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Identification of Arbuscular Mycorrhizal Fungi from *Botrychium ternatum* Native in Korea

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Arbuscular mycorrhizal fungi were observed in *Botrychium ternatum* native in Korea. The partial small subunit (SSU) of ribosomal DNA gene from the fern roots was amplified with primers AM1/NS31. Nucleotides sequence analysis of the clones revealed that two fragments were close to *Glomus proliferum* and *G. sinuosum*. The other three DNA fragments were close to those of *G. proliferum* with the relatively low similarities (92~95%) and speculated to be originated from three different species of *Glomus* (GLA006, GLA016, and GLA032). Five different nucleotide sequences close to three AM fungal species were found in the roots of *B. ternatum* native in Korea.

KEYWORDS: ***Botrychium ternatum*, Fern, *Glomus*, Mycorrhiza, PCR, Symbiosis**

Botrychium ternatum (grape fern) has been reported to be different from other ferns (mainly, Leptosporangiate fern), having fertile shoot (Eusporangiate ferns; Ophioglossaceae). It was also called as "mycorrhizal fern" because of its distinctive root features. This plant was observed to grow during the winter and spring, as producing the young sterile shoots and, after then, to produce the fertile shoot during late spring. It was reported that the life cycle of *B. ternatum* was similar to those of lily or orchids (Schmid and Oberwinkler, 1993; Kelly, 1994). Two kinds of mycorrhizal types, orchid and arbuscular mycorrhizae, were also found in the roots of *B. ternatum* collected from several sites in Korea (Lee *et al.*, 2001). These facts indicate that mycorrhizae might play important roles in growth and life cycle of *B. ternatum*.

In fern and its relatives, it was difficult to understand evolutional relationships between two species of fern, because any differences between them were not found in the stage of gametophyte or sporophytes. Previous studies found that symbiotic relationship between fern and fungi played an important role in the growth of fern (Gemma *et al.*, 1992; Zhao, 2000; Lee *et al.*, 2001). Boullard (1957) found that many species among 420 species of fern collected had symbiotic relationship with fungi, and concluded that advanced ferns have less symbiotic relationship with fungi than lower ferns. Also, Kelly (1994) investigated the presence of mycorrhizal relationships in the roots of 101 species of fern native in New Zealand, and concluded that his observation was consistent with Boullard's conclusion. The eusporangiate fern, especially grape ferns (*B. lunaria* and *Ophioglossum reticulatum*), was suspicious of being symbiotic with the AM fungi by morphological study (Schmid and Oberwinkler, 1995, 1996), but it has not been confirmed by the molecular techniques until recently (Helgason *et al.*, 1998, 1999; Daniell *et al.*, 2001). Further, it was not clearly demonstrated what species of AM fungi were involved in the roots of *Botrychium* species. In attempt to try and elucidate these problems, we investigated the grape fern native in the areas of Chungbuk, in particular; i) to clone DNA fragments synthesized by specific primers, ii) to display the RFLP pattern of them, and iii) to analyze the DNA sequences of them.

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Materials and Methods

Plants. *B. ternatum* were collected from various habitats, two sites of Cheongwon, and one site of Mt. Worak in Chungbuk, from April 1998 to September 2000. The plant specimens were carefully preserved and deposited in the herbarium of Korea National University of Education. The plants used in this study were identified with a manual (Park, 1975) and confirmed with the specimens previously collected in the herbarium of Seoul National University. Roots (rhizomes) of the plant collected were carefully cut from the plants and stored in the polyethylene bags at 4°C.

DNA extraction. Roots were ground with liquid nitrogen and mixed with a 650 μl lysis buffer [50 mM Tris-HCl (pH 7.5), 30 mM EDTA, 3% SDS and 1% 2-mercaptoethanol; Brunel 1992]. This mixture was then incubated at 65°C for 1 h, and centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a new microtube and mixed with an equal volume of PCI solution (24 : 25 : 1, TE-saturated phenol: chloroform: isoamylalcohol; Brunel, 1992). After a brief vortexing for 1 or 2 min, the mixture was centrifuged at 12,000 rpm for

5 min at 4°C. The aqueous phase was transferred to a new microtube, and DNA was pelleted by isopropanol precipitation. This pellet was suspended in 500 μl of TEN buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl), then added to 50 $\mu g/\mu l$ RNase A and incubated at 37°C for 30 min. The suspension was treated again with PCI, and the DNA was pelleted by ethanol precipitation. This DNA pellet was re-suspended in 50 μl of TE buffer, and the concentration of DNA was estimated by absorbance at 260 nm.

