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Analysis of Cultural Characteristics and Phylogenic Relationships of Collected Strains of *Pholiota* species

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Cultural characteristics and phylogenic relationships were investigated and classified among collected strains in *Pholiota* spp. which contain *P. adiposa*, *P. squarrosa*, *P. nameko* etc. They were tested on the four different media (PDA, MCM, YM, MEA) and sawdust (Alder, Oak, Pine, Popular) substrates. There was a little variation according to the media and sawdust substrates, although PDA and popular sawdust substrate seemed to be better. Most strains showed white colonies, but some strains were brown. Mycelial growth length differed according to the strains. To classify species, the internal transcribed spacer regions (ITS) of the ribosomal DNA (rDNA) repeats from *Pholiota* spp. were amplified using polymerase chain reaction (PCR) and then sequenced. According to the analysis of ITS sequences, they were classified into five clusters. Their spacer regions were 644~700 nucleotides in length. The reciprocal homologies of each ITS region among these strains were ranged from 49.6~99.9%. The phylogenic analysis might give a criterion to classify species in the collected strains.

KEYWORDS: ITS, *Pholiota*, rDNA

The health benefits of mushroom have been well known for a long time and are widely recognized around the world (Sung, 1998). Nowadays, the demands for "healthy food" or "functional food", are increasing in many countries. The mushroom provides potentially beneficial effects for several of the most common diseases afflicting human beings, including cancer (Chung, 1982). Furthermore, a lot of attention has been recently directed to the development of natural antioxidants as biologically active substances that can exert considerable protection against aging and cancer caused by free-radicals in humans (Cutler, 1984).

Pholiota (Fr.) Kummer is a genus of saprophytic species. Most species in the genus are active wood destroyers and, sometimes, are parasitic. Some live on charcoal, soil or humus but no species forms ectotrophic mycorrhiza (Jacobsson, 1989). *Pholiota* genus is a Basidiomycota phylum, Homobasidiomycetes class, Agaricales order, Strophariaceae family (Lee, 1988). A species of *Pholiota* genus, *P. nameko*, is not only cultured as edible mushroom, but is also a well known health food in Japan. Recently, various *Pholiota* spp. components possessing biological characteristics such as anticarcinogens, antioxidatives, antitumor, high quality protein, organic acid and vitamin have received much attention from many researchers (Mizuno, 1994). However, the taxonomy of this edible mushrooms, however, is still confusing apparently due to expansion of special cultivation techniques and incorrect of naming of newly cultivated strains (Bae, 1996). Collected strains of *Pholiota* spp. are so far ambiguously or incorrectly named, leading to erroneous identification. Therefore, analysis of ITS is regard to a useful method to discriminate species in recently (Chung, 1999). The strong preservation of functioning genomic zones (5.8s, 18s, 28s) does not permit an intergeneric discrimination, whereas the large variability of the ITS region justified the choice of this intergene for collected *Pholiota* spp.

This study investigated the characteristics and phylogenic relationship among collected *Pholiota* spp. Results of this study could supply characteristics and taxonomical information.

Materials and Methods

The strains used. Forty-five collected strains were used in investigating cultural characteristics and phylogenic relationships (Table 1). These strains were cultured and maintained on potato dextrose agar (PDA).

Colony characteristics. Four different culture media (Table 2) were used to investigate a favorable growth of mycelia in *Pholiota* spp. In order to prepare the inoculum, the collected strains were grown on the PDA media. Then, the cultured mycelia of each strain were inoculated at the center of each media petri dish using a sterile cork

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Table 1. The list of the strains used in this study

