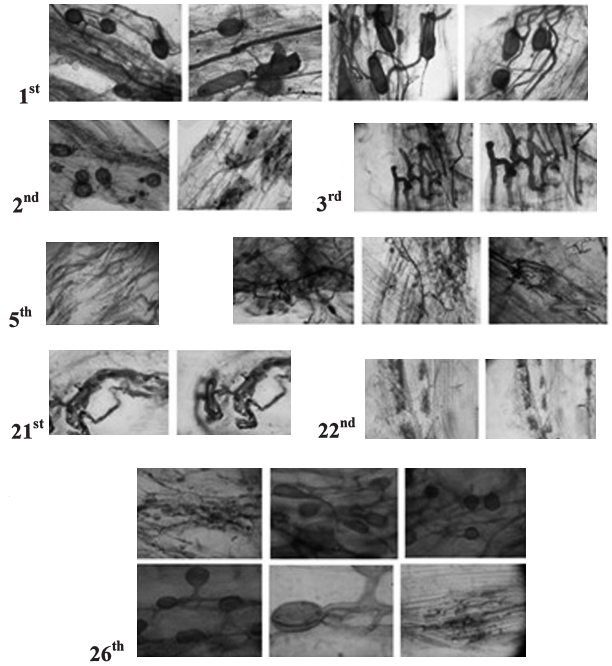

Fig. 5. Microscopic observaions of intracellular cells in the cortical layers of plants collected from Chungbuk– and Chungnam– provinces.
Sites 1st: Cheongwon1; 2nd: Cheongwon2; 3rd: Cheongwon3; 5th: Cheongwon4; 9th: Taean; 21st: March Hill; 22nd: Gongju; 26th: Jangsu

Species diversity

Morphogical identification based on spores

The fungi that form AM are currently all classified in the order Glomales (Morton, 1988). The taxonomy is further divided into suborders based on the presence of: (i) vesicles in the root and formation of chlamydospores (thick wall, asexual spore) borne from subtending hyphae for the suborder Glomineae or (ii) absence of vesicles in the root and formation of auxiliary cells and azygospores (spores resembling a zygospore but developing asexually from a subtending hypha resulting in a distinct bulbous attachment) in the soil for the suborder Gigasporineae. The term vesicular–arbuscular mycorrhiza (VAM) was originally applied to symbiotic associations 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 order Glomales is further divided into families and genera according to the method of spore formation. The spores of AM fungi are very distinctive. They range in diameter from 10 mm for *Glomus tenue* to more than 1,000 mm for some *Scutellospora* spp. The spores can vary in color from hyaline (clear) to black and in surface texture from smooth to highly ornamented. *Glomus* forms spores on the ends of hyphae, *Acaulospora* forms spores laterally from the neck of a swollen hyphal terminus, and *Entrophospora* forms spores within the neck of the hyphal terminus. The Gigasporineae are divided into two genera based upon the presence of inner membranous walls and a germination shield (wall structure from which the germ tube can arise) for *Scutellospora* or the absence of these structures for *Gigaspora*.

The diagnostic feature of arbuscular mycorrhizae (AM) 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. The general term for all mycorrhizal types where the fungus grows within cortical cells is endomycorrhiza. In this association neither the fungal cell wall nor the host cell membrane are breached. 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.

Other structures produced by some AM fungi include vesicles, auxiliary cells, and asexual spores. Vesicles are thin–walled, lipid–filled structures that usually form in intercellular spaces. Reproductive spores can be formed either in the root or more commonly in the soil. Spores produced by fungi forming AM associations are asexual, forming by the differentiation of vegetative hyphae. For some fungi (*e.g.*, *Glomus intraradices*), vesicles in the root undergo secondary thickening, and a septum (cross wall) is laid down across the hyphal attachment leading to spore formation, but more often spores develop in the soil from hyphal swellings.

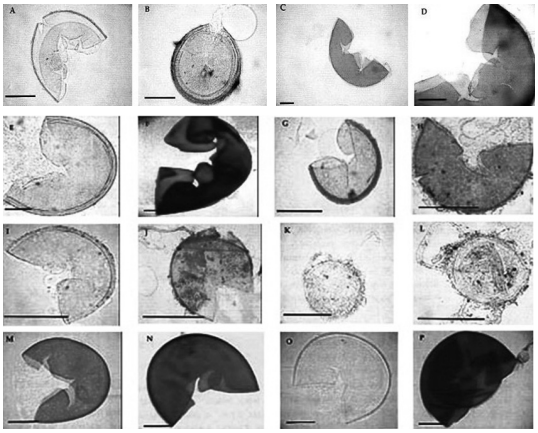

Fig. 6. Identified AMF spore based on morphological features (Scale bar 50 μm)
A: *Acaulospora longula*; B: *Achaeospora leptoticha*; C: *Gigaspora gigantea*; D: *Gigaspora gigantea*; E: *Gigaspora margarita*; F: *Gigaspora margarita*; G: *Glomus etunicatum*; H: *Glomus etunicatum*; I: *Glomus lamellosum*; J: *Glomus mosseae*; K: *Paraglomus occultum*; L: *Paraglomus* sp.; M: *Scutellospora castanea*; N: *Scutellospora castanea*; O: *Scutellospora weresubiae*; P: *Scutellospora weresubia.*

