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Identification of Some *Phellinus* spp.

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Four strains of *Phellinus* spp. was identified based on internal transcribed spacer (ITS) region of rDNA sequence analysis and morphological characteristics. Basidiocarps of all strains were effused-reflexed and hymenial surface was poroid. Hyphal system was dimitic and basidiospore was globose to ellipsoid. The amplification of ITS regions produced a DNA fragment of 500 to 780 bp in all strains examined. The determined sequences were analyzed for the reconstruction of phylogenetic tree. From these results, *Phellinus* sp. KM-1, KM-2, and KM-4 was identified as *P. hartigii*, *P. baumii*, and *P. linteus*, respectively.

KEYWORDS: ITS region, Molecular systematics, Morphological characteristics, *Phellinus*

Bracket fungus, *Phellinus* was occurred parasitically on deciduous trees, especially willow, causing intensive white rot. About two hundred species in genus of *Phellinus* have been reported all over the world. Many species in the genus have basidiocarps whit various colors and shapes, and the characteristics would easily change as grow older. So, the use of these characteristics in classification of *Phellinus* would lead to incorrect taxonomic conclusions. Some species of these fungi have been used as a medicinal ingredient and Indian folk medicines (Vaidya and Rabba, 1993). Recently, effective component of these fungi are isolated and characterized. Polysaccharides purified from *P. linteus* mycelium strongly stimulated B-lymphocyte production (Song *et al.*, 1995), cell-mediated and humoral immunity (Kim *et al.*, 1996), and inhibited tumor growth and metastasis (Han *et al.*, 1999). However, the precise identification of *Phellinus*, at the level of species, has not been reported and it has been in a controversy until now what species of these fungi are effective in cancer therapy.

On the basis of morphological and molecular properties, the identification of four species of *Phellinus*, which are popularly used, was reported in the present paper.

Materials and Methods

Strains and culture conditions. The fungal strains used in this experiment were supplied from FCCDU (Fungal Culture Collection of Daejeon University) and purchased from KCTC (Korean Collection for Type Cultures). The species whose sequences were analyzed and examined in this study were listed in Table 1 with related information. Basidiocarps were cut into pieces smaller than half a centimeter cube with a sterile scalpel and two or three pieces were inoculated on PDA plates and incubated at 30°C under dark condition for two weeks. Two or three colonies were subcultured on a new PDA plate with a sterile cellophane disc on it for the preparation of DNA templates. According to the method of Lecellier and Silar (1994), the mycelium is directly recovered from the cellophane disc covering.

Table 1. List of strains used in this study

Strains	Source	Amplicon size (bp)	Accession No.
Phellinus linteus	KCTC 6190	770	-
Phellinus linteus	KCTC 6719	750	-
Phellinus sp. KM-1	North Korea	550	AF357601
Phellinus sp. KM-2	Yeonbeon (China)	780	AF357602
Phellinus sp. KM-3	Unnam (China)	500	AF357603
Phellinus sp. KM-4	Tibet (China)	750	AF357604

Total genomic DNA extraction. DNA extraction was done by the rapid method for nucleic acids extraction from petri dish-grown mycelia (Lecellier and Silar, 1994). About 10 cm^2 of maginal growing mycelia were harvested in an Eppendorf tube, then 600 μl extraction buffer [100 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS] was added. After vortexing for 30 s, frozen in liquid nitrogen for 30 s and incubated in a dry incubator for complete thawing at 70°C for 30 s, which process was repeated three times. Extracted DNA was purified with classical phenol-chloroform extraction steps: phenol, phenol-chloroform (1 : 1), and chloroform extraction. The purified DNA was precipitated with 1 volume of isopropanol and centrifuged immediately at 12000 rpm for 10 min at room temperature. The pellet was dry in air and resuspended in 50 μl of deionized water.

