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Original Ariticle

Evaluation of arbuscular mycorrhizal fungi using 18S rDNA sequence from root of *Botrychium ternatum* *

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Abstract

The symbiosis with the arbuscular mycorrhiza (AM) was observed in the roots of *Botrychium ternatum*. The fungal partial 18S ribosomal DNA (rDNA: approximately 450/1800 bp) were amplified from the DNA extracts of fern roots using fungal specific primer, and subcloned to cloning vector (pGEM-T). According to the RFLP pattern of subclone, some clones were selected and their inserts were sequenced. One of them (GLA-006) was predominant in this analysis, however, four (or potentially five) different clone were found in the root of *B. ternatum*. To infer the phylogenetic relationship, sequences were donated on BLAST Search. According to the result of BLAST Search, neighbor joining analysis was performed. The partial sequencing data of 18S rDNA indicate that these isolates closely related to genus *Glomus* (AM fungi), especially *G. proliferum* and *G. sinuosum*. Three of them were 92-95 % identical to *G. proliferum*.

Key words: *Botrychium ternatum* · fern mycorrhiza · *Glomus* · 18S rDNA · symbiosis

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1. Introduction

The grape fern (*Botrychium ternatum*) was reported to be different from the other ferns (mainly, Leptosporangiate fern), having the fertile shoot (Eusporangiate ferns; Ophioglossaceae), and also called to "mycorrhizal ferns" because of its distinctive features of root. 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; this life cycle of *B. ternatum* was reported to be much similar to those of lily or orchid plants (Kramer, 1990; Kelly, 1994; Schmid and Oberwinkler, 1994; Parihar, 1996). Two kinds of mycorrhizae were also found in the roots of *B. ternatum* collected from several sites in Chung- Puk; Orchid and arbuscular mycorrhizae (AM) (Schmid and Oberwinkler, 1996; Lee et al., 2001). These regarding mentioned above indicated that mycorrhizae might play an important roles in growth or life *cycle* of *B. ternatum.*

In fern and relative plants, it was much difficult to understand the evolutional relationships between species, because any differences were not found in the stage of gametophyte (very simple) or sporophytes. Some researchers found symbiotic interactions between fern plant and fungi play an important role in the growth of fern (Gemma et al., 1992; Zhao, 2000; Lee et al., 2001). Boullard (1957) found many fern symbiotisis with the fungi among the 420 species of fern, and concluded that the advanced ferns have less symbiotic relationship with fungi than the lower ferns. Kelly (1994) investigated the presences of mycorrhizal relationships in the roots of the 101 species of fern in New Zealand, and concluded that his observation was consistent with the results of Boullard (1957). The eusporangiate fern, especially grape ferns (*B. lunaria* and *Ophioglossum reticulatum*), was suspicious of being symbiotic with the AM fungi with morphologic observations (Schmid and Oberwinkler, 1994; 1996), but was not confirmed by the molecular techniques (Helganson 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 as above, we have begun research on the native grape fern in the areas of Chung-Puk in particular to 1) clone the DNA fragments amplified with the fungal specific primer, 2) perform the restriction fragment length polymorphism analysis (RFLP) and 3) analyze phylogenetic relationship using the partial 18S rDNA sequence. Morphological observation was also performed.

2. Materials and methods

2.1. Plants

Botrychium ternatum were collected from various habitats, substrates and geobotanical zones of Korean peninsula; two sites of BuYoung, Chong-Won Kun, and

a site of Temple SeGye, Mt. WolAk in Chong-Puk, from April 1998 to September 2000. The specimens were carefully preserved and deposited in the herbarium of Korea National University of Education. The plants used in this work were identified with several manuals (Park, 1975; Lee, 1985; Kramer, 1990; Parihar, 1996) and confirmed with the specimens previously collected in the herbarium of Seoul National University. Collected roots or rhizomes of the plant were carefully cut from the plants and stored in the polyethylene bags at 4 ℃, until used.