AM fungi spores were obtained from soil samples. As shown in Fig. 6, twelve morphological species were recorded as spores in the soil samples. Within the AM fungi spores, about 18% were with diameter less than 80–100 μm diameter range, 18% in the 100–150 μm, 19% in the 150–200 μm and 45% larger than 200 μm. Some spores were identifiable to species level through their morphological characters (Fig. 6). Among these, *Acaulospora longula*, *Acaulospora leptoticha*, *Glomus etunicatum* and *Gigaspora margarita* were the most commonly encountered species. *Acaulospora longula* was the species most frequently found in the soil samples. Six genera of AM fungi were identified, among which *Acaulospora*, *Gigaspora* and *Glomus* were dominant with frequencies of 57%, 20 and 16%, respectively. The frequencies of AMF from the other three genera were low.

Molecular characterization (DNA sequencing)

The molecular characterization of AM fungi has been a complicated task in the past because of the considerable degree of variation detected among the copies of ribosomal DNA within single spores, which brought into question the validity of these methods (Calvente *et al.*, 2004). However, recent reports indicated that SSU rRNA sequence analysis is a suitable tool to infer phylogenetic relationships among AM fungi since the expected sequence difference could be more easily linked to a taxonomic entity than the possibly multi–allelic ITS sequences (Schwarzott and Schüßler, 2001).

Both morphology–based and novel molecular approaches of the spores from host plant root–associated soils were applied to characterize the AM fungi spores isolated from root–associated soil. This is the first step to analyse the diversity of AM fungi in the target fields of Chungbuk– and Chungnam– provinces, Korea. By following these approaches, only four distinguishable spore morpho–types, six genera of AM fungi, were found. In Chungbuk province, total 9 species of 6 genera, such as 3 species of *Glomus*, 1 species of *Paraglomus*, 1 species of *Gigaspora*, 2 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were identified. Based on further corroborated by molecular techniques, the six AM fungi morphotypes were genetically characterised by PCR amplification and sequence analysis of a portion of their SSU rRNA to verify their identification.

As shown in Table 5, the accession number for the new sequences, as deposited in the EMBL database are as follows: *Glomus mosseae* (AJ306438), *Paraglomus*

Table 5. BLAST search on NCBI with analyzed sequences for Identification of AMF (Chungbuk–province)

Isolate	Morphological Identification	Results of BLAST search on NCBI		
		Species	Accession number	Sequence similarity (%)
CB01005–1	*Glomus mosseae*	*Glomus mosseae*	AJ306438	713/717 (99%)
CB01005–2	*Glomus mosseae*	*Glomus mosseae*	AJ306438	748/755 (99%)
CB01005–4	*Glomus mosseae*	*Glomus. mosseae*	AJ306438	635/650 (97%)
CB01006–5	*Paraglomus occultum*	*Paraglomus occultum*	AJ276081	743/751 (98%)
CB02009–4	*Paraglomus occultum*	*Paraglomus occultum*	AJ276081	693/711 (97%)
CB01006–7	*Glomus etunicatum*	*Glomus etunicatum*	Y17644	759/763 (99%)
CB02015–2	*Glomus etunicatum*	*Glomus etunicatum*	Y17639	681/686 (99%)
CB02036–3	*Glomus etunicatum*	*Glomus etunicatum*	Y17644	724/725 (99%)
CB01006–9	*Gigaspora margarita*	*Gigaspora margarita*	AJ567844	332/356 (93%)
CB02020–4	*Gigaspora margarita*	*Gigaspora margarita*	AJ567844	450/471 (95%)
CB01007–2	*Scutellospora castanea*	*Scutellospora castanea*	AJ418851	220/230 (95%)
CB01007–3	*Archaeospora leptoticha*	*Archaeospora leptoticha*	AB047306	708/720 (98%)
CB02012–1	*Scutellospora calospora*	*Acaulospora longula*	AJ306439	731/736 (99%)
CB02020–5	*Scutellospora weresubiae*	*Scutellospora weresubiae*	AJ306444	701/724 (96%)
CB02040–2	*Glomus lamellosum*	*Glomus etunicatum*	Y17639	743/747 (99%)
CB02040–3	*Scutellospora heterogama*	*Glomus lamellosum*	AJ276087	744/749 (99%)

Table 6. BLAST search on NCBI with analyzed sequences for Identification of AMF (Chungnam–province)