Amplification and sequencing of ITS region. ITS region

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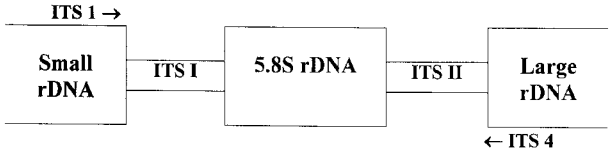

Fig. 1. Locations of primers for the amplification and sequencing of ITS regions. ITS 1(forward primer): 5'-TCCGTAGG-TGAACCTGCGG-3' (19mer), ITS 4(reverse primer): 5'-TCCTCCGCTTATTGATATGC-3' (20mer).

of *Phellinus* was amplified with the primer ITS1 and ITS4 (White *et al.* 1990, Fig. 1). Amplification was performed in a 50 μl reaction mixture containing each 50 pmol of primers, 2.5 unit of Taq polymerase, each 2.5 mM of dNTP mixture, 5 μl of 10× PCR buffer, and 10 ng of template DNA. The reaction was performed as follows; denaturation for 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, with final extension of 10 min at 72°C. The PCR products were analyzed by electrophoresis with 1.5% agarose (in 0.5% TBE) and purified using Gel Extraction Kit-spin (NucleoGen, Korea) using the procedure recommended by the supplier. The purified PCR product was sequenced with an automated DNA sequencer (ABI PRISM™ 377, Perkin Elmer). Homologous sequences were found by BLAST Search. The sequences determined in this study was deposited in the GenBank under accession number AF357601 to AF357604 (Table 1).

Reconstruction of phylogenetic tree. Sequences generated from materials and retrieved from GenBank were visually aligned after initial alignment by the program CLUSTAL X (Thompson *et al.*, 1997). Ambiguously aligned regions were excluded. Phylogenetic relationships were estimated from the aligned sequences for each data set using PAUP*4.0b4a (Swofford, 1999), treating all alignment gaps as missing data. Phylogenetic analyses of data sets were done using both distance and parsimony methods. Neighbor-joining method (Saitou and Nei, 1987) with distance option and parsimony method with heuristic option were applied for phylogenetic analyses. Strengths of internal branches found in distance and parsimony analyses were statistically tested by the bootstrap analyses of 1000 replications (Felsenstein, 1985; Hillis and Bull, 1993).

Results and Discussion

Morphological description. The basidiocarps of examined strains showed typical *Phellinus* characters. The fruitbodies were effused-reflexed and hymenial surfaces were poroid (Fig. 2).

***Phellinus* sp. KM-1.** The shape of pores is circular with 5~6 per mm, thick and entire dissempiments. Hyphal system is dimitic, generative hypha with 2~3 μm, skeletal hypha with 3.4~5.7 μm in diam. Setae was not seen. Basidiospores are globose, 5.1~6.3×4.7~5.5 μm.