2.2. DNA extraction

Root was placed with liquid nitrogen in a mortar and ground with a pestle into a fine powder. The root powder was transferred to another mortar and grounded with a 650 $\mu\ell$ lysis buffer [50 mM Tris- HCl, 30 mM EDTA, 3 % SDS and 1 % 2-mercaptoethanol: pH 7.5] (Brunel, 1992). This mixture was then incubated at 65℃ for 1 hr, and centrifuged at 10,000 g for 15 min. The lysate was extracted with an equal volume of PCI solution (24:25:1, TE-saturated phenol: chloroform: iso-amylalcohol (Brunel, 1992). The same volume of 2-propanol was added to the aqueous layer to precipitate DNA. Precipitated pellet was dissolved with TEN buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl), then added to 50 $\mu g/\mu\ell$ RNase A and incubated at 37 ℃ for 30 min. The suspension was treated again with PCI, and the DNA was precipitated with ethanol. This DNA pellet was re-suspended in 50 $\mu\ell$ of TE buffer, and the concentration of DNA was estimated by absorbance at 260 nm.

2.3. DNA amplification

Partial fungal 18S rDNA fragments were amplified by PCR using both a universal eukaryotic primer NS-31 (5' - TTG GAG GGC AAG TCT GGT GCC -3' ; Simon et al., 1992) and a general fungal primer AM-1 (5' - GTT TCC CGT AAG GCG CCG AA -3' ; Helgason et al., 1999; Daniell, 2001) that were designed to exclude plant DNA sequences (Helgason, 1999). For amplification, 10 ng of the genomic DNA was added to an *Accupower* PCR PreMix™ Kit (Bioneer Co.) with 10 p mole of each primer (Lee and You, 2000). PCR was carried out for 30 cycles (10 cycles at 95℃ for 1 min, 58℃ for 1 min, and 72℃ for 2 min, 19 cycles at 95℃ for 30 sec, 58℃ for 1 min, and 72℃ for 3 min, and 1 cycle at 95℃ for 30 sec, 58℃ for 1 min, and 72℃ for 10 min) on Personal Cycler™ (Biometra Co.; Helgason et al., 1999). PCR products were observed by electrophoresis on 1.2 % (w/v) agarose gel containing ethidiumbromide in Tris-acetate EDTA (TAE) buffer, and photographed under UV-illumination.

2.4. Cloning, RFLP and sequencing

PCR products were separated on an agarose gel, and the expected approximately 550 bp band was collected by *AccuPrep*™ gel purification kit (Bioneer Co.). After purification, the sticky ended PCR product was cloned into the pGEM T

easy Vector™ (Promega, USA) and transformed into *Escherichia coli* JM109 using as the competent cell. The positive transformed cells were screened by blue-white selection using X-Gal. And plasmid was extracted using *AccuPrep*™ plasmid extraction kit (Bioneer Co.). To confirme the subcloning was successful, plasmids were checked by PCR using the same primer (AM1/NS31). Restriction fragment length polymorphism (RFLP) pattern of every clone were checked using restriction enzymes *Hinf* I and *Alu* I (Helgason et al., 1999). The RFLP patterns of all clones were compared by electrophoresis described as above except for using 4 % (w/v) gel. The RFLP pattern present of each clone was compared with those of the others, and clones were grouped that have a same RFLP pattern. DNA sequencing was performed on an ABI PRIZM™ 377 Genetic Analyzer (Perkin-Elmer, USA), using cycle sequencing with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, 100 ng of purified PCR fragment, and 3.2 pmol primer (AM1/NS31) per reaction.

2.5. Data analysis

Reverse sequences were complemented and aligned with forward sequences by GENEDOC program. After making the consensus sequences by GENEDOC program, sequence was donated on BLAST Search provided by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast/index.html). CLUSTAL X was employed for multiple alignment and neighbor joining (NJ) phylogeny (Saitou and Nei, 1987), using *Geosiphon pyriforme* as an outgroup of order *Glomales* (Gehrig et al., 1996). To evaluate the strength of support for the branches of the NJ trees, 1000 bootstrap replicates analysis were performed. Tree was displayed using TreeView PPC 1.5.3 program.