DNA amplification (PCR). Partial fungal small-subunit ribosomal DNA fragments were amplified by PCR using both a universal eukaryotic primer NS31 (5'-TTG GAG GGC AAG TCT GGT GCC-3'; Simon *et al.*, 1992) and general fungal primer AM1 (5'-GTT TCC CGT AAG GCG CCG AA-3'; Helgason *et al.*, 1998) that were designed to exclude plant DNA sequences. For amplification, 10 ng of the genomic DNA previously extracted was added to an *Accupower* PCR PreMix™ Kit (Bioneer Co., Korea) with 10 pmole of each primer. PCR was carried out for 30 cycles (10 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, 19 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 3 min, and 1 cycle at 95°C for 30 s, 58°C for 1 min, and 72°C for 10 min) on Personal Cycler™ (Biometra Co.). DNA amplified by PCR were displayed on the 1.2% agarose gels, stained with ethidium bromide, and photographed under UV-illumination.

Cloning, RFLP and sequencing. PCR products were separated on an agarose gel, and approximately 550 bp band was eluted by *AccuPrep*™ gel purification kit (Bioneer Co.). After purification, the PCR-products were cloned into the pGEM-T easy Vector™ (Promega, USA) and transformed into *Escherichia coli* JM109. The positive transformed cells were screened by X-gal and plasmids including insert-DNA were excluded from the cell using *AccuPrep*™ plasmid extraction kit (Bioneer Co.). These

