Mycobiology 31(1): 23-31 (2003)
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ITS Primers with Enhanced Specificity to Detect the Ectomycorrhizal Fungi in the Roots of Wood Plants

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(Received September 27, 2002)

With universal primer ITS1-F, the specific DHJ2 primer was developed to detect the Ectomycorrhizal (ECM) root tips in soil and to identify the species of ECM fungi, as based on DNA sequences of rDNA stored in GeneBank of NCBI. This primer was designed with the common sites of rDNA of *Amanita* and *Boletus*, and was also designed with several DNA programs provided by NCBI. The DNA fragments synthesized by PCR were calculated to be 1,000 to 1,200 bps of DNA located to 18s to 28s rDNA to contain two variable sites of ITS, indicating much diversities for specific species or ecotypes of ECM fungi. The primer DHJ2 reacted with the genomic DNA's extracted from the tissues of basidiocarp at the rate of 73 of 80 fungi collected produced single bands with a 1,100 bps length. The DNA fragment synthesized with the genomic DNA that extracted from eight ECM tips of *Pinus densiflora* was confirmed and analysized to the rDNAs of ECM in full sequences, and informed to be a ECM fungal species in the forest.

KEYWORDS: ***Amanita*, *Boletus*, Ectomycorrhizal (ECM) fungal species, ECM root tips, Specific primer**

The plants as autotrophic organisms are reported to be associated with the microorganisms (Harley and Smith, 1983) in their roots and to have very complicated ecological webs with the animals in the forestry. Ectomycorrhizae (ECM) were known to be a complex of the plant root and mass fungi and to help the growth of wood plants. The physiological roles of ECM were not clearly understood in the roots of many forest plants. However, many ECM researches reported only that ECM plant survives well in nature, as compared with the non-ECM plants grown under artificial conditions (Horton and Bruns 2001; Smith and Read 1997; Tibbett *et al.*, 1998). The ECM fungi form the mantle, and Hartig nets were clearly confirmed to be associated or symbiotic with the plant roots, and to protect the roots from other pathogenic organisms (Barker *et al.*, 1998; Harley and Smith, 1983; Martin and Tagu, 1999). The ECM fungi produce the mushrooms edible for men or animals, dispersing their spores during summer or fall in forestry. This regarding indicated that ECM fungi play important roles in forestry between plants and animals. Particularly, the expensive species of mushroom as truffles in Europe (*Tuber* species; Isaac, 1992) and matsutake (pine mushrooms; Lee, 1991; Lee and Hong, 1998; Lee *et al.*, 1999, 2000a, b) or its related mushrooms in Eastern-Asia were intensively focused to work about ECM roots of oak or pine, respectively.

Identification of biological species was mostly carried out with the morphological features in specific orders or families of fungal species. In the recent works related to molecular biology, the sequences of rDNA or its related information made it easy to identify the ECM fungal species, especially symbiotic organisms in the tissues of plant infected with the pathogenic or symbiotic fungi (Agerer, 1991; Egger 1995). Much information about the sequences of rDNA of species was accumulated in a gene bank of NCBI (http://www.ncbi.nlm.nih.gov), and also able to confirm the biological species with specific primers. Several techniques of PCR-Randomly amplified polymorphism of DNA (RAPD) and PCR-Restriction fragment length polymorphism (RFLP) (Gardes and Bruns, 1993; Pristsch *et al.*, 1997) were developed by using polymerase chain reactions (PCR) with DNA polymerase™, including with the analyzing the sequences of 18s rDNA by the universal primers (Gardes *et al.*, 1991; White *et al.*, 1990). The region of ribosomal repeat unit is known to be generally informative for differentiation of species and genera of fungi (Kikuchi *et al.*, 2000). The analysis of internal transcribed spacer (ITS) regions by PCR was thought to be useful because it is accessible with universal primers or its specific primers designed and its DNA sequence is variable at both inter or intra-specific levels of rDNA analyses. Recently, species-specific ITS primers have been reported to be reacted with some fungal species (Amicucci *et al.*, 1998). However, it was posed on our works what kinds of primer were employed to react with the mixed DNAs extracted from the plant roots.