2.6. Microscopy observation

Roots or rhizomes were washed with tap water and placed in 10 bottle containing FAA solution. The roots were cut into 1 to 3 ㎝ section and stained with trypan blue (Phillip and Hayman, 1970; Koske and Gemma, 1989; Brundrett et al., 1996). Some roots of *B. ternatum* were cross-sectioned by freezing microtome (Leica) and stained with same staining solution (Lee et al., 2000). The stained mycorrhizal roots from the plant of *B. ternatum* were observed under a light microscope. For SEM (Scanning electron microscope), washed roots were cut to 3 ㎜ length in 100 mM phosphate buffer (pH 6.8), transferred to phosphate buffer containing 2.5 % glutaraldehyde, and incubated for 2 hr at 23℃. 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 hr (for post fixation) and rinsed three times with phosphate buffer (McArthur and Knowles, 1993). After post-fixation, dehydration was conducted in a graded ethanol series from 70 % to 100 % (absolute alcohol). After dehydration, samples were critical point 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 (Kal Zeiss) scanning electron microscope.

3. Results

3.1. DNA isolation

The primer (AM1/NS31) were successfully amplified the basidiomycetous DNA from the genomic DNA extract of the roots of *B. ternatum* collected from three different sites of Chong- Won Areas. Length of the PCR fragments were approximately 550 bp. Subsequently, we successfully obtained 32 positive colonies by transformation using TA- vector containing PCR fragments, and these subclones were named as a series of GLA-series. PCR-RFLP was carried out using selected 23 clones (Fig. 1). Many of them had same RFLP pattern but some clone had different pattern (Fig. 1, lanes 7, 9, 10 and 23).

3.2 DNA sequences

The DNA fragments of AM fungi have variation from 461 to 470 (Fig. 2). The partial sequences showing the dark color were showed consensus sequence, and grey color shows partially heterogeneous sequence at the rate of 30-40 of 470 bps in Fig. 2. The partial 18S rDNA sequences were subject to BLAST Search to infer the phylogenetic relationships with other fungi, and the DNA sequences producing significant homology were listed in Table 1.

After blasting of the DNA fragment of GLA-006 with the genes stored in NCBI storage, the five genes similar were selected and listed with the rate matched DNA sequences in Table 1. The GLA-006 was similar to *Glomus proliferum* at matching rate of .947, and were grouped to those of GLA-016 (.961) and GLA-032 (.917). The GLA-011 was similar to *G. sinuisum* at the matching rate of 0.961, and was grouped to that of GLA-016. The results of BLAST search showed all isolate (or clone) were closely related to genus *Glomus*, and to be, at least, divided into two groups of *G. sinuisum* and *G. proliferum*. According to the results of BLAST search, subsequent phylogenetic analysis was carried out. Figure 3 shows phylogenetic tree obtained from NJ analysis running 100 bootstrap replicates, using cloned fragment and the genus *Glomus* and the related genera. The DNA fragments of our cloned AM fungi were clustered in the Fig. 3 and arranged to be the result similar to those of Table 1.

3.3. Morphology

The morphological feature of *B. ternatum* was shown in Fig. 4A, which was collected from the site of Chong-Won Kun, Chung-Puk 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-symbiotic relationship in Fig. 4, and arranged at the different resolutions; arbuscular haustorium (Fig. 4B, C, D), intrahyphae (Fig. 4E, F) and vesicular mass (Fig. 4H, I) 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. 4G) and rarely observed in other plants colonized with AM fungi. These inflated tips in branches of arbuscle in the plant of *B. ternatum* were mentioned to "distinctive tip or mycorrhizae" in several works (Lee et al., 2001; Schmid and Oberwinkler, 1996; 1998).

