J. Life Science. (2001), 11(2), 126~134

JOURNAL OF
Life Science

Phylogenetic Analysis of *Phellinus linteus* and Related Species Comparing the Sequences of rDNA Internal Transcribed Spacers

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Received:

Abstract The phylogenetic tree displayed the presence of five groups in the *Phellinus* genus, which were distinguished based on their morphology. Most of the *P. linteus* appeared a cluster which was highly significant with the exception of *P. linteus* KACC 500122 and KACC 500411. They formed the sister taxa of *P. linteus* where *P. baumii*, *Phellinus* sp. MPNU 7003, MPNU 7007, and MPNU 7010 had similar morphological characteristics. Also, *P. nigricans* IMSNU 32024 and *P. pini* var. *carniformans* IMSNU 32031 were grouped in the same cluster with *P. igniarius* KCTC 6227, KCTC 6228, and *P. chrysoloma* KCTC 6225 extracted from the GenBank database. *P. torulosus* IMSNU 32028 and *Phellinus* sp. MPNU 7011 formed a closed group, however, these species had a distant taxa when compared with the other *Phellinus* species. The nucleotide sequences of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) including the 5.8S rDNA were determined from 24 strains of the *Phellinus* genus in order to analyze their phylogenetic relationship. These fungi were divided into two basic groups based on their ITS length, however, this grouping was different from that based on their morphological characteristics. Although, various ITS sequences were ambiguously aligned, conserved sites were also identified. Accordingly, a neighbor-joining tree was constructed using the nucleotide sequence data of the conserved sites of the ITS regions and the 5.8S rDNA.

Key words: Phylogeny, rDNA, *Phellinus*, ITS

Introduction

P. linteus, belongs taxonomically to the family of Hymenochaetaceae, is saprotrophic and lives on broad-leaf trees such as *Morus* and *Fagales* etc [2]. Hymenochaetaceae is white-rotters that lack clamp connections and often process setae [11]. At an ultrastructural level, the septal pore is an important criterion for the members of Aphyllophorales [7]. One other feature, color, often facilitates the recognition of the hymenochaetaeous species in the field. The basidiocarps of all species are golden brown to reddish brown in color, and the tissues are permanently darker with a KOH application, the xanthochroic reaction characteristic of the family [5]. These features can sometimes be mistaken as characteristic of the members of Polyporaceae [8].

P. linteus is phenotypically similar to *P. igniarius*, *P. nigricans*, *P. laevigatus*, *P. robustus*, *P. hartigii*, and especially *P. baumii*. Since Teng merged *P. baumii* with *P. linteus*, the differences between *P. linteus* and *P. baumii* have not been critically discussed [27]. Moreover, a study of the *Phellinus* species in Northeast Asia demonstrated that the concept of the species had been misinterpreted and that the Asian fungus was in fact *P. baumii* rather than *P. linteus* [6].

Various culture studies have been conducted to classify the genus *Phellinus* [3,4,23]. The classification by using Restriction Fragment Length Polymorphism (RFLP) analysis has identified new characteristics on the species level [18]. However, the results were inconsistent and showed various band pattern. The method cannot be used on a lower taxonomical level.

In this work, the 5.8S ribosomal RNA coding gene (rDNA) and internal transcribed spacers (ITS) were studied to determine their applicability to the systematics of *Phellinus*. The ribosomal RNA genes were chosen because they form a mosaic pattern of conserved and variable regions which make them attractive for taxonomic investigation at many levels [1,12]. However, Taylor et al. has shown that the levels of the sequence variability in a given region are quite different in various fungal taxa [26]. It means that there is no unique region that can be used to identify or address the phylogenetic relationship among all fungi. The most variable

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regions can be used for systematics at lower taxonomic levels. These include the intergenic regions (IGR), internal transcribed spacers (ITS), and divergent domains of a large ribosomal subunit (28S rDNA), named D1-D7 in the model of Michot and Bachellerie [17]. In this study, the 5.8S rDNA and flanking internal transcribed spacer regions (ITS1 and ITS2) were used to study the phylogenetic relationship of various *Phellinae*.