Various types of ECM roots collected for several years

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in our laboratory (Chung *et al*., 2002; Lee *et al*., 2000a, b) were found to be from a root of pine or oak trees in Korean forestry. Particularly, the ECM roots attached to the base of basidiocarps were collected and compared with the fungal specificity with the forest plants described in several manuals related to mushrooms. In most cases of ECM roots, it was very difficult to identify the ECM fungi, even the ECM roots attached to basidiocarps. In other word, eventhough various ectomycorrhizal roots were collected from a root of a single plant species, but difficulty to observe the ectomycorrhizal fungal species in the fields. In an attempt to elucidate the fungal species, we have designed the specific primer for rDNA of ectomycorrhizal fungi, and identified the species of mushrooms related to the ectomycorrhizal roots.

Materials and Methods

ECM fungi. The fruiting bodies or basidiocarps inhabiting under the communities of pine or oak trees have been collected and identified for the last five years in our laboratory (Lee, 1991; Lee and Hong, 1998; Lee *et al*., 1999, 2000a, b); from the areas of mainly Forest Station of ChungBuk National University and Temple of DeokJu in Mts. WolAk and MoonKyoung SeoJae and JeolGol in Mts. JoReong (near Resting Areas of JoReong) in City of ChungJu, ChungBuk Province. Occasionally, mycorrhizal and non-mycorrhizal fungi were collected from the foerstry around two temples of KongLim (Mts. NagYang) and BeobJu (Mts. SogRi) in BoEun Kun, ChungBuk. The plant roots attached to the bases of basidocarps were collected and carefully observed under dissection microscopes. Various types of ectomycorrhizal roots were observed as shown in Figs of articles of Lee *et al*. (2000a, b), but not clarified what kind of plant roots were involved under the soil. However, the basidiocarps attached with ectomycorrhizal roots were regarded or classified as an ectomycorrhizal fungus as shown in Table 1. Various fungi, approximately 80 species of basidiocarp collected and identified as shown in Table 1, were employed for this

Table 1. The basidiocarps of ectomycorrhizal fungi collected for DNA extraction[a]

	Scientific Name		Scientific Name		Scientific Name
1	*Boletus violaceofuscus*	28	*Russula rosacea*	55	*Cantharellus pallidus*
2	*B. edulis*[c]	29	*R. flavida*	56	*C. friesii*
3	*B. granulatus*	30	*R. violeipes*	57	*Ramaria flaccida*[c]
4	*Boletellus obscurecoccineus*[c]	31	*R. senecis*	58	*R. botrytis*
5	*Suillus granulatus*	32	*R. alboareolata*	59	*Hygrophorus russula*
6	*Leccinum extremiorientale*	33	*R. compacta*	60	*Boletopsis leucomelas*
7	*Strobilomyces confusus*	34	*R. cyanoxantha*	61	*Sarcodon scabrosusm*[c]
8	*Heimiella japonica*	35	*R. nigricans*	62	*Craterellus cornucopioides*
9	*Tylopilus felleus*	36	*R. virescens*	63	*Polyozellus multiplex*
10	*Gyroporus purpurinus*	37	*Lactarius volemus*[c]	64	*Naematoloma fasciculare*
11	*Amanita vaginata*	38	*L. scrobiculatus*	65	*Pyophyllum shimegi*
12	*A. vaginata*	39	*L. piperatus*	66	*Coprinus atramentarius*
13	*A. nivalis*	40	*L. hygrophoroides*	67	*C. atramentarius*
14	*A. pantherina*[c]	41	*L. violascens*	68	*Armillaria mellea*
15	*A. phalloides*[c]	42	*L. volemus*	69	*Tricholoma matsutake*
16	*A. griseofarinosa*	43	*L. subzonarius*	70	*Pisorithus tinctorius*
17	*A. virosa*	44	*L. obscutus*	71	*Tricholomopsis platyphylla*
18	*A. spissacea*	45	*Agaricus silvaticus*[c]	72	*Coriolus versicolor*
19	*A. muscaria*	46	*Rhodophyllus rhodopolius*[c]	73	*Coltricia cinnamomea*
20	*A. agglutinata*	47	*R. crassipes*[c]	74	*Pluteus atricapillus*
21	*A. longistriata*	48	*R. quadratus*[c]	75	*Pholiota spumosa*
22	*A. rubescens*	49	*Collybia bytyracea*	76	*Cortinarius variecolor*
23	*Laccaria amethystina*	50	*Hericium erinaceum*	77	*Sclerotinia sclerotiorum*
24	*Mycena pura*	51	*Panaeolus papilionaceus*	78	*Dictyophora indusiata*
25	*Clitocybe clavipes*	52	*Dictyophora indusiata*	79	*Lycoperdon mammaeforme*
26	*Marasmiellus chamaecyparidis*	53	*Cryptoporus volvatus*	80	*Polyporellus brumalis*
27	*Leucopaxillus giganteus*	54	*Cantharellus lutescens*		