Strain no. (ASI)[a]	Scientific name	Geographical origins	Strain no. (ASI)	Scientific name	Geographical origins
24001	*Pholiota adiposa*	Korea	24024	*P. adiposa*	Korea
24002	*P. squarrosa*	Korea	24025	*P. squarrosa*	Korea
24003	*P. adiposa*	Korea	24026	*P. flammans*	Korea
24004	*P. adiposa*	Korea	24027	*P. adiposa*	Korea
24005	*P. squarrosa*	Korea	24028	*P. aurivella*	Korea
24006	*P. squarrosa*	Korea	24029	*P. adiposa*	Korea
24007	*P. squarrosa*	Korea	24030	*P. adiposa*	Korea
24008	*P.* sp	Korea	24031	*P. aurivella*	Korea
24009	*P. squarrosa*	Korea	24032	*P. carbonaria*	Korea
24010	*P. adiposa*	Korea	24033	*P. carbonaria*	Korea
24011	*P. carbonaria*	Korea	24034	*P. lucifera*	Korea
24012	*P. adiposa*	Korea	24035	*P. lucifera*	Korea
24013	*P. adiposa*	Korea	24036	*P. nameko*	Korea
24014	*P. malicola*	U.S.A	24037	*P. nameko*	Korea
24015	*P. malicola*	U.S.A	24038	*P. squarrosa*	Korea
24016	*P. aggericola*	U.S.A	24039	*P. squarrosa*	Korea
24017	*P. terrestris*	Korea	24040	*P. squarrosa-adiposa*	Korea
24018	*P. adiposa*	Korea	5008	*P. nameko*	Japan
24019	*P.* sp	Korea	5010	*P. nameko*	Japan
24020	*P. highlandensis*	Korea	5011	*P. nameko*	Japan
24021	*P. highlandensis*	Korea	5019	*P. nameko*	Korea
24022	*P. adiposa*	Korea	5020	*P. nameko*	China
24023	*P.* sp	Korea			

[a]ASI: Agricultural Science Institute, Suwon.

Table 2. Composition of media used

Ingredient	Medium (g/*l*)			
	PDA[a]	MCM	MEA	YMA
$MgSO_4 \cdot 7H_2O$		0.5		
KH_2PO_4		0.46		
K_2HPO_4		1.0		
Peptone		2.0	5.0	5.0
Yeast extract		2.0		3.0
Malt extract			20.0	3.0
Dextrose		20		10.0
PDA (Difco)	21.0			
Agar	20.0	20	20.0	20.0

[a]PDA (Potato Dextrose Agar), MCM (Mushroom Complete Medium), MEA (Malt Extract Agar), YMA (Yeast Malt Agar).

borer (10 mm dia.). After 14 days of incubation, the mycelial growth was measured.

Selection of optimal media for cultivation. Four different sawdust (Alder, Oak, Pine, Popular) were used to test characteristics of mycelia growth length. For inoculums, each sawdust media was poured into test tube (length 20 cm, dia. 3 cm) to 15 cm. All of those media were autoclaved for 40 minutes at 121°C. The mycelia of the five strains were inoculated in the test tube, and then monitored for mycelial growth length four times a week (Table 3).

DNA preparation and ITS sequencing. Total genomic DNA was prepared using a modification of the simplified method described by Graham (Graham, 1994). For PCR-amplification of ITS I-II region, the sequences of two primers ITS 1 and ITS 4 (Primer ITS 1 : 5'-TCC GTA GGT GAA CCT GCG G-3', ITS 4 : 5'-TCC TCC GCT TAT TGA TAT GC-3') were chosen from the known sequence of the 3'-end of 18s rRNA gene and the 5'-end of 28s rRNA gene of rDNA repeat unit and were synthesized using automatic DNA synthesizer (Applied Biosystems Model/9600) (Choi, 2000). The PCR-amplified fragments were subcloned into pGEM T vector (Promega) and the nucleotide sequences were determined by dideoxy chain termination method with forward sequencing and reverse sequencing primer (Park, 1999). Alignment of nucleotide sequences was determined using DNA Star program.

Results and Discussions

Colony characteristics and selection of optimal media for cultivation. Four different culture media were used to investigate colony characteristics in *Pholiota* spp. (Fig. 1). The mycelial growth of *Pholiota* spp. on four different media was observed in the range of 8.0~63.7 mm and 19~87.0 mm for 7 days and 14 days incubation, respectively (data not shown). It was observed that the average mycelia growth were good on PDA media. But there were some variations according to strains in different media.