The present study was done to construct the phylogenetic relationship of *P. linteus* and closely related species by using the ITS1 and ITS2 regions, and the 5.8S ribosomal DNA that distinguish. To address these aims 5.8S rDNA and the internally transcribed spacers ITS1, and ITS2 were all amplified and sequenced.

Materials and Methods

Fungal strains and cultivations

The strains used in this study were obtained from Korean mountains, farms, and various culture collections (Table 1). For comparison, sequence data of 15 strains of *Phellinus* were used in this study [13].

The strains were cultured with shaking in 100 ml of Yeast-Malt extract (YM), at 24℃ for two or three weeks. The mycelium was harvested by filtration or centrifugation, and stored in a freezer at -20℃ with no agitation for one week before use.

DNA extraction

Fungal DNA was extracted from each sample according to the procedure adapted from the benzyl chloride method [13]. Approximately 0.05 g of the fungal pellets was suspended in 500 μl of a Tris buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 μl of 10 % (w/v) sodium dodecyl sulphate (SDS) and 300 μl of benzyl chloride and then incubated at 55℃ for 30 min. Treatment with phenol : chloroform : isoamylalchol (25 : 24 : 1) and RNase (1 mg/ml) was carried out for the purification of the DNA. The DNA was then precipitated by adding 2.5 volumes of 100% ice-cold ethanol. The pellet was washed with 2 volumes of 70% ethanol and resuspended in distilled water. The purified DNA was kept at -20℃.

PCR amplification and DNA sequencing

The nuclear rDNA region spanning the ITS1 and ITS2 regions, and the 5.8S rDNA was amplified by a polymerase chain reaction (PCR) from each strain, as described in Table 1. The primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') were derived from the conserved regions of 18S and 28S

Table 1. List of species and GenBank accession number of *P. linteus* and its allies used in this study

Species	Strains*	Geographical origins	Accession No.
P. linteus	ATCC 26710	-	AF153010
P. linteus	IFO 6989	-	AF200226
P. linteus	MPNU 7001	Hongcheon, Korea	AF200227
P. linteus	MPNU 7002	Hongcheon, Korea	AF200228
P. linteus	MPNU 7016	Japan	AF153009
P. baumii	MPNU 7004	Andong, Korea	AF200229
P. baumii	MPNU 7005	Changwon, Korea	AF200230
P. baumii	MPNU 7006	Incheon, Korea	AF200231
Phellinus sp.	MPNU 7003	Andong, Korea	AF200232
Phellinus sp.	MPNU 7007	China	AF200235
Phellinus sp.	MPNU 7008	Andong, Korea	AF200233
Phellinus sp.	MPNU 7009	Andong, Korea	AF200234
Phellinus sp.	MPNU 7010	Andong, Korea	AF153007
Phellinus sp.	MPNU 7011	Andong, Korea	AF200236
Phellinus sp.	MPNU 7012	Andong, Korea	AF153008
P. baumii	MPNU 7013	Chuncheon, Korea	AF153011
P. ribis f. *ulicis*	IMSNU 32011	-	AF200237
P. nigricans	IMSNU 32024	-	AF200239
P. torulosus	IMSNU 32028	-	AF200240
P. tremulae	IMSNU 32029	-	AF200241
P. pini var. *carniformans*	IMSNU 32031	-	AF200242
P. conchaetus	IMSNU 32017	-	AF200238
P. linteus	KACC 500122	-	AF200243
P. linteus	KACC 500411	-	AF200244