[a]The ectomycorrhizal roots attached on the bases of basidiocarps were confirmed to be an ectomycorrhizal fungi by the microscopic observations.
[b]Some of these fungal species were checked for Professor Heyser's work.
[c]All fungal species were employed for this work and some species of above worked by Carsten Harm in University of Bremen; *Agaricus silvaticus* (AF438556), *Amanita pantherina* (AF438561), *Amanita phalloides* (AF438560), *Amanita vaginata* (11; AF438562), *Amanita virosa* (AF438563), *Boletus edulis* (AF438566), *Boletellus obscurecoccineus* (AF438567), *Heimiella japonica* (AF438576), *Lactarius volemus* (AF438578), *Rhodophyllus crassipes* (AF438599), *Rhodophyllus quadratus* (AF438600), *Ramaria flaccida* (AF438554), *Sarcodon scabrosusm* (AF438602), *Naematoloma fasciculare* (AF438590), *Coprinus atramentarius* (AF438568), *Tricholoma matsutake* (AF438605).

work; 4~6 species of *Boletus* around the plant of *Pinus densiflora*, 13 species of *Amanita* around the plant species of *Quercus*, 10 species of *Russula* and 9 species of *Lactarius* around the mixed communities of plant *Pinus* and *Quercus* species, 3 species of *Cantharellus* and two species of *Ramaria*. The genomic DNA were extracted from the 80 species of basidocarps for PCR-RAPD identification.

Identification. Various basidiocarps collected were identified with the keys published by Lee (1988) and Park and Lee (1996) written into Korean. The species of mushroom were also compared to the descriptions made by Smith and Smith (1980) for the species of *Amanita* and *Boletus*, Breitenbach and Kranzlin (1984) for those of *Boletus* and its related genera, Lincoff (1997) and Miller (1997) for the relationships of ECM roots. The species of plant related to the ECM roots in the forestry were also compared and identified with the descriptions made by Lee (1988). The basidiocarps of two *Sarcodon* (Hydnaceae) species were compared with the descriptions of *Sarcodon* species made by Johannsson *et al.* (1999). The other gilled basidiocarps were also compared with the detailed descriptions made by Miller (1997). The funnel and clubed shaped basidiocarps were easily identified using Park and Lee's descriptions (1996) and compared with other descriptions of cantharelloid fungi in Pacioni and Lincoff's descriptions (1981). All fungal DNAs were directly obtained from the basidiocarps identified and collected from the various mountains located in ChungBuk, South Korea.

PCR. Total genomic DNA was directly extracted from several tips of ECM roots rather than the isolates according to the procedure described by Bruns *et al.* (1993). Nucleic acids were precipitated in isopropanol, re-suspended in TE (1.5 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5). The total genomic DNA extracted above was amplified with the primers as mentioned in previous work by Lee and You (2000). The genomic DNA was reacted and amplified by Polymerase Chain Reaction (PCR) using the four specific primers; ITS1-F (5'-CTT GGT ATT TAG AGG AGT AA-3') for universal primer of fungal rDNA, DHJ2 (5'-ACT GAC ATC AAG CGT TTC CC-3'; designed in this work), and ITS1 (TCC GTA GGT GAA CCT GCGG) and ITS8 (ACA GGC ATG CTC CTC GGA A) employed in laboratory of Professor Heyser (Dr. Kim, SJ; kim@uni-bremen.de.or Dr. Carsten Harms; carsten@uni-bremen.de). These four primers were also reacted with these DNA templates directly extracted from ECM roots. The site specific primers were added to genomic DNA in the buffer/enzyme mixture (10× PCR buffer), the reaction mixture kept at 95°C for 5 min and then subjected to 30 cycles each of 95°C for 1 min, 43°C for 1 minute and 72°C for 1 minute. The final reaction mixture was kept at 72°C for 8 minute to stabilize the amplified DNA and then stored at 4°C. A sample of each amplification reaction was electrophoresed through 1.2% agarose gel (TAE buffer) at 100 volts.