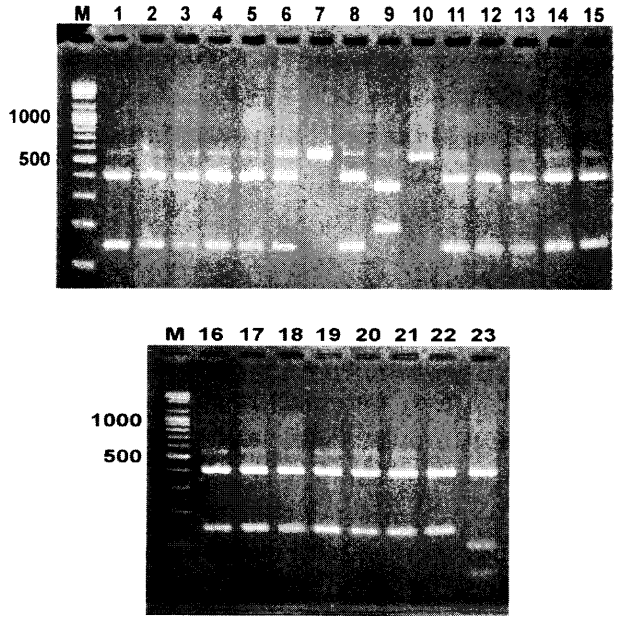

Fig. 1 Restriction fragment length polymorphism patterns of PCR products of 18S rDNA fragment from subclone. PCR products of insert-DNA were digested with Hinf I. and electrophoresis on 4 % (w/v) agarose gel. Lane M, molecular size marker (100-bp ladder); The 23 lanes of cloned PCR-product were selected from 32 colonies cloned from pGEM-T Easy Vector. Lane 1 (DNA fragment corresponded to the clone 3); 2 (clone 5); 3 (clone GLA006); 4 (clone 7); 5 (clone 9); 6 (clone 10); 7 (clone GLA011); 8 (clone 13); 9 (clone GLA014); 10 (clone 15); 11 (clone GLA016); 12 (clone 17); 13 (clone 18); 14 (clone 19); 15 (clone 20); 16 (clone 21); 17 (clone 22); 18 (clone 23); 19 (clone 25); 20 (clone 28); 21 (clone 29); 22 (clone 30); and 23 (clone GLA032).

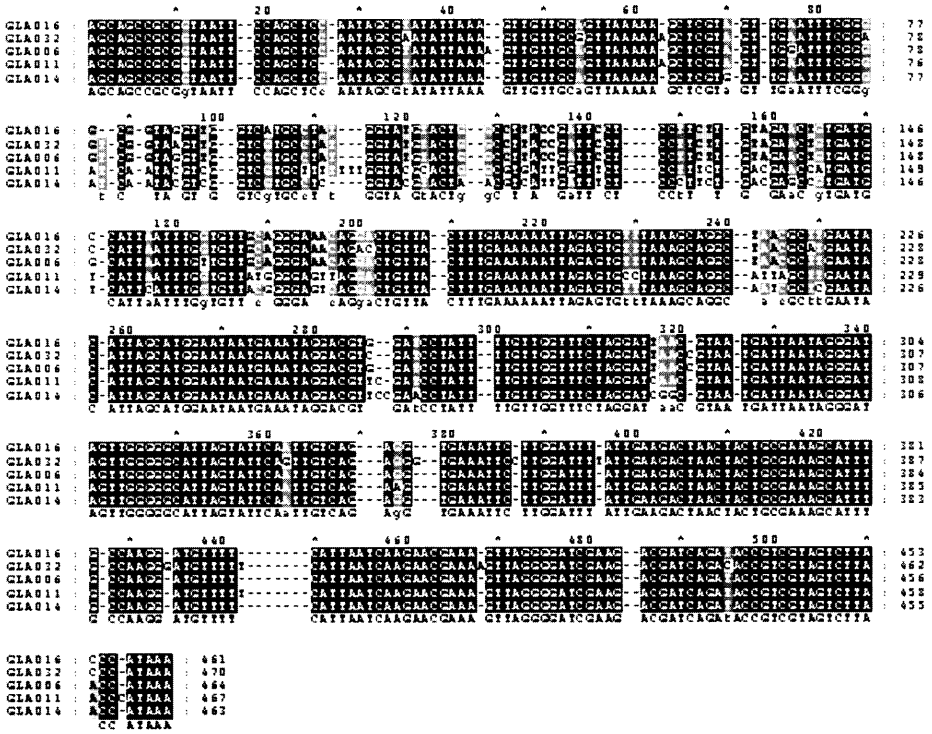

Fig. 2 Sequence alignment of the partial 18S rDNA sequence from clones that isolated from the roots of *Botrychium ternatum* collected in Korea. All of these clones were sequenced in this study.