*ATCC : American Type Culture Collection, Manassas, U.S.A.
IFO : Institute for Fermentation, Osaka, Japan
IMSNU : Culture Collection Center Institute of Microbiology Seoul University, Seoul, Korea
KACC : Korea Agricultural Culture Collection
MPNU : Mycological lab. of Pusan National University

rDNA, respectively (Fig. 1) [29]. PCR was carried out using a Perkin-Elmer model 480 thermocycler with the following program : initial denaturation for 3 min at 95℃, 30 cycles of amplification (denaturation for 30 sec at 95℃, annealing for 30 sec at 50℃, and extension for 1 min at 72℃ and final extension of 5 min at 72℃. The PCR products from the amplification were subjected to preparative electrophoresis in a 1.6% agarose gel in a TBE buffer. All PCR products yielded only a single visible band (approximately 700 bp). The PCR products were then excised from the ethidium bromide-stained gel and purified using a QIAGEN gel elution kit (Qiagen, Warfworth CA). The direct sequencing of the PCR products was performed using a Perkin-Elmer Applied Biosystems ABI 377A sequencer with a PRISM Dye Dideoxi Terminator Cycle Sequencing kit (Perkin Elmer) following the manufacturer's protocol [22]. Two primers, ITS 4F and ITS 5R, were used for sequencing in both directions and the DNA sequences were edited and assembled using the program CLONE MANAGER version 4.0 (Scientific & Educational Software, Stateline, PA.).

Data analysis

The determined ribosomal DNA sequences were deposited in the European Molecular Biology Laboratory (EMBL) data library (Heidelberg, FRG) and the accession numbers were indicated in Table 1. The new sequences were initially aligned with the sequences of related genera from the EMBL data library using the multiple alignment program Clustal W [28]. The phylogenetic relationships were inferred by the neighbor-joining method [20]. The strength of the internal branches from the resulting trees was statistically tested by a bootstrap analysis based on 1,000 bootstrap replications [9]. The distance matrix was calculated using NucML, and the initial tree based on the neighbor-joining method was reconstructed using NJdist from the PHYLIP 3.5 software package.

Results

Sequence alignments

The ITS1 sequences were more variable than the ITS2 ones. Among the different species, the length of ITS1 varied more than that of ITS2. It was different length in *P. linteus*, *Phellinus* sp. MPNU 7003, and *P. baumii* MPNU 7004, even though these species form a species complex. The morphologically related taxa of *P. linteus* included many insertion sites in ITS1 and ITS2 sequences. The ITS1 of *Phellinus* sp. MPNU 7003 had a large insertion site than that of the other *Phellinus*. Overall, there were 468 variable sites and 284 conserved sites making a total of 752 possible sites. ITS1 contained 247 variable sites and 73 conserved sites, while ITS2 had 215 variable sites and 61 conserved sites. In contrast, the 5.8S rDNA sequence was highly conserved across all species and contained only 7 variable sites and 149 conserved sites. The 5.8S rDNA sequence was similar in *P. linteus*, *P. weirianus*, *P. baumii*, and *Phellinus* sp. MPNU 7003 reflecting the similarity of their morphological characteristics. However, *P. torulosus* IMSNU 32028 and *Phellinus* sp. MPNU 7011, distant species compared with *P. linteus*, had many more deletion sites in ITS1 than in ITS2.

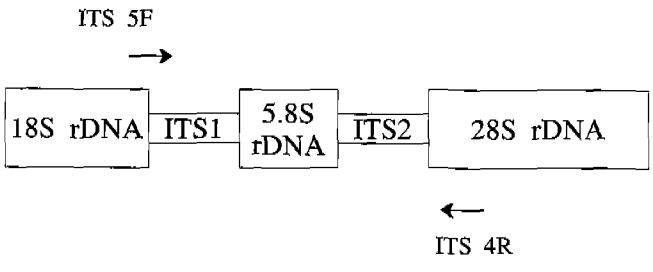

Fig. 1. Map of ribosomal DNA region containing ITS1 and ITS 2, and 5.8S rDNA coding gene. Arrows indicate the positions of the primers used for the PCR and sequence analysis.