Cloning and sequencing. PCR products were separated on an agarose gel, and the expected approximately 900 to 1,000 bp band was eluted by *AccuPrep*™ gel purification kit (Bioneer Co., Korea). After purification, the sticky (-A) -ended PCR-product was cloned into the pGEM-T easy Vector™ (Promega, USA) and transformed into *Escherichia coli* (JM109; using as the competent cells). 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., Korea). These plasmids were screened using the (IT1F/DHJ2) primer amplification. After selection, every clone was digested with restriction enzyme *AflII*, according to manufacturer's manual (Promega, USA). The restriction fragment length polymorphism (RFLP) pattern present in each clone was compared, and clones were grouped based on RFLP patterns. Then, one selected clone from each group and purified plasmids were automatically sequenced on an ABIPRISM™ 377 (Perkin-Elmer, USA). For sequencing of insert-DNA regions, designed primer (AM1/NS31) was used and the analyses of DNA sequence were sent to the DNA-lab charged (http;//www.eugentech.com).

Data analysis. DNA sequences were analyzed or read 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. After making the concensus sequences using GENDOC program, Tree View (3.2) and CLUSTAL X (v1.81; Thompson *et al.*, 1994) was used for multiple alignment and neighbor-joining phylogeny (Saitou and Nei, 1987), using rDNA sequences of fungal species selected from gene bank of NCBI (Gehrig *et al.*, 1995: Tae *et al.*, 2002).

Results

Primer. The information related to the genomic DNAs of 70 subspecies or species of Amanitaceae and 101 lines (subspecies) or species of Boletaceae were being searched in the GeneBank of NCBI (http://www.ncbi.nlm.nih.gov). The species of Boletaceae were composed of various species of *Suillus*, *Boletus*, *Leccinum*, *Boletinus*, *Pulveroboletus*, and *Strobilomyces*, and drawn to a dendrogam of phylogenic tree like that the lines (subspecies) or species of Amanitaceae shown in Fig. 1 as based on the

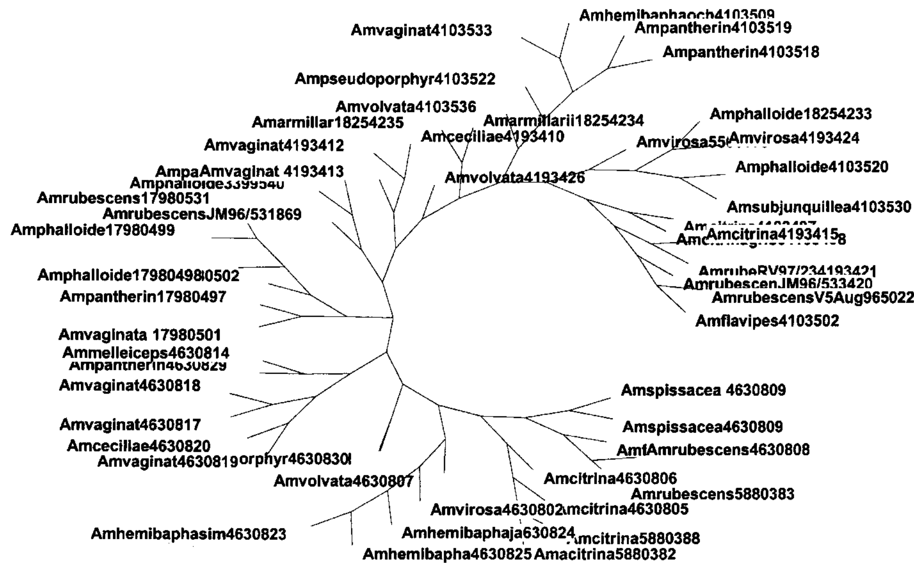

Fig. 1. Phylogenic dendrogram of the species of *Amanita* searched throughout NCBI as based on the information of rDNA. See the DNA information of *Amanita* in NCBI (http://www.ncbi.nlm.nih.gov) in detailed.