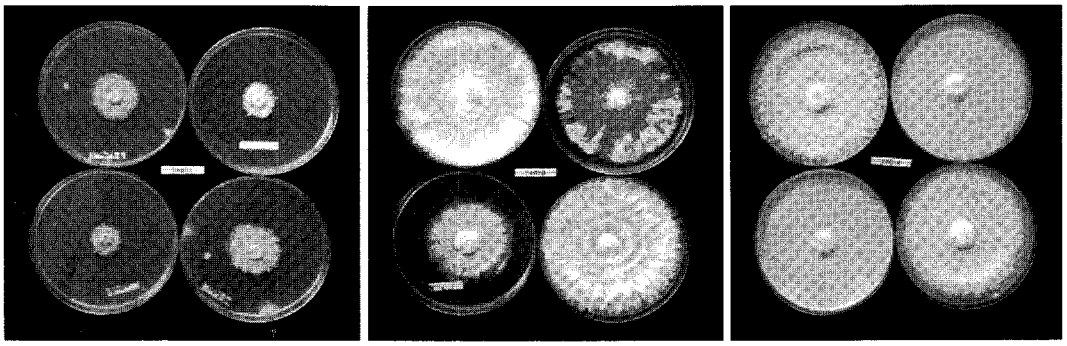

Fig. 1. Comparison of culture characteristics among *Pholiota* strains on different media. They are arranged in clockwise rotation with PDA, YM, MEA and MCM from upper left.

Table 3. Grouping according to different colony morphology

Group		Growth length (mm)[a]		Strains No. in this group
		7 days	14 days	
A	A1	46±4.9	80±6.4	24001, 24003, 24004, 24010, 24012, 24013, 24018, 24022, 24024, 24027, 24015, 24008, 24002, 24005, 24007, 24025, 24017
	A2	34±7.0	63±15.6	24011, 24020, 24009, 24040
	A3	32±11.6	60±19.3	24030, 24028, 24031
	A4	36±10.9	68±19.4	24029, 24035, 5010
B		39	87	24019
C		42±10.7	74±11.8	5008, 5011, 5019, 5020, 24036, 24037
D	D1	57	87	24033
	D2	13±0.7	23±0.5	24038, 24039
	D3	20	30	24021
	D4	24	45	24034
E		60±5.2	87±0.0	24014, 24016

[a]Growth length : Mean±S.D.

Most strains showed white colonies, but some strains were brown. After the strains were identified by colony morphology on PDA and taken into account phylogenetic tree (Fig. 3), they were divided into 11 small groups (Table 3). Most of *P. adiposa* strains belonged to A1 group and *P. nameko* strains belonged to C group.

The study selected five representative strains in A, C, D and E group, which were tested on four different sawdust media. They showed different response to substrate demands. The results revealed that popular sawdust was more favorable for the mycelial growth of *Pholiota* spp. than *P. nameko* (ASI 5019), which grew better on pine tree sawdust.

Forty-five collected strains in *Pholiota* spp. were used in this study (Table 1). According to the phylogenic tree (Fig. 2), the collected strains of *Pholiota* spp. were classified five groups. As the result of ITS analysis, the length variation for the entire ITS region (including 5.8S) ranged

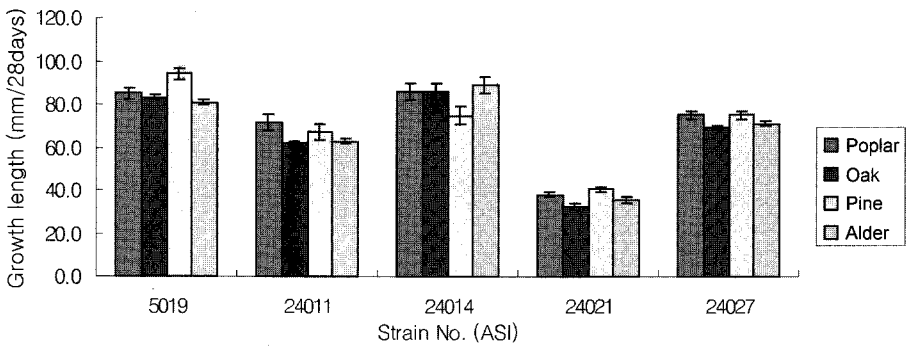

Fig. 2. Mycelial growth length according to different sawdust media.