Isolate	Morphological Identification	Results of BLAST search on NCBI		
		Species	Accession number	Sequence similarity (%)
CN–02–032–3	*Gigaspora margarita*	*Gigaspora margarita*	AJ567844	450/471 (95%)
CN–02–048–1	*Gigaspora gigantean*	*Gigaspora gigantea*	Z14010	392/399 (98%)
CN–03–025–1	*Glomus etunicatum*	*Glomus etunicatum*	Y17639	211/213 (99%)
CN–02–028–1	*Acaulospora longula*	*Acaulospora longula*	AJ306439	321/332 (96%)
CN–02–028–2	*Glomus etunicatum*	*Glomus etunicatum*	Y17644	276/286 (96%)
CN–02–057–1	*Acaulospora longula*	*Acaulospora longula*	AJ306439	514/528 (97%)
CN–03–005–1	*Gigaspora gigantean*	*Gigaspora gigantea*	Z14010	245/252 (97%)
CN–03–002–1	*Gigaspora gigantean*	*Gigaspora gigantea*	Z4010	571/576 (99%)
CN–03–002–5	*Archaeospora leptoticha*	*Archaeospora leptoticha*	AB047308	697/712 (97%)
CN–03–005–3	*Archaeospora leptoticha*	*Archaeospora leptoticha*	AB047306	708/720 (98%)
CN–02–057–3	*Scutellospora weresubiea*	*Scutellospora weresubiea*	AJ306444	679/684 (99%)

occultum (AJ276081), *Glomus etunicatum* (Y17644), *Glomus etunicatum* (Y17639), *Gigaspora Margarit* a(AJ567844), *Scutellospora castanea* (AJ418851), *Acaulospora leptoticha* (AB047306), *Acaulospora longula* (AJ306439), *Scutellospora weresubiae* (AJ306444) and *Glumus lamellosum* (AJ276087). Comparisons of the obtained sequences with those present in the database revealed that they show more than 97–99% identity to the SSU rRNA gene of different AM fungal isolates. Only three *Scutellospora calospora*, *Glumus lamellosum*, and *Scutellospora heterogama* did not matched with BLAST search results.

In Chungnam province, total 6 species of 5 genera, such as 2 species of *Gigaspora*, 1 species of *Glomus*, 1 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were identified. As shown in Table 6, the accession number for the new sequences, as deposited in the EMBL database are as follows: *Glomus mosseae* (AJ306438), *Paraglomus occultum* (AJ276081), *Glomus etunicatum* (Y17644), *Glomus etunicatum* (Y17639), *Gigaspora margarita* (AJ567844), *Scutellospora castanea* (AJ418851), *Archaeospora leptoticha* (AB047306), *Acaulospora longula* (AJ306439), *Scutellospora weresubiae* (AJ306444) and *Glomus lamellosum* (AJ276087). Comparisons of the obtained sequences with those present in the database revealed that they show more than 97–99% identity to the SSU rRNA gene of different AM fungal isolates. Only three *Scutellospora calospora*, *Glomus lamellosum*, and *Scutellospora heterogama* did not matched with BLAST search results.

CONCLUSIONS

This study was carried out to perform the collection of AM fungi from various sites of Chungbuk– and Chungnam– provinces in Korea, and identification of arbuscular mycorrhizal fungi which are associated to symbiotic relation with plants including crops. Those approaches include thorough investigation of physiological and morphological characteristics and molecular biological techniques.

In terms of height growth of *Sorghum bicolor*, the soil in Cheongwon site (host plant *Fagopyrum esculen-*

tum) resulted in the best growth, and the order of height growth was *Platycodon grandiflorus*, *Miscanthus sinensis*, *Sesamum indicum*, and *Capsicum annuum*. It refers that AM fungi species are having host–specific differences. Concerned to AM fungi colonization of host plant, *Sorghum bicolor*, AM fungi colonization in the roots were observed by Microscope, and there was some increasing colonization tendency with the increase of culture periods. At the initial culture stage, colonization upto 10 days was rather slow, and afterwards the rates were increased. This colonization increase has leveled off more than 40 days culture.

Both morphology–based and molecular approaches of the spores from various soils of Chungbuk– and Chungnam– provinces in Korea were applied to characterize the AM fungi. By following these approaches, in Chungbuk province, total 9 species of 6 genera, such as 3 species of *Glomus*, 1 species of *Paraglomus*, 1 species of *Gigaspora*, 2 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were identified. In Chungnam province, total 6 species of 5 genera, such as 2 species of *Gigaspora*, 1 species of *Glomus*, 1 species of *Scutellospora*, 1 species of *Acaulospora*, and 1 species of *Archaeospora*, were confirmed.

In conclusion, this study emphasizes the importance of exploring and exploiting the natural diversity of AM fungi, particulary in Chungbuk– and Chungnam–provinces, Korea, as a starting point to formulate inoculants to be applied for the production of appropriate plant varieties with optimized quality.

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