G+C content and nucleotide length of rDNA ITS regions

The G+C content and nucleotide length of the ITS1, ITS2, 5.8S rDNA, and total (ITS1-5.8S-ITS2) are shown in Table 2 and 3. Positive correlations in the G+C content and nucleotide length were found between the ITS1 and the ITS2 (Fig. 2). The ITS1 of a taxa with a GC-rich ITS2 also had a long ITS1 sequence. The total G+C content of the ITS1-5.8S-ITS2 ranged from 41.8% in *Phellinus* sp. MPNU 7011 to 48.5% in *Phellinus* sp. MPNU 7003, 7008, and 7009. *Phellinus* sp. MPNU 7011 and *P. torulosus* IMSNU 32028 had a lower G+C content (41.8% and 42.9%, respectively) in the total sequences than any other *Phellinus* species. The G+C content of the 5.8S rDNA was not variable (45.5~47.4%) among the all taxa investigated. Especially, *P. linteus*, *P. baumii*, and *Phellinus* sp. MPNU 7003 forming a species complex, had an identical G+C content of 47.4%. In contrast, the ITS regions showed relatively broad G+C contents : 42.2~50.1% in ITS1 and 37.9~50.8% in ITS2.

Among the 24 taxa received from the Korean Agricultural Culture Collection (KACC) and sequenced in this study, the shortest size of the ITS1-5.8S-ITS2 was 615 nucleotides in *P. torulosus* IMSNU 32028, and the longest was 752 nucleotides in *Phellinus* sp. KACC500122 misidentified as *P. linteus* (Table 3 and Fig. 3). This size variation was derived from a variation in the ITS regions, because the size of the 5.8S rDNA was identical (156 nucleotides) among all the tested taxa. Most of the tested taxa could be divided into two groups depending on the ITS length. The group with short ITS regions having below 649 nucleotides included *P. nigricans* IMSNU 32024, *P. torulosus* IMSNU 32028, and *P. tremulae* IMSNU 32029, while the group with long ITS regions above 700 nucleotides included many *P. linteus* species; farm cultivated *P. baumii*, strains isolated in the Korean mountains, *Phellinus* sp. MPNU 7007 imported from China and *P. conchaetus* IMSNU 32017. The length of the ITS1-

Table 2. G+C contents of ITS1, ITS2, and 5.8S rDNA sequences

Fungal strain	ITS1	5.8S rDNA	ITS2	Total
P. linteus ATCC 26710	47.3*	47.4	47.9	47.5
P. linteus IFO 6980	47.0	47.4	47.9	47.4
P. linteus MPNU 7001	47.0	47.4	47.9	47.4
P. linteus MPNU 7002	47.3	47.4	47.9	47.5
P. linteus MPNU 7016	47.0	47.4	47.9	47.4
P. baumii MPNU 7004	50.1	47.4	49.4	47.0
P. baumii MPNU 7005	50.1	47.4	49.4	47.0
P. baumii MPNU 7006	50.1	47.4	49.4	47.0
Phellinus sp. MPNU 7003	47.1	47.4	50.8	48.5
Phellinus sp. MPNU 7008	47.1	47.4	50.8	48.5
Phellinus sp. MPNU 7009	47.1	47.4	50.8	48.5
Phellinus sp. MPNU 7007	49.2	47.4	47.5	48.2
Phellinus sp. MPNU 7010	49.2	47.4	47.6	48.4
Phellinus sp. MPNU 7011	42.2	46.2	37.9	41.8
Phellinus sp. MPNU 7012	49.1	47.4	46.9	48.0
P. baumii MPNU 7013	49.5	46.8	46.9	48.0
P. ribis f. *ulicis* IMSNU 32011	49.8	46.2	46.2	47.7
P. conchaetus IMSNU 32017	46.4	46.2	49.4	47.3
P. nigricans IMSNU 32024	46.5	47.4	46.1	46.5
P. torulosus IMSNU 32028	45.1	45.5	41.8	43.9
P. tremulae IMSNU 32029	49.4	47.4	47.1	48.1
P. pini var. *cancriformans* IMSNU 32031	46.4	47.4	49.0	47.5
P. linteus KACC 500122	48.1	46.8	50.6	48.5
P. linteus KACC 500411	48.1	46.8	50.6	48.5

*shows mol %.

