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Phylogenetic Relationships Among *Pleurotus* species Inferred from Sequence Data of PCR Amplified ITS II Region in Ribosomal DNA

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rDNA의 ITS II 부위의 염기서열분석에 의한 느타리 버섯 종간의 근연관계

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ABSTRACT: This study was carried out to identify the phylogenetic relationship among several isolates of *Pleurotus* species by comparing ITS II region of ribosomal DNA(rDNA) repeat unit. Two primers from ribosomal DNA sequences were chosen to amplify the specific internal transcribed spacer (ITS) II region of *Pleurotus* spp. The exact ITS II region with an unique band from six species of *Pleurotus* genus could be amplified using the two primers taken from at the 3'-end of 5.8S rDNA and 5'-end of 28S rDNA. Six representative species of the *Pleurotus* genus were easily characterized according to the length differences of ITS II region. Furthermore, within *P. ostreatus* species, different sizes of ITS II region could be observed in the isolates of ASI 2025 and ASI 2095 although they were classified as *P. ostreatus* by the conventional observation. The nucleotide sequence analyses of PCR-amplified ITS II region indicated that the isolates ASI 2025 and ASI 2095 were different from other *Pleurotus* spp. When the nucleotide sequences of six *Pleurotus* species were compared, three typical ITS II regions were highly variable especially at both ends of this region. The phylogenetic tree obtained by the Neighbor program of Felsenstein PHYLIP package with all the nucleotide sequence of *Pleurotus* spp. indicated that *P. ostreatus*, *P. florida*, *P. sajor-caju* and *P. eryngii* were closely related to one phylogenetic branch and *P. cystidious* was related to other branch with *P. cornucopiae*. The isolates ASI 2025 and 2038, however, were not closely related to any other *Pleurotus* spp. and formed their own individual branches.

KEYWORDS: ITS II, Phylogenetic tree, *Pleurotus* spp., rDNA

Oyster mushroom (*Pleurotus* spp.) is very popular as one of the special foods having unique flavour, taste and nutrient values. The worldwide production of fresh oyster mushroom was 917,000 metric tons in 1991 which was the second largest amount followed by *Agricus bisporus* (Chang and Miles, 1991). The taxonomy of this edible mushrooms, however, is still confusing apparently due to expansion of special cultivation techniques and incorret naming of newly cultivated strains. Commercial strains so far are ambiguously or incorrectly named, leading to erroneous identification. During the

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last two decades, isozyme studies of the fungi focused primarily on general protein patterns, utilizing some specific enzymes (Zervakis and Labarere, 1992; Paranjpe *et al.*, 1979). The electrophoretic phenotypes were evaluated on the presence or absence of particular bands without regard to the genetic bases of these bands. For example, different isozyme patterns of esterase or peroxidase among *Pleurotus* spp. were observed by Park *et al.* (1988). Such phenotypic markers, however, are fluctuated by environmental and physiological conditions unlike the constant gene sequences and the DNA fingerprints of each species. Many attempts using molecular techniques such as restriction fragment length polymorphism (RFLP) (Summerbell *et al.*, 1989), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990; Kerrigan *et al.*, 1993) were used to classify mushroom species.

The rDNA are repeated hundreds to thousands times and arranged in a tandem array in the specific chromosomes (Garber *et al.*, 1988; Jorgensen and Cluster, 1988). Since the structure of rDNA is relatively well characterized and rDNA repeat unit has component sequences evolved at different rates (Russell *et al.*, 1984; Appels and Honeycutt, 1986), the molecular studies of rDNA is of importance to obtain taxonomical and phylogenetical information. In this regard, we report the results of length polymorphism as well as sequence variation of PCR-amplified ITS II region from different *Pleurotus* species.

Materials and Methods

Strains

Worldwide collection of *Pleurotus* species were used as follow. Nine isolates of *P. ostreatus*, 4 isolates of *P. sajor-caju*, 4 isolates of *P. florida*, 3 isolates of *P. cystidious*, 2 isolates of *P. cornucopiae* and 1 isolate of *P. eryngii* were collected from several countries as in Table 1. The isolates were grown on potato dextrose broth (PDB; Difco) in a stationary condition for 7 days. Mycelia were harvested by filtering through nylon mesh for freeze drying.