sequences of rDNA stored in Genebank of NCBI, but not shown here for the species of Boletaceae. All proceduces were made from the programmings of ClusterX (v1.81) and TreeView (v3.2) supplied by NCBI. The dendrogram shown in Fig. 1 was extracted from the species or subspecies (line numbers) of Amanitaceae (Am vaginata 4103533 described as a line or an isolate (unique numbers of AF4103533 of *Amanita vaginate* worked);

Amanita spissacea (AB015683), *A. vaginata* (AF097374), *A. phalloides* (AF261435), *A. phalloides* (AF024469), *A. pilosella* (AF024470), *A. pantherina* var. *lutea* (AF024468), *A. virosa* (AF097386), *A. virosa* (AF159086), *A. armillariiformis* (AF024487), *A. armillariiformis* (AF261436), *A. ceciliae* (AF097372), *A. ceciliae* (AF024444), *A. citrina* var. *grisea* (AF024447), *A. flavipes* (AF024451), *A. hemibapha* var. *ochracea* (AF024458), *A. pseudoporphyria* (AF024471), *A. rubescens* (AF097383), *A. rubescens* (AF097382), *A. rubescens* (AF042607), *A. volvata* (AF097388), *A. volvata* (AF024485). The individual or species shown in Fig. 1 was, for example, Am(anita) plus name of species and

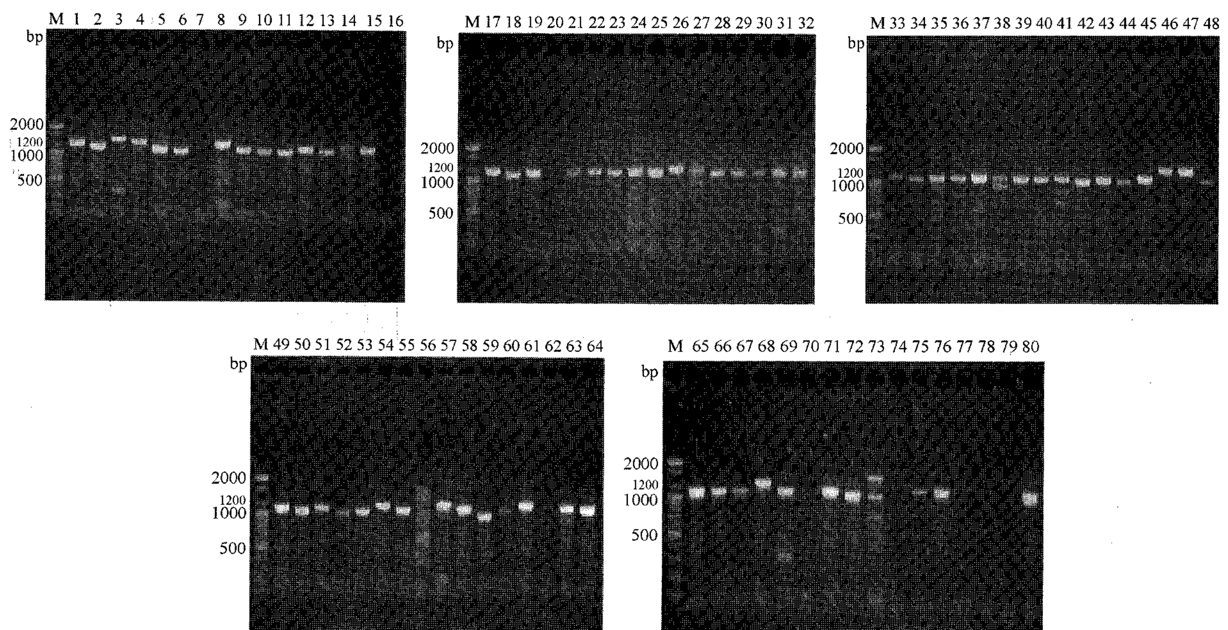

Fig. 2. DNA bands synthesized with the primers of ITS1F and DHJ2 designed in this work. The numbers listed indicated the genomic DNA of basidiocarps listed in Table 1.

accession number stored in Genebank of NCBI.