Table 3. Nucleotide length of ITS1, ITS2, and 5.8S rDNA sequences

Fungal strain	ITS1	5.8S rDNA	ITS2	Total
P. linteus ATCC 26710	300*	156	250	706
P. linteus IFO 6980	300	156	250	706
P. linteus MPNU 7001	300	156	250	706
P. linteus MPNU 7002	300	156	250	706
P. linteus MPNU 7016	300	156	250	706
P. baumii MPNU 7004	328	156	263	747
P. baumii MPNU 7005	328	156	263	747
P. baumii MPNU 7006	328	156	263	747
Phellinus sp. MPNU 7003	327	156	264	747
Phellinus sp. MPNU 7008	327	156	264	747
Phellinus sp. MPNU 7009	327	156	264	747
Phellinus sp. MPNU 7007	328	156	249	733
Phellinus sp. MPNU 7010	325	156	263	744
Phellinus sp. MPNU 7011	245	156	248	649
Phellinus sp. MPNU 7012	328	156	263	747
P. baumii MPNU 7013	327	156	263	746
P. ribis f. *ulicis* IMSNU 32011	289	156	243	688
P. conchaetus IMSNU 32017	362	156	230	748
P. nigricans IMSNU 32024	247	156	244	647
P. torulosus IMSNU 32028	220	156	239	615
P. tremulae IMSNU 32029	243	156	232	631
P. pini var. *cancriformans* IMSNU 32031	283	156	233	672
P. linteus KACC 500122	320	156	267	752
P. linteus KACC 500411	320	156	267	752

*shows the length of the nucleotide sequence (bp)

5.8S-ITS2 in the former group ranged from 615 to 649 nucleotides, whereas that in the latter ranged from 706 to 752 nucleotides. *P. ribis* f. *ulicis* IMSNU 32011 and *P. pini* var. *carniformans* IMSNU 32031 fell between the two groups (688 and 672 nucleotides).

DNA similarity

The DNA similarity between the ITS1 and ITS2 is reported

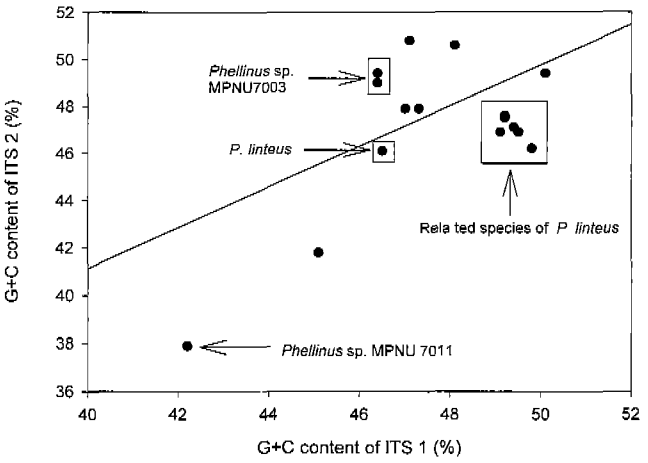

Fig. 2. Positive correlations of G+C content between ITS1 and ITS2.

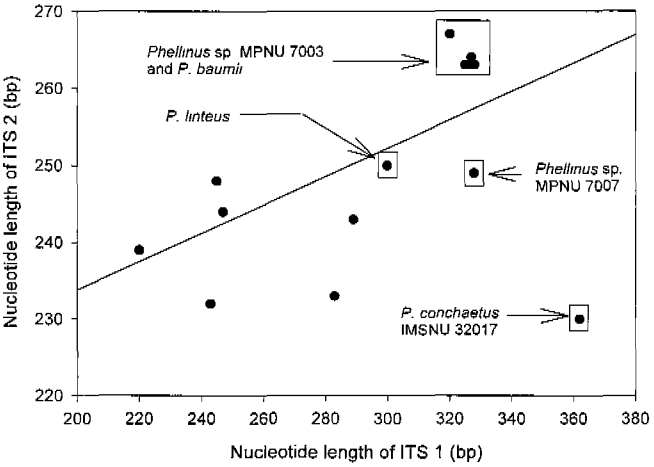

Fig. 3. Positive correlations of the nucleotide length between ITS1 and ITS2.