Table 1. Isolates of *Pleurotus* spp. used for DNA extraction

Species	Isolates No.[a]	Geographic Origin	Species	Isolates No.[a]	Geographic Origin
P. ostreatus	ASI 2001	Korea	*P. sajor-caju*	ASI 2091	Japan
	ASI 2003	Japan		ASI 2096	Thailand
	ASI 2017	Denmark	*P. florida*	ASI 2013	Hong Kong
	ASI 2018	Korea		ASI 2014	German
	ASI 2025	Korea		ASI 2015	German
	ASI 2028	Korea		ASI 2016	German
	ASI 2072[b]	Korea	*P. cystidious*	ASI 2019	Taiwan
	ASI 2095[c]	Taiwan		ASI 2049	Japan
	ASI 2180	Korea		ASI 2079	Taiwan
P. sajor-caju	ASI 2070	India	*P. cornucopiae*	ASI 2011	Japan
	ASI 2085	Hong Kong	*P. eryngii*	ASI 2038	Japan
				ASI 2125	U.S.A.

a: The identification No. of the species was followed by the stock list of the Agricultural Sciences Institute, Suwon Korea.

b: ASI 2072 was mated between *P. ostreatus* and *P. florida*, through mycelial fusion.

c: ASI 2180 is a hybrid between *P. ostreatus* and *P. florida* through protoplast fusion.

DNA extraction

Total DNA was extracted from the lyophylized mycelial powder using the procedure described by Graham *et al.* (1994). The samples (1 g) were ground to fine powder in liquid nitrogen. One ml of extraction buffer [2% (wt/vol) CTAB, 100 mM Tris-HCl (pH 7.0), 1.4 M NaCl, 20 mM EDTA] was added and mixed by gentle inversion. After incubation at 55°C for 20 min the sample was centrifuged for 5 min at 15,000×g. The supernatant was collected and 1 volume of phenol : chloroform (1:1) was added and mixed by gentle inversion followed by centrifugation. The DNA was precipitated with 2.5 volume of ice-cold absolute ethanol containing 2% potassium acetate. The final precipitated DNA was harvested by centrifugation and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] for further PCR amplification.

Polymerase Chain Reaction

For PCR-amplification of ITS II region, the sequences of two primers ITS 3 and ITS 4 (Primer A: 5'-GCATCGATGAAGAACGAGC-3', B: 5'-TCCTCCGCTTATTGATATGC-3') were chosen from the known sequence of the 3'-end of 5.8S rRNA gene and the 5'-end of 28S rRNA gene of rDNA repeat unit (White *et al.*, 1990) (Fig. 1) and were synthesized using automatic DNA synthesizer (Applied Biosystems Model/381A). The PCR-amplifiction reaction was carried out for 5 min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and polymerization at 74°C for 3 min. After phenol extraction, one tenth of the amplified products was loaded on the 6% denatured polyacrylamide gel to analyze the PCR products.

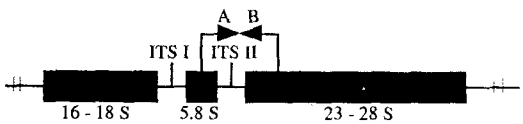

Fig. 1. The location of primers used for the amplification of ITS II region.

Cloning and Sequence Analysis

At the end of PCR-amplification, two unit of T4 polynucleotide kinase (Pharmacia) was added to the reaction tube for making efficient ligation into cloning vector. The PCR-amplified fragments were subcloned into pTZ 19U vector (Pharmacia) and the nucleotide sequences were determined by dideoxy chain termination method with forward sequencing and reverse sequencing primer (Sanger *et al.*, 1977). Alignment of nucleotide sequences was determined using Boxer program (kindly provided by Dr. H. S. Jung at Seoul National University).

Statisitical Analysis

Primary sequences of each *Pleurotus* spp. were determined and compared with each other. The number of different bases between the two compared sequences was evaluated by calculating the corresponding Knuc values. From the comparison of every sequence data, a distance matrix was calculated by Kimura's two parameter method (Kimura, 1980). A phylogenetic tree was constructed using the Neighber program of the Felsenstein PHYLIP package with the Neighber-joining option.

Results

We have collected 6 species, each from a different country, which were morphologically identified. Among those 6 representative species, considerable variations were observed by comparing the length differences of ITS II region (Fig. 2). From PCR am-