The DNA band synthesized with the primers of ITS1-F and DHJ2 would be speculated to be 900 to 1,100 base pairs of rDNAs (middle of 18s rDNA to 28s rDNA) containing two variable ITS's. The 80 genomic DNAs extracted from the ectomycorrhizal basidiocarps mentioned in Table 1 were reacted with the two primers designed (PCR reactions in Materials & Methods), and produced one or few bands shown in Fig. 2. In other word, the 73 genomic DNAs reacted with the designed primers and produced the bands having around 1,000 bps of DNA weight, being a speculated above. Two species of *Amanita* included other three species of *Craterellus*, *Pluteus*, and species of Gasteromycetes (*Sclerotinia*, *Lycoperdon*, *Dictyophora*, *Pisorithus*) were not reacted with these two primers. Also, 18 species of ECM basidiocarp were reacted with other two primers of ITS1 and ITS8 and produced a single band of DNA employed in laboratory of Professor Heyser (in Table 1 marked; Dr. Kim, SJ; kim@uni-bremen.de or Dr. Harms; carsten@uni-bremen.de);

All fungal species were employed for this work and some species of above worked by Dr. Carsten Harm in University of Bremen; *Agaricus silvaticus* (AF438556; the name of basidiocarp was identified by our laboratory and this number was registered in NCBI by Dr. Carsten), *Amanita pantherina* (AF438561), *A. phalloides* (AF), (AF438560), *A. vaginata* (11; AF438562), *A. virosa* (AF438563), *Boletus edulis* (AF438566), *Boletellus obscurecoccineus* (AF438567), *Heimiella japonica* (AF438576), *Lactarius volemus* (AF438578), *Rhodophyllus crassipes* (AF438599), *R. quadratus* (AF438600), *Ramaria flaccida* (AF438554), *Sarcodon scabrosusm* (AF438602), *Naematoloma fasciculare* (AF438590), *Coprinus atramentarius* (AF438568), *Tricholoma matsutake* (AF438605).

The DNA bands obtained from the PCR reactions with the primers designed were confirmed in the agarose gel, and re-purified thoughtout the specific column sold for around 1,000 bps (using *AccuPrep*™ plasmid extraction kit made by Bioneer Co, Korea). After then, the band re-synthesized were inserted to the pGEM-T easy Vector™ (Promega, USA) and transformed into *Escherichia coli* (JM109; using as the competent cells). The plasmids

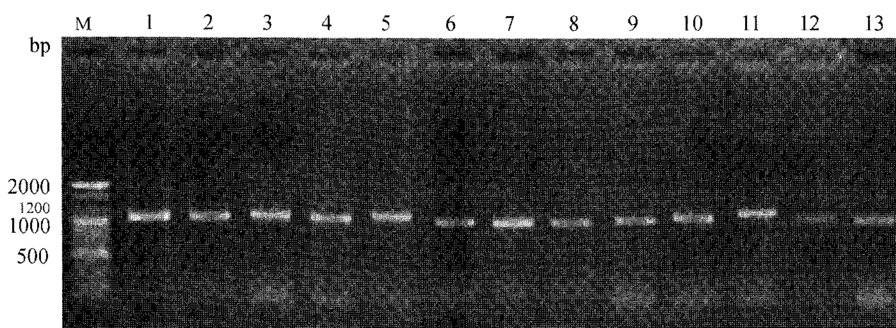

Fig. 3. PCR of the ectomycorrhizal DNA band extracted with the primers re-synthesized for cloning works by p-GEM T.

Table 2. The ectomycorrhizal root tips collected for this works[a]