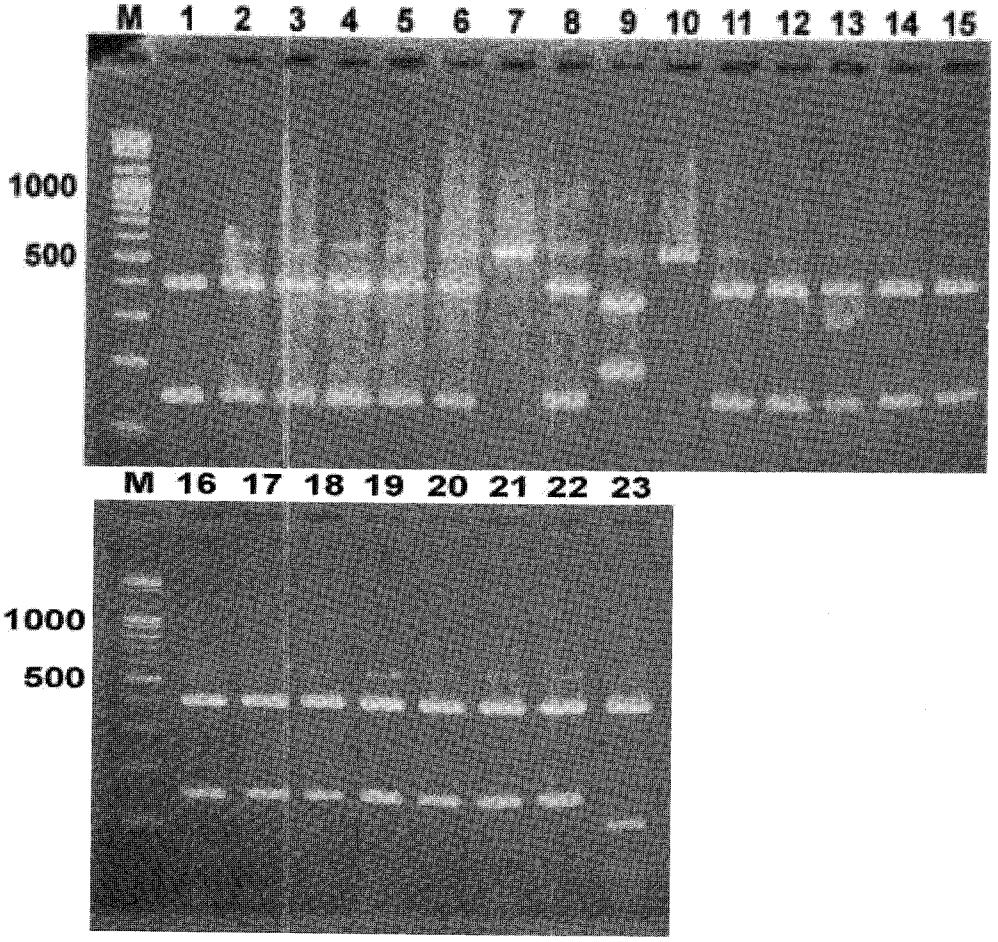

Fig. 1. Restriction fragment patterns of PCR products of 18s rDNA fragment made of the plasmid (pGEM-T Easy Vector™). PCR products of insert-DNA were digested with the enzyme *Hinf*I and run on 4% agarose gel. Lane M, molecular size marker (100 bp ladder); The 23 lanes of cloned PCR product were selected from 32 colonies cloned and grouped into 5 RFLP patterns. Five clones of DNA's shown in the lanes, 3 (GLA006), 7 (GLA011), 9 (GLA014), 11 (GLA016) and 23 (GLA032) were selected as representatives of each RFLP group for sequence analysis.

plasmids were amplified using the AM1/NS31 primer pair. Then, the amplified DNA was digested with restriction enzymes *Hinf*I and *Alu*I, according to manufacturer's manual. The restriction fragment length polymorphism (RFLP) pattern present in each clone was compared and clones were grouped into same RFLP pattern. Then, one clone selected from each RFLP group was sequenced on ABIPRISM™377 (Perkin-Elmer, USA).

Data analysis. The sequences were aligned with GENEDOC program (http://www.psc.edu/biomed/genedoc) and CLUSTAL X 1.81 (Thompson *et al.*, 1997) was used for multiple alignment and neighbor-joining phylogeny (Saitou and Nei, 1987), using *Geosiphon pyriforme* as an outgroup of order *Glomales* (Gehrig *et al.*, 1996).

Microscopy observation. Roots were washed with tap water and placed in 100 bottle containing formaldehyde-acetic acid-alcohol (FAA) solution. Then, the roots were cut into 1 to 3 cm section and stained with trypan blue (Koske and Gemma, 1989). Some roots of *B. ternatum* were cross-sectioned by freezing microtome and stained

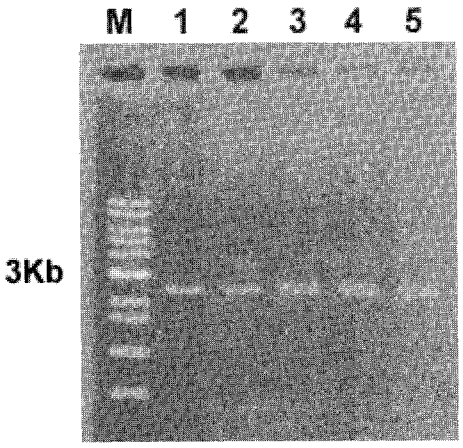

Fig. 2. The confirmation of the plasmid inserted with the fragment of 18s rDNA's amplified with the specific primers. Lane M, molecular weight marker (100-bp ladder); Lane 1 were corresponded with the lane of 3 in Fig. 1 and to the colony GLA006; Lane 2 with lane 7 in Fig. 1 and to the colony GLA011; Lane 3 with 9 in Fig. 1 and to the colony GLA014; Lane 4 with 11 in Fig. 1 and to the colony GLA016 and Lane 5 with 23 in Fig. 1 and to the colony GLA032, respectively.