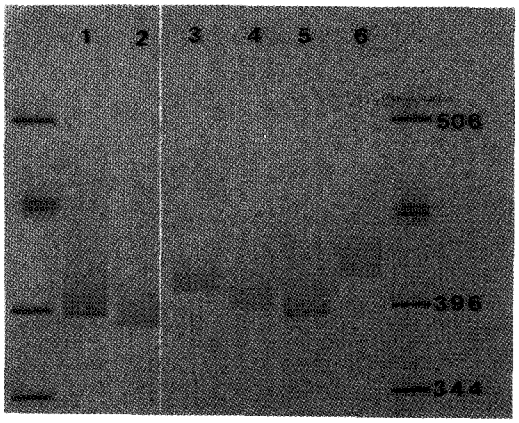

Fig. 2. Amplification of internal transcribed spacer (ITS) II between 5.8S and 28S rDNA genes of *Pleurotus* spp. lane 1, *P. eryngii* ASI 2125; lane 2, *P. sajor-caju* ASI 2070; lane 3, *P. cystidious* ASI 2079; lane 4, *P. florida* ASI 2014; lane 5, *P. ostreatus* ASI 2001; lane 6, *P. cornucopiae* ASI 2011.

plification, one unique band including ITS II region was produced at around 396 bp and the sizes of obtained ITS II were different one another. Two hundred and thirteen bp as the longest ITS II region amplified by PCR was the one extracted from *P. cornucopiae* and 189 bp as the shortest one was from *P. sajor-caju*. The sizes of *P. cystidious, P. florida, P. eryngii* and *P. ostreatus* were slightly different one anther as well.

To examine the variation within a same species, 9 isolates of *P. ostreatus* were chosen and the ITS II regions were amplified by PCR with their own genomic DNA. In general, there were no variations in the length of ITS II region except the isolates ASI 2095 and ASI 2095. They contained 5-11 bp longer ITS II region than that of others (Fig. 3).

We also examined 4 isolates of *P. sajor-caju*, 4 isolates of *P. florida*, 3 isolates of *P. cystidiosus* and 2 isolates of *P. cornucopiae*. All the isolates had the same size of ITS II region (Fig. 4) to their own representative species which had been confirmed by morphological

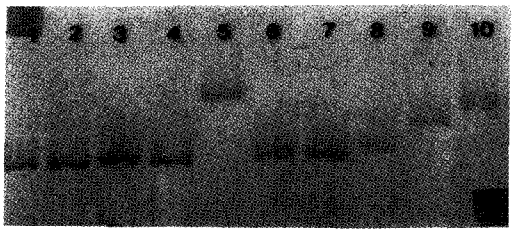

Fig. 3. Amplification of ITS II region of *Pleurotus ostreatus* which were classified by morphological studies. lane 1, ASI 2001 (Korea); lane 2, ASI 2003 (Japan); lane 3, ASI 2017 (Denmark); lane 4, ASI 2018 (Korea); lane 5, ASI 2025 (Korea); lane 6, ASI 2028 (Korea); lane 7, ASI 2072 (Korea); lane 8, ASI 2180 (Korea); lane 9, ASI 2095 (Taiwan); lane 10, *P. cornucopiae* ASI 2011 (Japan).

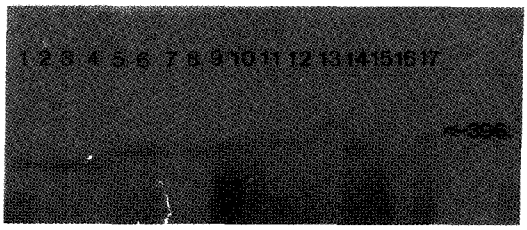

Fig. 4. Amplifiction of ITS II region between 5.8S and 28S rDNA genes of ***Pleurotus*** spp. which were classified by morphological studies. *P. sajor-caju*: lane 1 and 16, ASI 2070 (India); lane 2, ASI 2085 (Hong Kong); lane 3, ASI 2091 (Japan); lane 4, ASI 2096 (Thailand); *P. florida*: lane 5 and 14, ASI 2013 (Hong Kong); lane 6, ASI 2014 (German); lane 7, ASI 2015 (German); lane 8, ASI 2016 (German); *P. cystidious*: lane 9 and 17, ASI 2019 (Taiwan); lane 10, ASI 2049 (Japan); lane 11, ASI 2079 (Taiwan); *P. cornucopiae*: lane 12, ASI2038 (Japan); lane 13, ASI 2011 (Japan).

studies. However, the isolate of ASI 2038 had 8 bp shorter ITS II region than that of ASI 2011 (Fig. 4, lanes 12, 14) and both of those had bean classified as *P. cornucopiae* by conventional methods according to thier morphological phenotype. Therefore, in this study, more accurate analysis was carried out by comparing nucleotide sequences of