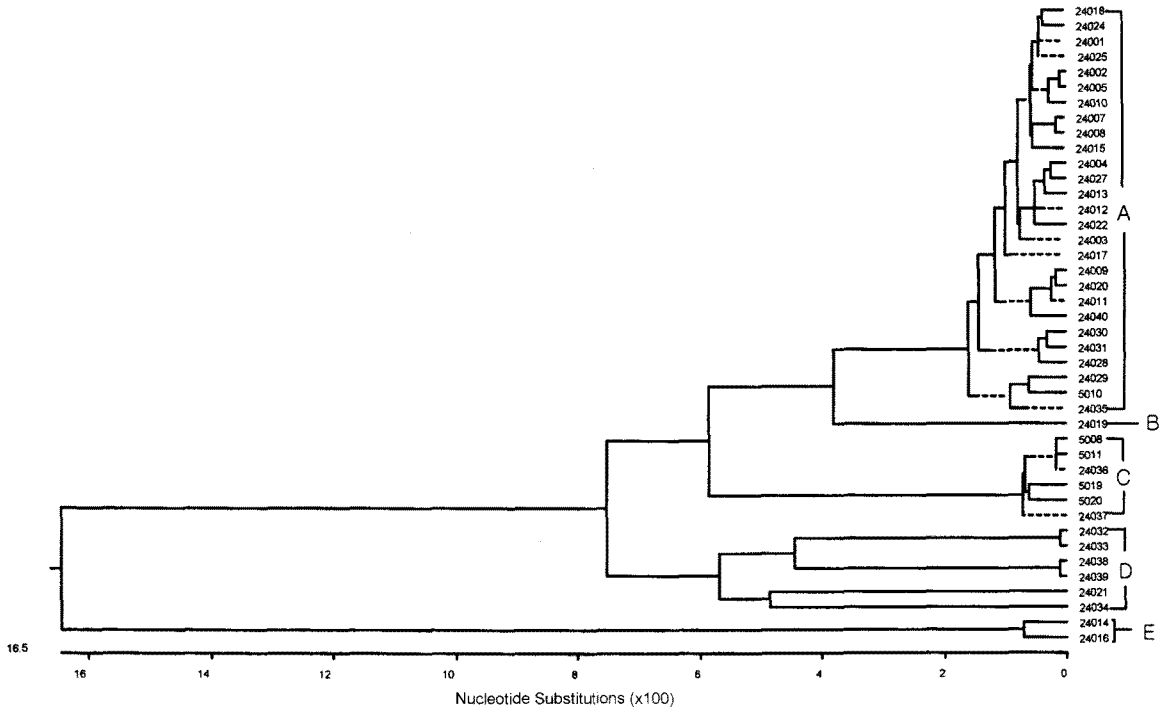

Fig. 3. The Phylogenetic tree based on ITS region sequences of 42 *Pholiota* spp.

from 644~700 bp. The nucleotide sequence similarity of ITS region among *Pholiota* spp. tested was 49.6~99.9%. The nucleotide sequence homology was 95.3~100% among strains in group A, 98.8~99.9% in group C, 76.3~99.9% in group D, 97.6% in group E, respectively. In case of comparison between groups, the genetic homologies of ITS sequences were 52.9% between group A and B, 49.6% between group A and E, respectively. The components of group A, D, and E consisted of several species. However, the species of group C was the same with *Pholiota nameko*. This result showed that the phylogenic analysis could give a criterion to classify species in the collected strains, especially, *Pholiota* nameko.

In the confirmed sequences tested, ASI 24027 (Genbank. AY251300), 24032 (Genbank. AY251301), 24038 (Genbank. AY251302), 24040 (Genbank. AY251303), 24036 (Genbank. AY251304), 24028 (Genbank. AY251305), 24034 (Genbank. AY251306) were registered to National center for Biotechnology Information (NCBI) in U.S.A.

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