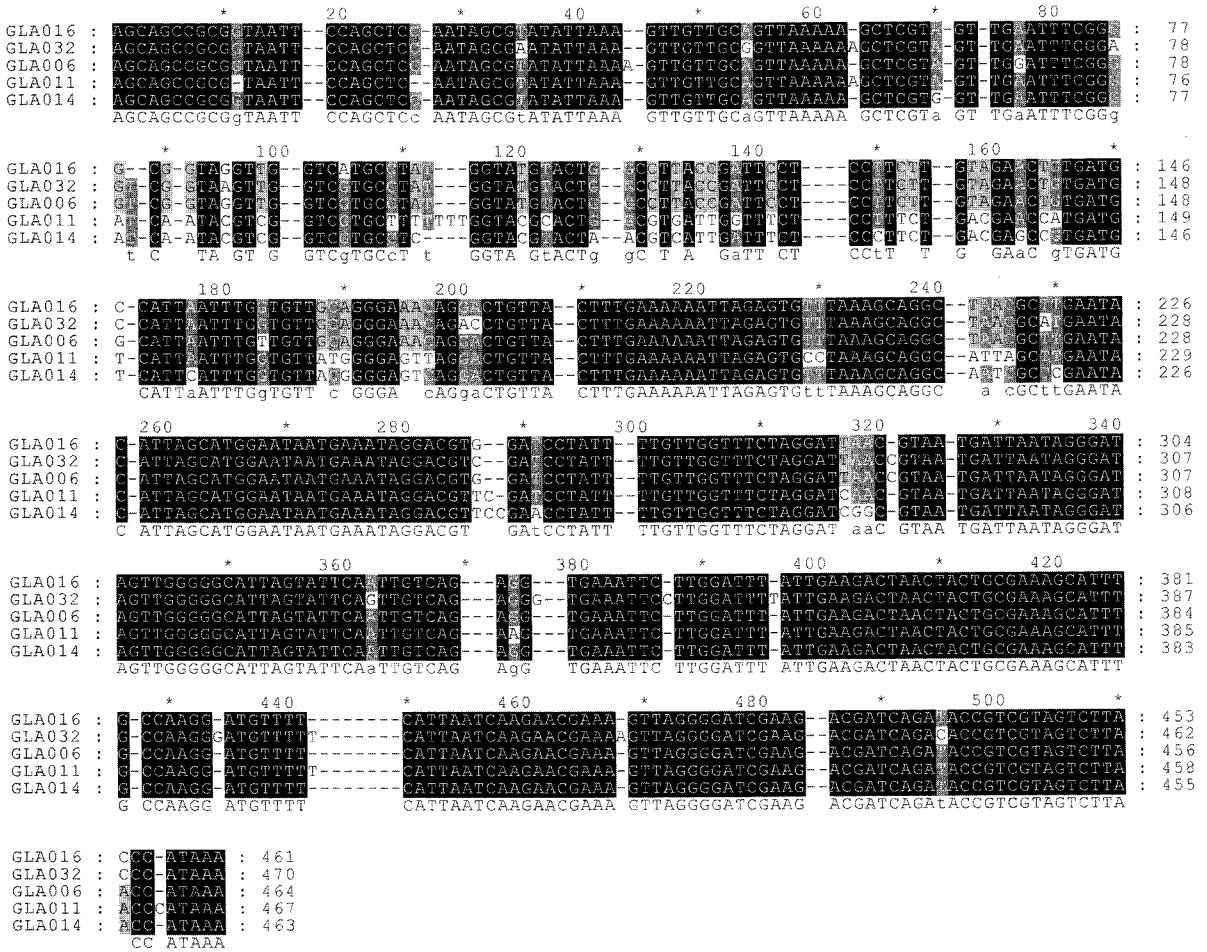

Fig. 3. Sequence alignment of partial SSU ribosomal DNA (18s rDNA) regions of the clones isolated from the roots of *Botrychium ternatum* collected in Korea. All of these clones were sequenced in this study.

with same staining solution. The stained mycorrhizal roots from the plant of *B. ternatum* were observed under a light microscope. For SEM (Scanning electron microscope), roots were cut to 3 mm length in 100mM phosphate buffer (pH 6.8), transferred to phosphate buffer containing 2.5% glutaraldehyde, and incubated at 23°C for 2 h. Roots were then rinsed three times with phosphate buffer. Roots were frozen in liquid nitrogen and quickly fractured. Roots were incubated in phosphate buffer containing 2% OsO_4 for 2 h (for post fixation) and rinsed three times with phosphate buffer. After post-fixation, dehydration was conducted in a graded ethanol series from 70%

Table 1. The DNA sequences producing significant alignments from the NCBI and fungal species similar to AM fungal isolates with matching rates.