4. Discussion

The DNA fragments amplified with AM1/NS31 primers from the roots of *B. ternatum* were revealed to be similar to AM fungi (Simon et al., 1992; Helgason et al., 1998; 1999). All isolate (or clone) shows close relationships to the genus *Glomus*, especially *G. proliferum* and *G. sinuosum* (Table 1). The DNA sequences of AM fungi listed in Table 1 and Fig. 3 were provided by NCBI (Helgason, 1998; 1999; Daniell, 2001). Subcloned isolates were categorized into four group by the *Hinf* I RFLP pattern (Fig. 1), whereas these four groups were divided into two groups by BLAST search (Table 1) and phylogenetic analysis (Fig. 3). Five DNA fragment (GLA 006, 011, 014, 016 and 032) were aligned in approximately 500 positions, of which were different. As shown in Table 1, the matching rate of *G. sinuosum* with GLA-011 or GLA-014 were different from *G. proliferum*. The result that our three DNA fragment were identified to the DNA sequence of was speculated to be due to little information stored in Genebank.

Table 1 The DNA sequences producing significant identity with the data on Genbank by BLAST search.

DNA lines[a]	Accession No. of NCBI storage	Organisms	Identities[c]
GLA006	1[b]gi|9863867|gb|AF213462.1|AF213462[d]	*Glomus proliferum*	378/400
	2 gi|17063936|gb|AF437722.1|AF437722	*Glomus* sp. Glo3	377/400
	3 gi|16945285|emb|AJ418894.1|GSP418894	*Glomus* sp. MO-G2	377/400
	4 gi|6448859|gb|AF131053.1|AF131053	Glomus sp. Glo11	375/400
	5 gi|14275538|emb|Y17640.2|GFA17640	*Glomusfasciculatum*	374/400
GLA011	6 gi|6687601|emb|AJ133706.1|GSI133706	*Glomus sinuosum*	367/382
	7 gi|14252955|emb|AJ301857.1|GMA301857	*Glomus* sp. W3347	489/511
	8 gi|17063895|gb|AF437681.1|AF437681	*Glomus* sp. Glo9	475/491
	9 gi|16945247|emb|AJ418856.1|GSP418856	*Glomus* sp. MO-G7	469/491
	10 gi|16945283|emb|AJ418892.1|GSP418892	*Glomus* sp. MO-G1	463/491
GLA014	11 gi|6687601|emb|AJ133706.1|GSI133706	*Glomus sinuosum*	357/365
	12 gi|14252955|emb|AJ301857.1|GMA301857	*Glomus* sp. W3347	360/369
	13 gi|9863867|gb|AF213462.1|AF213462	*Glomus* proliferum	355/363
	14 gi|14275538|emb|Y17640.2|GFA17640	*Glomus fasciculatum*	347/355
	15 gi|437339|gb|L20824.1|GLOSEQA	*Glomus vesiculiferum*	346/355
GLA016	16 gi|9863867|gb|AF213462.1|AF213462	*Glomus proliferum*	487/507
	17 gi|14275538|emb|Y17640.2|GFA17640	*Glomus fasciculatum*	491/513
	18 gi|16945274|emb|AJ418883.1|GSP418883	*Glomus* sp. MO-G2	471/491
	19 gi|17063935|gb|AF437721.1|AF437721	*Glomus* sp. Glo3	471/492
	20 gi|2743|emb|X58725.1|GI18SRRNA	*Glomus intraradices*	515/547
GLA032	21 gi|9863867|gb|AF213462.1|AF213462	*Glomus proliferum*	455/496
	22 gi|16945274|emb|AJ418883.1|GSP418883	*Glomus* sp. MO-G2	436/474
	23 gi|17063935|gb|AF437721.1|AF437721	*Glomus* sp. Glo3	445/486
	24 gi|14275538|emb|Y17640.2|GFA17640	*Glomus fasciculatum*	451/496
	5 gi|6448859|gb|AF131053.1|AF131053	*Glomus* sp. Glo11	444/486

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

[b] The arrangements of the DNA sequences ordered with the homologies of those of the five AM isolates.