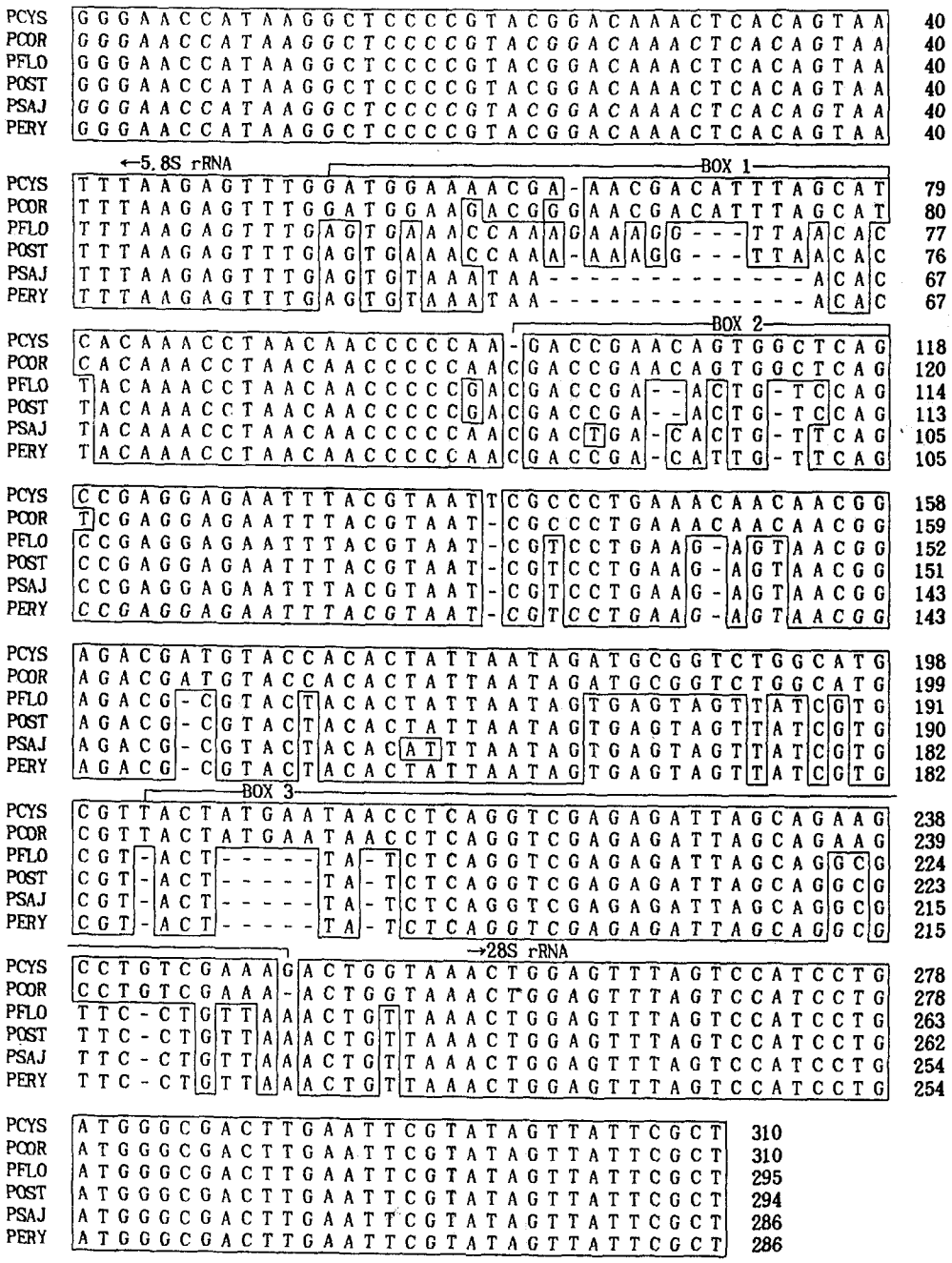

Fig. 5. Comparison of nucleotide sequence at the ITS II region of *Pleurotus* spp. PCYS, *P. cystidious* ASI 2079 (Taiwan); PCOR, *P. cornocupiae* ASI 2011 (Japan); PFLO, *P. florida* ASI 2014 (German); POST, *P. ostreatus* ASI 2001 (Korea); PSAJ, *P. sajor-caju* ASI 2070 (India); PERY, *P. eryngii* ASI 2125 (U.S.A)

ASI 2011 and ASI 2038 (Fig. 7). All the PCR amplified fragments were cloned into pTZ 19U plasmid vector and their nucleotide sequences were analyzed by dideoxy chain ter-