Isolates[a]	Accession Number of sequences in NCBI	Fungal species	Sequence similarity
GLA006	AF213462	*Glomus proliferum*	378/400
GLA011	AJ133706	*Glomus sinuosum*	367/382
GLA014	AJ133706	*Glomus sinuosum*	357/365
GLA016	AF213462	*Glomus proliferum*	487/507
GLA032	AF213462	*Glomus proliferum*	455/496

[a]DNA lines of AM fungi obtained from the roots of Korean Grape fern (*Botrychium ternatum*).

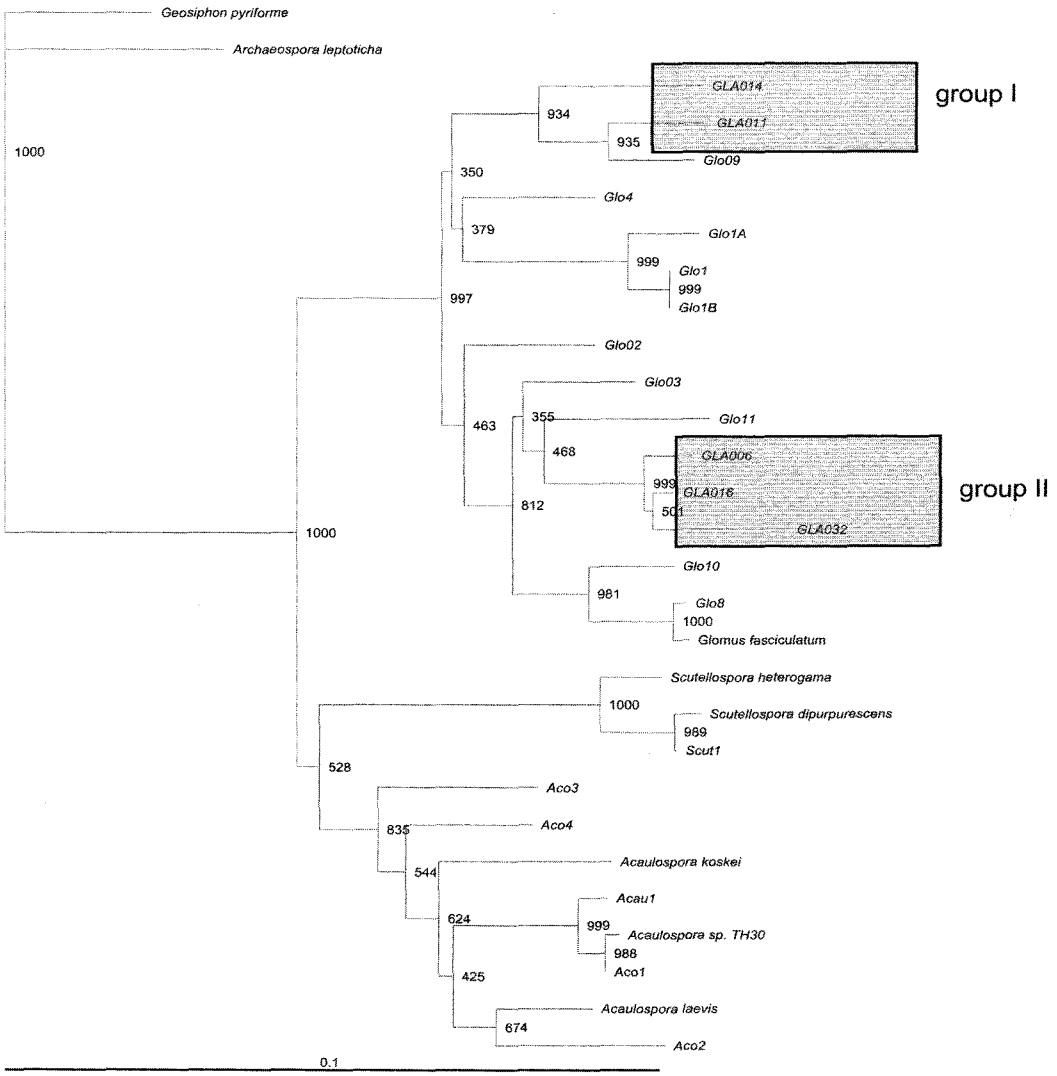

Fig. 4. Neighbor-joining tree showing the relationships of the fungi identified in *B. ternatum* roots and the other fungi. Various bootstrap values were shown (1000 replicates) on branches. Gray colored boxes indicate groups of the colonies isolated from the roots of *B. ternatum* in this study, group I (similar to the sequence of *G. sinuosum* in NCBI) and group II (similar to *G. proliferum*). Glo1 (accession number AF074358 in NCBI), Glo2 (AF131045), Glo3 (AF131049), Glo4 (AJ309460), Glo8 (AJ309462), Glo9 (AF074356), Glo10 (AJ309410), Glo11 (AF131053), Glo1A (AJ309414), Glo1B (AJ309438), *Glomus fasciculatum* (AF231760), Aco1 (AF131036), Aco2 (AF074346), Aco3 (AF074349), Aco4 (AF074351), Acau1 (AJ309439), TH30 (AF074372), *Acaulospora koskei* (AF231762), *Acaulospora laevis* (AF074347), Scut1 (*Scutellospora* sp.; AF131022), *Scutellospora dipurpurenscens* (AF131027), *Geosiphon pyriforme* (X86686), and *Archaeospora leptoticha* (AJ301861).

to 100%. After dehydration, samples were dried in a Polaron E 3000 critical point dryer using CO_2 as the carrier gas. The materials were mounted on double-sided sticky tape on stub, gold-palladium, and viewed with a LEO 1420 (Karl-Zeiss) scanning electron microscope.

Results

AM fungi. AM1/NS31 primers were reacted with the genomic DNA extracted from the roots of *B. ternatum*. The DNA fragments of the approximately 550 bp from the reaction were confirmed separated on an agarose gel. The purified DNA fragment was inserted into pGEM-T easy Vector™ and transformed into *E. coli* JM109. Then, the transformed cells positive in X-gal and resistant on the ampicillin LB agar were screened and stored in E-tube until next step. The 32 transformed cells of *E. coli* JM 109 were isolated, marked as numbers of GLA-series for