Marks[a]	ECM Type[b] (colored)	Collected site[c]	NCBI searchings[d]
1(P1)	Single (White and Black)	KNUE, back hill of 3rd college	Mycorrhizal isolate BE1-db2 (AF104988)
2(P2)	Y-shaped (Brwon)	KNUE, back hill of 2nd college	*Tomentella sublilacina* (AF323111)
3(P3)	Y-shaped (Brwon)	KNUE, in front hill of 3rd college	Thelephoraceae sp. (AF272941)
4 (JR)	Y-shaped (Brwon)	JR (with Ericoid mycorrhizal)	*Russula gracillima* (AY061678)
5	Single (Red Brwon)	Mt Seong Du in TaeJeon City	No works[e]
6	Y-shaped (Brwon)	Mt Seong Du in TaeJeon City	No works[e]
7(SD3)	Y-shaped (Brwon)	Mt Seong Du in TaeJeon City	*Tylospora fibrillosa* (AF052565)
8	Y-shaped (Brwon)	Mt Seong Du in TaeJeon City	No works[e]
9	Y-shaped (Brwon)	GongLim Temple	No works[e]
10(KL2)	Y-shaped (Brwon)	GongLim Temple	*Russula* sp. (AF335442)
11(KL3)	Y-shaped (Brwon)	GongLim Temple	*Tomentella stuposa* (AY010277)
12(KL4)	Y-shaped (Brwon)	GongLim Temple	*Tomentellopsis submollis* (TSU410771)
13	Y-shaped (Brwon)	GongLim Temple	No works[e]

[a]Ectomycorrhizal root tips collected under the community of *Pinus densiflora*.
[b]Gross morphologies of ectomycorrhizal roots.
[c]The sites collected; Korea National University of Education (KNUE), Mt JoRyeong (JR) located in the YeongPuk Myeon, GwiSan Kun, and GongLim Temple (Mt. NakYoung) located at SaDam Ri, GwiSan Kun, ChungBuk in Korea, Mt. Seong Du located in the Daejeon City.
[d]The possible DNA sequences provided from NCBI searching.
[e]Not worked for transformation of DNA bands in this moment.

(pGEM-T) were selected to be transformed to the cells of *E. coli* JM109 and shown in Fig. 3, after the plasmid were reacted with the above primers with PCR similar to the procedures made to be synthesized by these two primers (Tae *et al.*, 2002). Only ten bands indicating around 1,000 bps as weight base were confirmed among 13 ectomycor-

Fig. 4. DNA sequences of band synthesized with two primers and analyzed from the ectomycorrhizal tips collected under the soil of *Pinus densiflora*.

rhizal tips collected (Fig. 3). Out of ten DNA bands, eight DNA bands mentioned in Table 2 (C, D, E) were transformed to competent cells of *E. coli* for this work and analyzed for DNA sequences. The DNA bands were resynthesized with the two primers from the plasmid cloned, again, as mentioned above, and sent to DNA analyses, after purified using the *AccuPrep*™ kit. The DNA band was hardly read at the ending or stating site, because the analyzer read DNA sequence within 1,000 bps. The PCR products were read forward and then reverse directions with proof read. The sequences of DNA band were analyzed or read at the ranges of 884 to 1,099 bps as showed in Fig. 4. Those represented common sites or regions painted by the dark color (green color) and different site by pale color at the sequences of DNA with GeneDoc program. From results of NCBI searching, the sequence of DNA band P1 obtained from ectomycorrhizal tips was synthesized to 1,031 bps and revealed to be similar to that of unidentified ectomycorrhizal fungi; 95% of mycorrhizal isolate BEl-db6 (AF104988) and 96% of mycorrhizal isolate LH-db2 (AF104990) and 94% of *Tomentella sublilacina* (AF323111). The sequence of DNA band P2 obtained from ectomycorrhizal tips was also matched with 92% the species of *Tomentella lapidum* (AF272941) or 96% of unidentified species of ectomycorrhizal fungi (ectomycorrhizal root tip, AF476974). By the same way, the sequence of DNA band P3, KL3 and KL4 (in Table 2) were also matched with 92~93% the species of Thelephoraceae species (AF272922), 94~95% the species of *Tomentella stuposa* (AY010277) and *Tomentellopsis submollis* (TSU410771). Specially, DNA sequence of ECM root tips JR showed 98% matching result with *Russula* sp.

Discussion

ECM roots. The root tips of ECM contained brown and white Y-type (coral form), and pyramid type was collected from the communities of *Pinus densiflora* around the areas of ChungBuk and ChungNam. We had very hard time to extract the fungal genomic DNA from the root tips for two years with several repeating and with various methods. The methods described in Materials and Methods were adequate and very simple to our purpose and also able to extract various fungal genomic DNAs from various root tips (with an approximately rate of 100%, the root tips were reacted with these primers and produced the single band around 1,000 bps). The ten root rips showed the single band among 13 different root tips of ectomycorrhiza collected under the plants of *P. densiflora* in Table 2.