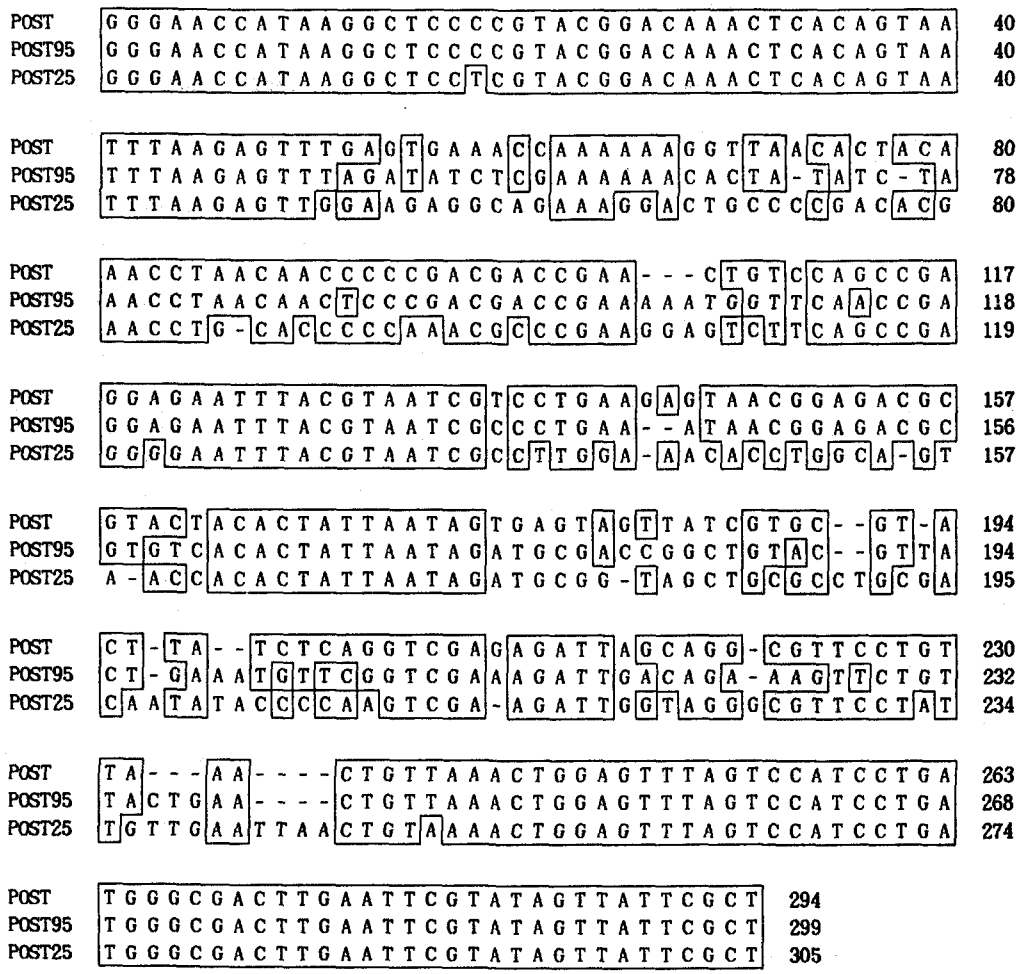

Fig. 6. Comparison of nucleotide sequence of ITS II region of *P. ostreatus*. POST, *P. ostreatus* ASI 2001 (Korea); POST25, *P. ostreatus* ASI 2025 (Korea); POST 95, *P. ostreatus* ASI 2095 (Taiwan).

mination method. Because 98 bp of 3'-end of 5.8S rDNA and 11 bp of 5'-end of 28S rDNA were completely homologus among the six species of *Pleurotus* genus, these sequences were eliminated in Fig. 5. The 5'-end and 3'-end of amplified DNA fragments were homologous to the 5.8S and 28S rDNA sequence of *Saccharomyces cerevisiae*, within the six representative species of *Pleurotus* genus. Even though the nucleotide sequences of the 5.8S rDNA and 28S rDNA coding region were completely homologous, those of the ITS II region were slightly different each other.

From the above results, the six representative species could be divided into 3 groups. In general *P. cystidious* (ASI 2079) and *P. cornucopiae* (ASI 2011) contained the longest ITS II region (213 bp). The next two species, *P. florida* (ASI 2014) and *P. ostreatus* (ASI 2001), had the middle size of ITS II region (198-197bp) and the third group, *P. sajor-caju* (189 bp) and *P. eryngii* (189 bp) contained the shortest region.