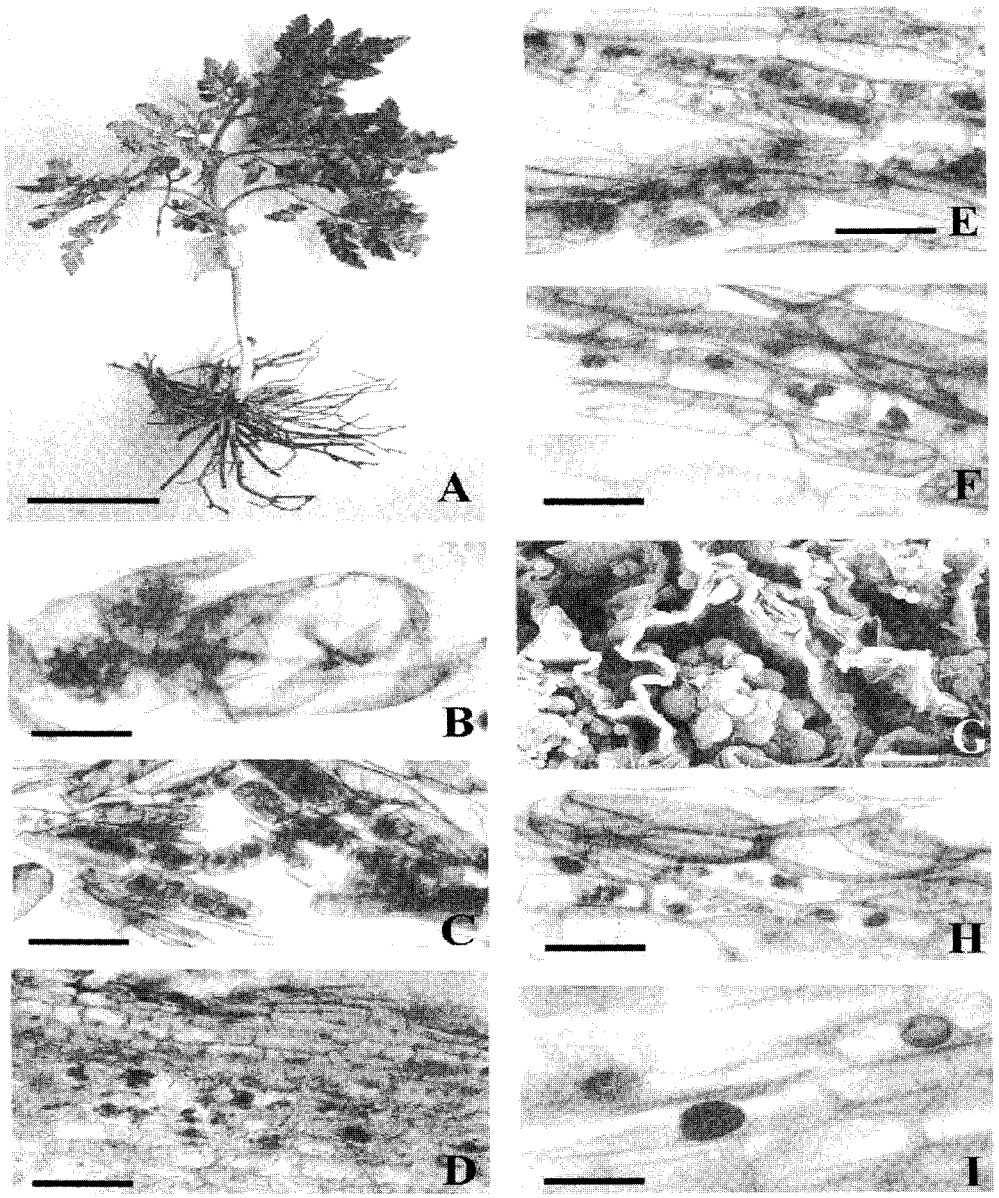

Fig. 5. The mature sporophyte of *B. ternatum* collected in Korea (A) and its unique type mycorrhizas (B to I): A; Sporophyte of *B. ternatum* (chlorophyllous diploid stage; but the fertile frond is not mature yet). There are no root hairs (strongly mycorrhizal). B, C, D, E, F, H and I; Light microscopic images of the cortical cells of the *B. ternatum* roots and its distinctive form mycorrhizae; B and H; arbuscules in cortical cells. C and E; ellipsoidal form cortical cells, netted and coiling hyphae. D; well-development of mycorrhizae (dark part) in the cortex layer and thin-epidermis of the root of *B. ternatum*. F; degenerated arbuscules and netted hypae in the old roots. G; Scanning electron microscopic image of arbuscule structure in the *B. ternatum* root and terminal hyphal swollen tips in haustorium. Scale bar: A = 7 cm; C, D = 200 μm; E, F = 100 μm; B, H, I = 50 μm, and G = 500 μm.

further works, and incubated in the culture broth. The plasmids of pGEM-T with 550 bp of DNA fragment were extracted and these plasmids were amplified using fungal specific primer pair AM1/NS31 and reconfirmed. Finally, 23 cloned DNA fragments were selected and displayed on agarose gel (Fig. 1) after the DNA were reacted with the restriction enzymes, *Hinf*I. RFLP pattern present in each clone was compared and clones were grouped into same RFLP pattern (Fig. 1). The five clones were selected as based on RFLP pattern and the plasmids were re-amplified (ca 2,800 bps, Fig. 2); GLA-006, GLA-011, GLA-014, GLA-016, and GLA-032. Then, these clones were purified and sequenced.