***Phellinus* sp. KM-2.** The shape of pores is circular with

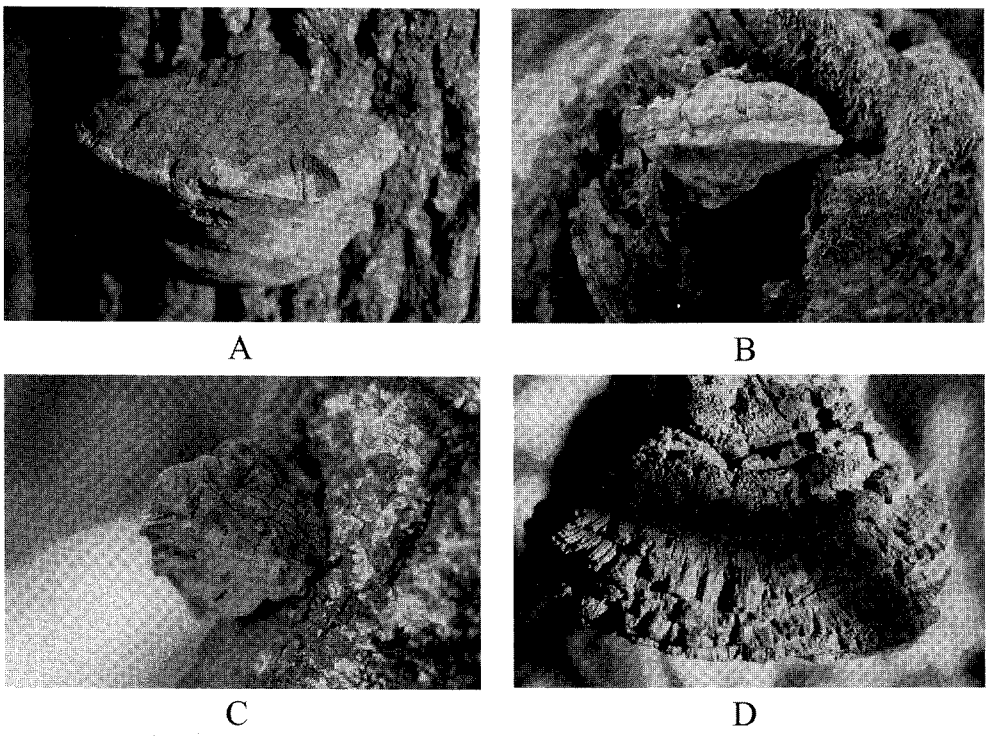

Fig. 2. Photographs of basidiocarps of *Phellinus* spp. KM-1 (A), KM-2 (B), KM-3 (C), and KM-4 (D).

8~9 per mm. Hyphal system is dimitic, generative hypha with 2~3 μm, skeletal hypha with 4~6 μm in diam. Setae is abundant, thick-walled, dark brown in KOH, 19.4~32× 6.8~7.9 μm. Basidiospores are ellipsoid, 4.1~4.3×3.0~3.2 μm.

***Phellinus* sp. KM-3.** The shape of pores is circular with 7~8 per mm. Hyphal system is dimitic, generative hypha with 2~4 μm, skeletal hypha with 3~6 μm in diam. Setae was not shown. Basidiospores are ellipsoid, 4.1~5.2×3.2~ 3.5 μm.

***Phellinus* sp. KM-4.** The shape of pores is circular with 6~8 per mm. Hyphal system is dimitic, generative hypha with 2~3 μm, skeletal hypha with 4.2~6.7 μm in diam. Setae is abundant, thick-walled, dark brown in KOH, 26~ 27×9.14~11 μm. Basidiospores are ellipsoid, 4.0~4.3×3.4 μm.

Molecular systematics. Segments of ITS rDNA encompassing 500 to 780 bp of 15 taxa were analyzed. The amplicon size of strain KM-2 (780 bp) and KM-4 (750 bp) was similar to that of *P. linteus* KCTC 6190 (770 bp) and KCTC 6719 (750 bp). The PCR product of KM-1 showed a homology of 98 and 97% with ITS rDNA of *P. hartigii* and *P. robustus*, respectively. The DNA fragment of strain KM-2 showed 93% similarity with ITS rDNA of *P. baumii* MPNU 7006 and KM-4 showed a 99% homology with ITS rDNA of *P. linteus* MPNU 7002 and *P. linteus* IFO 6980. Alignment of sequences was initially accomplished by clustal X. Parsimony analysis with the data set produced one parsimonious trees of 1006 steps (CI = 0.7495, RI = 0.6415, RC = 0.4808). The tree was rooted with the sequence of *Schizopora radula* that was deduced by previously studies from various rDNA sequences analysis (Hibbett and Donoghue, 1995). The maximum parsimony cladogram is presented in Fig. 3 along with the bootstrap values. The *Phellinus* was separated in three groups, *hartigii-robustus-igniarius* group, *linteus-baumii* group, and *pini-Inototus* group. This result was similar to the previous work, which showed at least six separated clade, namely, group A (*P. linteus*, *P. baumii*), *P. rimosus* complex, *P. pini* complex, *P. igniarius* complex 1, *P. igniarius* complex 2, and *P. robustus* complex consisting *P. robustus* and *P. hartigii* (Jeong, 1999). Strain KM-1 was belongs to *P. robustus* complex that was supported by 91% bootstrap value and connected to *P. hartigii* with 100% bootstrap. KM-2 and KM-4 were formed *linteus-baumii* group with 100% bootstrap value and KM-3 was more or less separated with *linteus-baumii* group. From these results, *Phellinus* sp. KM-1, KM-2, and KM-4 was identified as *P. hartigii*, *P. baumii*, and *P. linteus*, respectively. The PCR product of strain KM-3 showed only a low homology with 18S rRNA of *I. radiatus* (78%) and