in Table 4. Strains of *P. linteus* shared an identical sequence in their 5.8S rDNA and diversified in 2 positions in the ITS1 and in 2 positions in the ITS2 (< 0.6%). Among the *P. linteus* species, the DNA similarity was very high over 99.4%. Three strains of *P. linteus* (*P. linteus* IFO 6989, MPNU 7001, and MPNU 7016) had the most identical sequence in all the regions sequenced. The *P. linteus* MPNU 7001 and MPNU 7002 isolated in South Korea had a variation of 3 nucleotides in the ITS regions (< 0.4%). This variation was presumably related to the differences in the geographic and environmental conditions. *P. baumii* exhibited a high similarity with *P. linteus*, about 95.5% excluding gaps and 90.8% including gaps. *P. baumii* had similar sequences with *P. linteus*, while there were many gaps in the ITS regions. The *P. baumii*, purchased from Korean farms (MPNU 7004, MPNU 7005, and MPNU 7006), shared identical sequences, while the wild South Korean isolates (MPNU 7010, MPNU 7012, and MPNU 7013) exhibited variations (< 0.5%) intraspecies. The two strains of *P. linteus* KACC 500122 and KACC 500411 received from the Korean Agricultural Culture Collection (KACC) were different from the sequences of the other *P. linteus*, yet the similarity between the two strains was above 96.8% excluding gaps and over 90.0% including gaps. These two strains had a high similarity at about 98.1% when compared with the sequences of *Phellinus* sp. MPNU 7003, *Phellinus* sp. MPNU 7008 and MPNU 7009 collected from the mountains. Other related species are *P. nigricans* IMSNU 32024 and *P. tremulae* IMSNU 32029 (94.8% / 92.5%), *P. torulosus* IMSNU 32028 and *Phellinus* sp. MPMU 7001 (70.4% / 75.8%), and *P. ribis* f. *ulicis* IMSNU 32011 and *P. pini* var. *carniformans* IMSNU 32031 (78.3% / 74.4%).

Phylogenetic analyses of ITS1, ITS2, and 5.8S rDNA sequences

Separate analyses based on the recorded ITS1 and ITS2 data sets produced topologically very similar trees, which are depicted in Fig. 4. The main difference between the two analyses was the placement of *P. conchaetus* IMSNU 32017, which could belong to either a cluster including the species of the *P. linteus* complex (ITS1 phylogeny) or a cluster including the species of the *P. chrysoloma* group (ITS2 phylogeny). However, in both cases the statistical support or branch was low in similarity (83% and 81%). In addition, the tree produced from the ITS1 data set indicated that *P. laevigatus*, *P. nigricans*, *P. igniarius*, and *P. tremulae* were monophyletic (87% confidence level), while the tree produced from the ITS2 data set showed a poor resolution of the internal branches.

752 changes were identified in the original data set composed of the 5.8S rDNA and both internal transcribed spacer sequences (Fig. 5). Most of the *P. linteus* screened a cluster which was highly significant with the exception of *P. linteus* KACC 500122 and KACC 500411. They formed the sister taxa of *P. linteus* where *P. baumii*, *Phellinus* sp. MPNU 7003, MPNU 7007, and MPNU 7010 had similar morphological characteristics. *P. nigricans* IMSNU 32024 and *P. pini* var. *carniformans* IMSNU 32031 were grouped in the same cluster with *P. igniarius* KCTC 6227, KCTC 6228, and *P. chrysoloma* KCTC 6225 extracted from the GenBank database. *P. torulosus* IMSNU 32028 and *Phellinus* sp. MPNU 7011 formed a closed group but these species had a distant taxa when compared with the other *Phellinus* species.