DNA sequences. The DNA fragments amplified with AM1/NS31 primers were sequenced as the pair bases of 461 to 470 (Fig. 3). The arrangements of DNA sequences were aligned with forward sequences by GENEDOC program and CLUSTAL X. The partial sequences showing the dark color were indicated to be homolog, but those showing the grey color to be partially heterogeneous at the rate of 30~40 of 470 bps in Fig. 3. The DNA sequences of these DNA fragments were blasted to the gene bank of NCBI and compared with other DNA sequences stored in NCBI. After blasting sequences of the DNA fragment from this study with the genes in NCBI, five genes were listed with rate matched DNA sequences in Table 1. The DNA fragment of GLA-006 were similar to sequences of *Glomus proliferum* in NCBI at matching rate of .947, and were grouped to those of GLA-016 (.961) and GLA-032 (.917). Also, the DNA fragment of GLA-011 was similar to that of *G. sinuosum* at the matching rate of 0.961, and was grouped to that of GLA-014. All DNA fragments in this study were belong to those of *Glomus*, and divided into two groups, *G. sinuosum* and *G. proliferum*. The 24 DNA sequences of AM and its related fungi listed in the gene bank of NCBI (http://www.ncbi.nlm.nih.gov/) were used to compare with 5 cloned fragments in this study (Table 1 and Fig. 4). The DNA fragments of cloned AM fungi in this study were clustered in the grey box indicated as group I and II in Fig. 4 and arranged to be the result similar to those of Table 1.