The ITS II region of *P. cyctidious* and *P. cornucopiae* had additional sequence such as "AACGACATTTA" and "ATGAA" compared

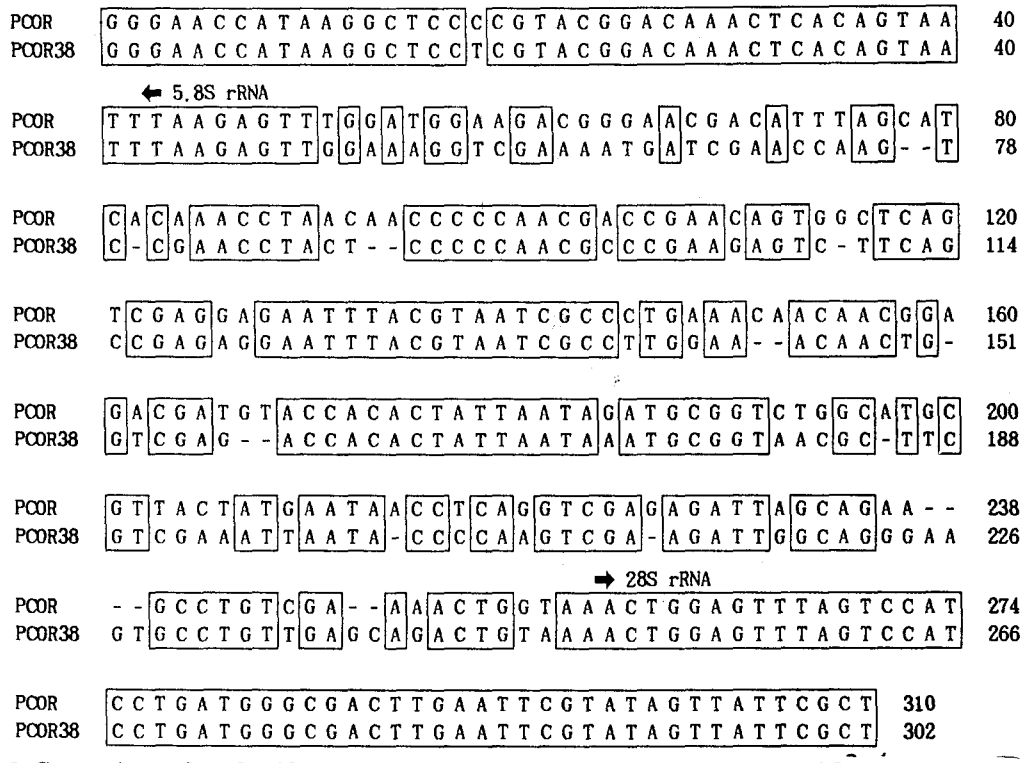

Fig. 7. Comparison of nucleotide sequence of ITS II region of *P. cornucopiae*. PCOR, *P. cornucopiae* ASI 2011 (Japan); PCOR 38, *P. cornucopiae* ASI 2038 (Japan).

to those of other species. *P. florida* had only one base insertion compared to that of *P. ostreatus*, while *P. sajor-caju* had 4 bases substitution compared to those of *P. eryngii*.

Since the expected sizes of PCR-amplified products were not obtained from the isolates ASI 2025 and ASI 2095 in comparison to their own representative species which had been classified by morphological studies, their nucleotide sequences were also analyzed to identify them. Fig. 6 shows nucleotide sequence variations among three isolates of *P. ostreatus* species (ASI 2001, ASI 2025 and ASI 2095). The nucleotide sequence homology of ITS II region was 72.4% between ASI 2025 and ASI 2095, 74.4% between ASI 2025 and ASI 2001, and 78.5% between ASI 2095 and 2001, respectively. The isolate ASI 2025 was 5 to 11 bp longer in ITS II region in com-

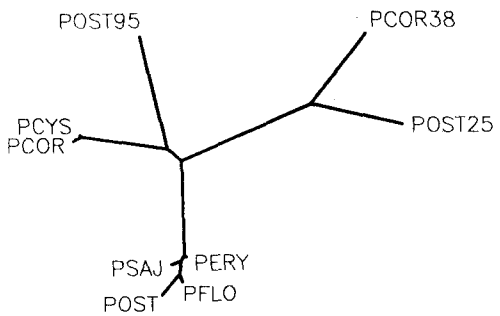

Fig. 8. Phylogenetic tree of *Pleurotus* spp. inferred by the Neighbor joining method. POST, *P. ostreatus* ASI 2001 (Korea); PFLO, *P. florida* ASI 2014 (German); PSAJ, *P. sajor-caju* ASI 2070 (India); PERY, *P. eryngii* ASI 2125 (U.S.A.); PCYS, *P. cystidious* ASI 2079 (Taiwan); PCOR, *P. cornucopiae* ASI 2011 (Japan); PCOR 38, *P. cornucopiae* ASI 2038 (Japan); POST 25, *P. ostreatus* ASI 2025 (Korea); POST 95, *P. ostreatus* ASI 2095 (Taiwan).