[c] (Identities) The nucleotide numbers matched with compared DNA.

[d] GenBank accession number (http://www.ncbi.nlm.nih.gov).

Fig. 3 Neighbor-joining tree which showing the relationships among the fungal sequences isolated from *Botrychium ternatum* roots and the other fungi. Confidence values from 1000 bootstrap replications that are higher than 350 are given in the node. Gray colored boxes (left side in this tree) are containing groups of the colonies isolated from the roots of *B. ternatum* in this study (Clade I, Clade II). Glo1 (AF074358: Genbank accession number), 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), *A. laevis* (AF074347), Scut1 (*Scutellospora* sp; AF131022), *S. dipurpurenscens* (AF131027), *Geosiphon pyriforme* (X86686), and *Archaeospora leptoticha* (AJ301861), respectively.

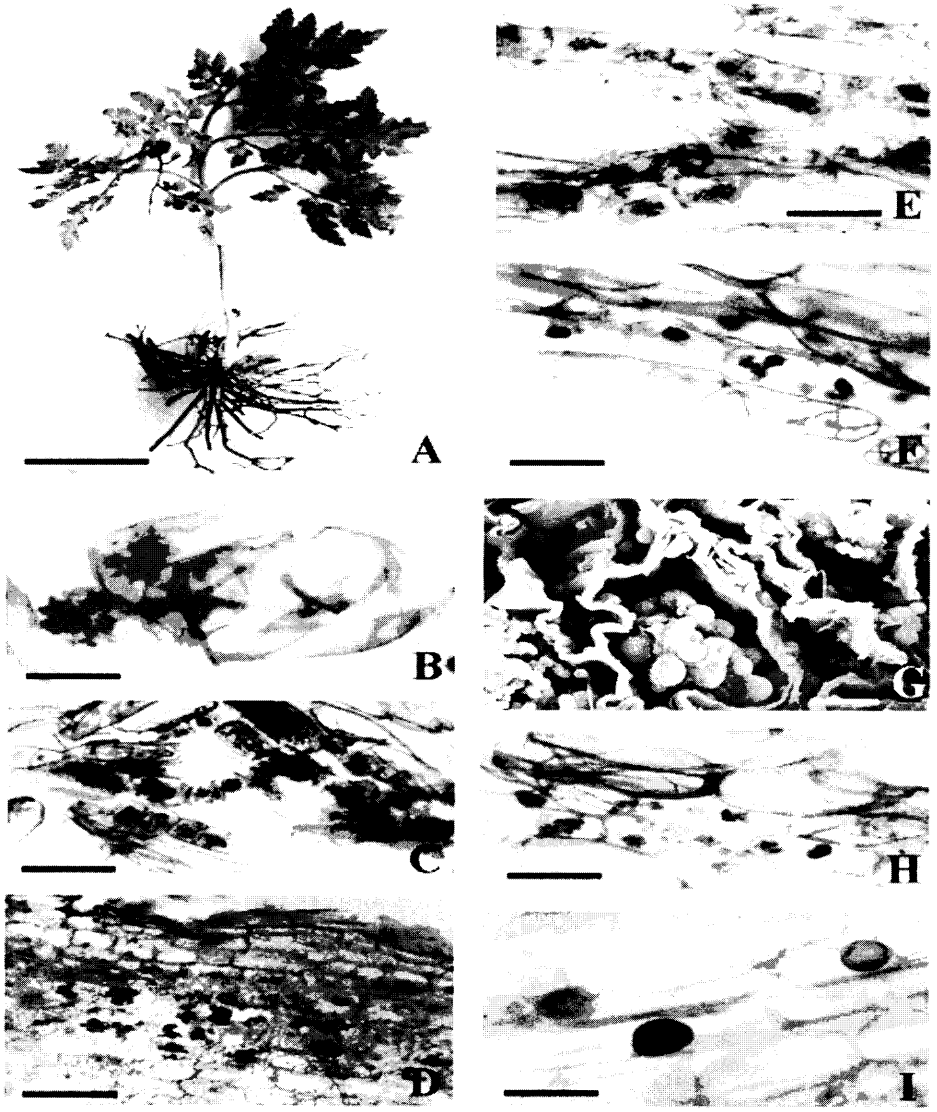

Fig. 4 The mature sporophyte of *Botrychium ternatum* collected in Korea (A) and its unique type mycorrhizas (B to I): A; Sporophyte of *B. ternatum* (chlorophyllous diploid stage; but the fertile frond is not mature yet). There are no root hairs (strongly mycorrhizal). B, C, D, E, F, H and I: Light 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 ㎛ ; E, F, 100 ㎛; B, H, I, 50 ㎛, and G, 500 ㎛

Any information related to the spore of *G. proliferum* was not reported for last 15 years. The spores of *G. sinuosum* were previously reported which associated with the roots of legume plants in the central Korea (near Chung-Puk area) (Eom et al., 1992). Our DNA fragments were aligned with or matched with partial sequences of *G. proliferum* at relatively low rate, as compared with those of *G. sinuosum*. Thus, our DNA fragments were speculated to be originated from the 18S rDNA different from that of *G. proliferum*, but similar to that of *G. proliferum*. As based on this speculation, the three DNA fragments (GLA-006, GLA-016, and GLA-032) were also considered to be originated in different species of *Glomus*.