The morphological feature of *B. ternatum* was shown in Fig. 5A, which was collected from the site of Cheongwon, Chungbuk in Korea. The yellow fertile shoot would be spouted directly from the roots, one or one and half month later. The microscopic observations were selected only from AM relationship in Fig. 5, and arranged at the different resolution; arbuscular haustorium (Figs. 5B, 5C and 5D), intra-hyphae (Figs. 5E and 5F) and vesicular mass (Figs. 5H and 5I) in the cortical cells. Particularly, the swollen and inflated tips in branches of haustoria were found in the cortical cells under scanning electron microscope (Fig. 5G) and rarely observed in other plants colonized with AM fungi.

Discussion

Identification. The sequences of DNA amplified with AM1/NS31 primers from the roots of *B. ternatum* were similar to those of 18s rDNA from AM fungi (Simon *et al.*, 1992; Simon, 1996; Helgason *et al.*, 1998, 1999). In other words, DNA sequences analyzed in this study were, at more than 92% rates, matched with those of AM fungi (Fig. 3). Also, these sequences were related to those of 18s rDNA originated from the species of *Glomus*, especially similar to those from *G. proliferum* and *G. sinuosum* (Table 1). Five DNA groups in the RFLP pattern by the restriction enzyme of *Hinf*I (Fig. 1) were divided into two groups in sequence analysis (Fig. 4) and in sequence alignments (Table 1). This indicates that the result shown in RFLP pattern by restriction enzymes was not consistent with those of sequence analysis. Three DNA fragments identified as *G. proliferum* in this study would be due to a little information in gene bank of NCBI. *G. proliferum* has not been reported in Korea. The AM fungal spores of *G. sinuosum* were reported to be associated with the roots of legume plants in the central Korea (Eom *et al.*, 1992). The three DNA fragments were aligned with partial sequences of *G. proliferum* at relatively low rate, as compared with those of *G. sinuosum* and they would be originated from the 18s rDNA different from those of *G. proliferum*, but similar to those of *G. proliferum*.

Molecular interpretation. Several studies indicated that various fern plants might be symbiotic with the AM fungi (Gemma *et al.*, 1992; Schmid and Oberwinkler, 1996; Zhao, 2000). Particularly, mycorrhizal formation was focused on the roots of Ophioglossaceaeous fern, because their distinctive roots were similar to those of lily or orchid plants. Also, swollen and inflated tips in branches of haustoria (arbuscules) found in the cortical cells (Fig. 5G) were suspected to be a new type of mycorrhizae (Schmid and Oberwinkler, 1996; Zhao, 2000). However, Lee (2002) found that they could be a kind of AM fungi. On the basis of analysis of DNA sequences, five different AM fungi were found in the roots of *B. ternatum* plants and would be three or four different species of *Glomus*. The GLA006 similar to *G. proliferum* were pre-dominant in the roots of *B. ternatum* (at rate of 19/23 in RFLP pattern in Fig. 1). An ophioglossaceaeous fern was reported to have strongly mycorrhizal relationship during their whole life cycle. Their sporophytes were reported to have two kinds of mycorrhizae; orchid and arbuscular mycorrhizae (Schmid and Oberwinkler, 1996; Lee *et al.*, 2001, 2002). The most gametophytes of ferns (achlorophyllous prothallus) would be associated with symbiotic fungi

because its achlorophyllous prothallus didn't have any energy sources. Therefore, the question about which fungus stimulates to the germinations of fern spores and help the gametophyte grow was posed on our minds. This study might give us some clues for this question.

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