Table 2. Distance matrix of *Pleurotus* spp.

	PCOR	PCOR38	PCYS	PERY	PFLO	PSAJ	POST95	POST25	POST
PCOR	0.0000	0.2169	0.0078	0.1296	0.1429	0.1397	0.1366	0.2028	0.1499
PCOR38	0.2169	0.0000	0.2140	0.1843	0.2092	0.1996	0.2047	0.1127	0.2231
PCYS	0.0078	0.2140	0.0000	0.1231	0.1401	0.1332	0.1308	0.2069	0.1472
PERY	0.1296	0.1843	0.1231	0.0000	0.0195	0.0139	0.1515	0.1955	0.0310
PFLO	0.1429	0.2092	0.1401	0.0195	0.0000	0.0224	0.1555	0.2159	0.0219
PSAJ	0.1397	0.1996	0.1332	0.0139	0.0224	0.0000	0.1620	0.2108	0.0340
POST95	0.1366	0.2047	0.1308	0.1515	0.1555	0.1620	0.0000	0.2203	0.1631
POST25	0.2028	0.1127	0.2069	0.1955	0.2159	0.2108	0.2203	0.0000	0.2251
POST	0.1499	0.2231	0.1472	0.0310	0.0219	0.0340	0.1631	0.2251	0.0000

PCOR, *P. cornucopiae* ASI 2011 (Japan); PCOR38, *P. cornucopiae* ASI 2038 (Japan); PCYS, *P. cystidious* ASI 2079 (Taiwan); PERY, *P. eryngii* ASI 2125 (U.S.A.); PFLO, *P. florida* ASI 2014 (German); PSAJ, *P. sajor-caju* ASI 2070 (India); POST95, *P. ostreatus* ASI 2095 (Taiwan); POST25, *P. ostreatus* ASI 2025 (Korea); POST, *P. ostreatus* ASI 2001 (Korea).

Numbers are 1/2Knuc values between ITS II rDNA sequences of the both compared species

parison to those of ASI 2095 and ASI 2001. In case of *P. cornucopiae* species the nucleotide sequences of the ITS II region were quite variable between the isolates ASI 2011 and ASI 2038 whose their homology was 77.8% (Fig. 8). The nucleotide sequence of ITS II region of ASI 2011 was 8 bp longer than that of ASI 2038.

Discussion

In this study, we tried to classify 6 representative species of genus *Pleurotus* using PCR-amplified ITS II region of rDNA repeat unit. Our results confirmed that the two primers used in this study were useful for classification of the species. The two primers which were taken from the 3'-end of 5.8S rDNA coding region and the 5'-end of 28S rDNA coding region could amplify the exact position from the genomic DNA of *Pleurotus* spp. Furthermore, all the ITS II regions amplified using PCR from 6 different species could be easily differentiated on the polyacrylamide gel according to their length polymorphism. This technique appeared to be much more accurate and efficient than any other conventional biological method for species classification such as isozyme pattern and could be useful for differentiating other mushroom species as well.

To provide insight at molecular level, the nucleotide sequences of their PCR-amplified ITS II region were determined. The major sequence variations such as nucleotide insertion or substitution were identified at box I, box II and box III, by comparing the shortest and longest ITS II region (Figs. 5-7).

All the nucletoide sequences of ITS II region were compared with each other and the frequency of sequence variation was calculated by Kimura's two parameter method. The mean values were then arranged in a matrix table as shown in Table 2. The nucleotide sequence divergences were converted to 1/2 Knuc values between the nucleotide sequences of the ITS II region of both two compared species. This matrix table was utilized directly for a phylogenetic analysis using the Neighbor-joining method of the PHYLIP package. As shown in Fig. 8., *P. ostreatus* (ASI 2001), *P. florida* (ASI 2014), *P. sajor-caju* (ASI 2070) and *P. eryngii* (ASI 2125) were located on the same lineage and *P. cystidious* (ASI

2079), *P. cornucopiae* (ASI 2011) and *P. ostreatus* (ASI 2095) were located on another lineage, while ASI 2038 and ASI 2025 exhibited their own individual lineages. The two strains (ASI 2025 and ASI 2095) of *P. ostreatus* and one strain (ASI 2038) of *P. cornucopiae* were located at relatively large distance from other *Pleurotus* spp.