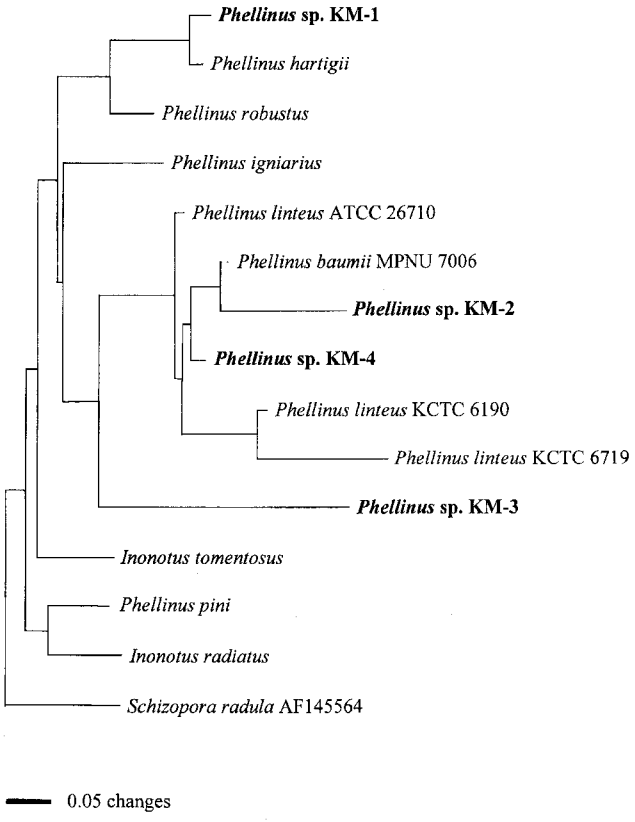

Fig. 3. Phylogenetic relationships of *Phellinus* inferred from ITS sequences. Parsimony analysis with the data set produced one parsimonious trees of 1006 steps (CI = 0.7495, RI = 0.6415, RC = 0.4808).

the morphological characteristics were similar to those of *P. baumii*. So, it will be needed to study further for the detailed phylogeny of strain KM-3.

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References

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Han, S. B., Lee, C. W., Jeon, Y. J., Hong, N. D., Yoo, I. D., Yang, K. H. and Kim, H. M. 1999. The inhibitory effect of polysaccharides isolated from *Phellinus linteus* on tumor growth and metastasis. *Immunopharmacology* **41**: 157-164.

Hibbett, D. S. and Donoghue, M. J. 1995. Progress toward a phylogenetic analysis of the Polyporaceae through parsimony analysis of mitochondrial ribosomal DNA sequences. *Can. J. Bot.* **73**: S853-S861.

Hillis, D. M. and Bull, J. J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* **42**: 182-192.

Jenong, W. J. 1999. Phylogenetic analysis of *Phellinus* based on internal transcribed spacer and mitochondrial small subunit

ribosomal sequences. Ph.D. Thesis. Seoul National University, Seoul, Korea.

Kim, H. M., Han, S. B., Oh, G. T., Kim, Y. H., Hong, D. H., Hong, N. D. and Yoo, I. D. 1996. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. *Int. J. Immunopharmac.* **18**: 295-303.

Lecellier, G. and Silar, P. 1994. Rapid methods for nucleic acids extraction from Petri dish-grown mycelia. *Curr. Genet.* **25**: 122-123.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Song, K. S., Cho, S. M., Lee, J. H., Kim, H. M., Han, S. B., Ko, K. S. and Yoo, I. D. 1995. B-lymphocyte-stimulating polysaccharide from mushroom *Phellinus linteus*. *Chem. Pharm. Bull. (Tokyo)* **43**: 2105-2108.

Swofford, D. L. 1999. PAUP*: phylogenetic analysis using parsimony (* and other method), version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids. Res.* **24**: 4876-4882.

Vaidya, J. G. and Rabba, A. S. 1993. Fungi in folk medicine. *Mycologist* **7**: 131-133.

White, T. J., Bruns, T., Lee, S. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322. *In*: Innis, M. A., Gelfand, D. H, Sninsky, J. J. and White, T. J. Eds. PCR protocols: A guide to methods and application. Academic Press. San Diego.