Primer. For last five years, we have collected various ectomycorrhizal roots in the forests located at different region and studied the edible mushrooms like the pine mushroom (*Tricholoma matsutake*) and few species of *Sarcodon* in our laboratory. It was, however, speculated what fungus of the ectomycorrhizal tips collected from the field was originated from various ectomycorrhizal tips, for example Y-type or single dark tip were found at the same base of basidiocarps collected in various Mountains. In practice, the mycelium isolated from the tissues of basidiocarp would speculated to be simple for handling the inoculation and comparison on the host plants rather than unidentified mycelia isolated from ectomycorrhizal tips (Chung *et al.*, 2002). The development of specific primers adequate for these aspects was speculated to be more important than any other work of ectomycorrhiza in our laboratory. Thus, we started to develop the primer specific for detection of the species of *Amanita* and Boletaceae, being known to be an ectomycorrhizal basidiocarp in Korean forestry (Table 1; Lee *et al.*, 2000b). The phylogenic tree of *Amanita* made from middle step (Fig. 1) was strictly based on the information of rDNA genes from 70 individual or species stored in NCBI, but not involved in any other morphological and physiological characteristics. Also, this was made under Cyber-system with the two programming of Cluster X and TreeView. At glance, few ramified branches (a group of branch containing 3 to 7 individuals) under a main branch were speculated to be distinguishable in the genus of *Amanita*, even though morphological and biochemical information of this was much limited in our knowledge for fungal systematic. Also, this figure was not focused for phylogenic aspects. The primer DHJ2 developed was hopefully able to distinguish the differences of the fungal species in the works of ectomycorrhizal fungi. First of all, the DHJ2 primer designed by these steps was revealed to react with 80 genomic DNAs and to produce the DNA band having the expected weight. In other word, this primer gave us much potential for catching the fungal information in the plant roots with simple techniques of molecular biology.

The DNA band synthesized with DHJ2 primer was revealed to react with the genomic DNAs extracted from the species of *Russula* and *Lactarius*, besides *Boletus* and *Amanita*, including some species of Aphyllopharales or Gasrteromyctes or Loculoascomycetes (Fig. 2). This band was characterized to be a DNA fragment having 1,000 to 1,200 bps by length, indicating the regions of 18s to 28s rDNA including the two variable sites and twice longer than other primer (as compared with the primers, ITS1 and ITS8; carsten@uni-bremen.de). The conserved sites of rDNA (18s, 5.8s, and 28s) would be speculated to give us much information of species or genus or family above the distinctions of species and the two variable sites of ITS to diversity of species or individuals of fungal organisms. Also this primer was revealed to react with the genomic DNA's extracted from the 73 per 80 basidio-

carps and to produce the DNA fragment having twice information than the other work mentioned. This DNA fragment synthesized with DHJ2 primer was believed to be most adequate for cloning of p-GEMT employed in our work and easy for analysis of DNA sequences.

Fungal identification. Among 13 ECM tips collected from communities of *Pinus densiflora* in various regions, only the DNA extracts originated from ten ectomycorrhizal root tips were reacted with DHJ2 primer. Also, only eight root tips were cloned and analyzed for DNA sequences. From the results of NCBI searching (Fig. 4), the ECM fungi matched with the root tips P1, P2 and P3 based on the DNA sequences of over 700 bps showed 92~96%. And other marks (JR, SD, KL) showed more than 90%, based on the DNA sequences of 309~598 bps. These results indicated that the matching rates be relatively low, as compared with the other work (Tae *et al.*, 2002). As based on these speculations, ECM root tips of P2, P3, and KL3 were hardly speculated to be a species of *Tomentella* or Thelephoraceae sp, closely related to the ectomycorrhizal species. Some species of *Tylospora fibrillosa* (SD3) were reported to be mycoflora, but those of *Tomentella* not in Korean forestry in Lee's or Park's manuals of mushrooms (Lee, 1988; Park and Lee, 1996). Otherwise, ECM root tip P1 would be speculated to be the fungus registered in NCBI, but unfortunately an unidentified mycelium matched in this work. This indicated that development of primer system would be needed to obtain much information about DNA sequences of ectomycorrhizal fungi in Korean forest.

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