Several workers reported indicated that various fern plants might be symbiotic with the AM fungi (Gemma and Koske, 1992; Schmid and Oberwinkler, 1996; Zhao, 2000) . Particularly, mycorrhizal formation was focused in the roots of Ophioglossaceaeous (grape) fern, because their distinctive roots were similar to those of lily or orchid plants (Parihar, 1996; Kramer, 1990). Also, the swollen and inflated tips in branches of haustoria (arbuscule) found in the cortical cells (Fig. 4G) were suspected to be a new type of mycorrhizae (Schmid and Oberwinkler, 1994; 1996; Zhao, 2000), but revealed to be a kind of AM fungi. Based on the DNA sequences, the five different AM fungi were found to inhabit in the roots of *B. ternatum* and it would be speculated to be three or four different species of *Glomus*. According to the results of RFLP, the GLA-006 seemed to be pre-dominant (19 of 23) species in the root of *B. ternatu* (Fig. 1), and it was similar but different species to *G. proliferum*. It was reported that an ophioglossaceaeous fern has the relationships with mycorrhizal fungi strongly in their whole life cycle (Krameer, 1990; Parihar, 1996); the sporophytes were reported that forms two kinds of mycorrhizae; orchid and arbuscular mycorrhizae (Schmid and Oberwinkler, 1996; Lee et al., 2001). The most gametophyte of fern (achlorophyllous prothallus) should be mentioned to be associated with some fungi because their achlorophyllous prothallus have no energy sources. Therefore, the question what fungus stimulates to the germinations of fern spores and helps to the gametophyte growth of gametophyte was posed on our minds. The present study might give us some clues to resolve the mysteries of fern gametophyte life. The species of AM fungi and ecological roles of specific species in the roots of plant were easily understood by using (AM1/NS31) primer and the technique employing pGEM-T, and giving us further step for fern ecology.

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遺伝学的手法を用いた*Botrychium ternatum*菌根菌の評価

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要　旨

*Botrychium ternatum*根茎の菌根菌について遺伝学的手法を用いて検討した．根茎より抽出したDNAを真菌類特異的18S rDNAプライマーにより増幅，クローニングし，得られたプラスミドのインサートの配列についてRFLP解析，シーケンシングを行った．塩基配列はBLAST Search を用いてGenBankのデータと比較後，近縁種のデータを用いてＮＪ法で系統解析を行った．解析の結果，DNAフラグメントGLA-006，GLA-016，GLA-032は*G. proliferum*と92-95%の相同性を示した．RFLP解析より数種のAM菌根菌が見出されたが，*B. ternatum*の根茎ではGLA-006が優占種であることが明らかになった．

キーワード：*Botrychium ternatum*・シダ類菌根菌・*Glomus*・18S rDNA・共生