There have been much debating about the classification of ASI 2095 and ASI 2025 isolates since they showed physiological variations according to their geographic origin. In gernral, *P. ostreatus* has a white stem and gill reaching into bottom of stem and grows usually in temperate zone. However, ASI 2025 collected at mountain area of Kangwon province, South Korea has a dark yellowish brown stem and gill reaching into only upper part of stem and ASI 2095 originally collected from Taiwan which is a subtropical area, showes higher optimal temperature for fruiting than that of a typical *P. ostreatus*. Although they were identified to be *P. ostreatus* physiologically different from other typical *Pleurotus ostreatus*. In this sense, We suggest that our results can provide a new criteria for the classification of such an ambiguous isolates.

Many similar studies have been reported with our results. For example, Hopple and Vilgalys (1994) recently reported that the seven species of *Coprinus* genus exhibited their own branch in the phylogenetic tree based on RFLP of ITS II region. Anderson and Stasovski (1992) also investigated the PCR-amplified intergenic region between the 3'-end of 26S rDNA gene and the 5'-end of 16S rDNA gene of northern hemisphere species of *Armillaria*. In case of *Armillaria* spp., *A. ostoyae, A. gemina* and *A. borealis* were more closely related to one another than to any other species. They suggested that the morphological divergence in *A. mellea* and *A. tabescens* relative to the majority of northern hemisphere species was roughly paralleled by divergence in the intergenic region (IGR). Our results also suggest that most of phenotypically similar species of *Pleurotus* formed a same phylogenetic lineage.

Another interesting result was also reported with the genus *Lyophyllum* based on the RFLP analysis of the PCR-amplified ITS II region by Monclavo (1992). He compared the result obtained by three different methods for taxonomic criteria; mycelial behavior, isozyme pattern for a typical enzyme, and molecular data from a PCR-amplified ITS II region of the ribosomal DNA. He suggested that all the methods are valuable in its own and the results supported each other for more meaningful interpretation. In this regard, we plan to investigate and compare taxomomic criteria such as isozyme pattern and other biochemical means. Nevertheless, our results of molecular biological studies of *Pleurotus* spp. seem to be valuable for further investigation.

Acknowledgement

We thank Dr. Hack Sung Jung of the Deparment of Microbiology, College of Natural Sciences, Seoul National University for his kind arrangement of computer analysis.

적 요

느타리 버섯속 6종 23균주에 대한 rDNA의 ITS II 부위의 DNA 염기서열을 비교 분석함으로서 종간 및 종내의 근연관계를 조사하였다. rDNA의 ITS II 부위를 증폭하고자 5.8S rDNA의 3'말단 부위와 28S rDNA의 5'말단 부위에 두개 프라이머를 이용하여 PCR 증폭을 행하였다.

느타리 버섯 6종의 게놈 DNA로 부터 ITS II 부위를 증폭한 결과 종에 따라 밴드 길이에 차이가 있었으며 ITS II 부위의 길이 차이로 느타리 버섯

6종을 구분할 수 있었다. 같은 종내 개체간 구분을 위하여 느타리 버섯 6종 23균주의 PCR 증폭 결과는 느타리 버섯(*P. ostreatus*) ASI 2095, ASI 2025와 노랑 느타리 버섯(*P. cornucopiae*) ASI 2038 균주외에 동일 종내 균주의 ITS II 길이는 동일한 양상을 보였다.

느타리 버섯 6종 및 ASI 2095, ASI 2025 그리고 ASI 2038에 대한 ITS II 부위의 염기서열을 비교해 보면 염기서열의 변이가 ITS II 부위가 시작되는 부분과 말단 부분에서 주로 존재하였다. ASI 2095와 ASI 2025 균주들은 동일 종내 느타리 버섯(*P. ostreatus*, ASI 2001) 균주와 ASI 2038와 ITS II 부위의 DNA 염기서열에 차이를 보였다. 이들 염기서열을 기초로 하여 Neighbor program을 이용한 균주간 유연관계는 느타리 버섯(*P. ostreatus*), 사철 느타리 버섯(*P. florida*), 여름 느타리 버섯(*P. sajor-caju*) 그리고 맛 느타리 버섯(*P. eryngii*)들이, 노랑 느타리 버섯(*P. cornucopiae*)과 호고 느타리 버섯(*P. cystidious*)이 각각 서로 가까운 유연관계를 나타내었으나ASI 2025와 ASI 2038 균주들은 특이한 계통분지를 나타내었다.

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