The phylogenetic tree from the ITS1 data set was similar to that from the ITS2 data set (Fig. 4). The topology of the individual ITS phylogeny was in agreement except for the placement of *P. conchaetus* IMSNU 32017. The ITS1 phylogeny and ITS2 phylogeny were in conflict in the placement of *P. conchaetus* IMSNU 32017. It was considered that the current data set was insufficient to resolve the correct placement of *P. conchaetus* IMSNU 32017 and that there was no incongruence in combining the different data sets. The overall combined tree was depicted in Fig. 5, and *P. conchaetus* IMSNU 32017 was placed in an isolated position representing a phylogenetic hypothesis based on rDNA se-

Table 4. DNA similarity between taxa in ITS regions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	-	99.7	99.4	99.7	99.7	91.1	91.1	90.8	90.8	90.8	90.9	90.9	90.9	90.2	90.9	66.3	90.7	91.8	72.4	72.8	77.0	61.2	75.7	71.3
2	99.7	-	99.6	100	100	90.0	90.0	91.0	91.0	91.0	90.9	90.9	90.9	89.6	90.3	67.1	91.0	90.6	71.9	70.0	74.6	59.1	74.1	76.1
3	99.4	99.6	-	99.9	99.6	90.7	90.7	90.4	90.4	90.4	90.5	90.5	90.5	89.9	90.3	66.2	90.0	90.5	72.0	70.0	74.7	58.5	73.7	71.3
4	99.7	100	99.6	-	100	90.0	90.0	91.0	91.0	91.0	90.9	90.9	90.9	89.6	90.3	67.1	91.0	90.6	71.9	70.0	74.6	59.1	74.1	76.1
5	99.7	100	99.6	100	-	90.0	90.0	91.0	91.0	91.0	90.9	90.9	90.9	89.6	90.3	67.1	91.0	90.6	71.9	70.0	74.6	59.41	74.1	76.1
6	96.8	96.2	96.7	96.2	96.2	-	100	88.6	88.6	88.6	97.5	97.5	97.5	87.1	88.0	64.6	88.1	88.6	71.3	69.3	69.1	62.3	69.3	70.5
7	96.8	96.2	96.7	96.2	96.2	100	-	88.6	88.6	88.6	97.5	97.5	97.5	87.1	88.0	64.6	88.1	88.6	71.3	69.3	69.1	62.3	69.3	70.5
8	96.2	96.0	95.7	96.0	96.0	91.6	91.6	-	100	100	90.6	90.6	90.6	96.2	99.5	56.7	99.4	99.9	71.3	69.8	76.2	55.8	70.1	70.0
9	96.2	96.0	95.7	96.0	96.0	91.6	91.6	100	-	100	90.6	90.6	90.6	96.2	99.5	56.7	99.4	99.9	71.3	69.8	76.2	55.8	70.1	70.0
10	96.2	96.0	95.7	96.0	96.0	91.6	91.6	100	100	-	90.6	90.6	90.6	96.2	99.5	56.7	99.4	99.9	71.3	68.8	76.2	55.8	70.1	70.0
11	96.3	96.5	96.0	96.5	96.5	98.1	98.1	93.1	93.1	93.1	-	100	100	88.4	90.7	66.2	90.5	91.0	72.5	68.7	68.8	57.8	70.9	70.7
12	96.3	96.5	96.0	96.5	96.5	98.1	98.1	93.1	93.1	93.1	100	-	100	88.4	90.7	66.2	90.5	91.0	72.5	68.7	68.8	57.8	70.9	70.7
13	96.3	96.5	96.0	96.5	96.5	98.1	98.1	93.1	93.1	93.1	100	100	-	88.4	90.7	66.2	90.5	91.0	72.5	68.7	68.8	57.8	70.9	70.7
14	96.4	95.8	95.7	95.8	95.8	90.7	90.7	99.7	99.7	99.7	92.3	92.3	92.3	-	96.9	57.9	96.9	97.1	70.8	69.6	76.0	70.0	70.9	71.1
15	95.8	95.2	95.2	95.2	95.2	90.9	90.5	99.7	99.7	99.7	92.8	92.8	92.8	99.2	-	57.1	99.7	99.7	71.2	68.6	69.5	55.7	69.8	70.5
16	75.7	75.6	75.5	75.6	75.6	75.4	75.4	66.4	66.4	66.4	70.3	70.3	70.3	68.3	66.8	-	57.5	57.3	64.6	54.2	69.1	75.8	68.7	63.5
17	95.9	95.3	95.3	95.3	95.3	91.0	91.1	99.7	99.7	99.7	92.9	92.9	92.9	99.3	99.7	66.6	-	99.5	71.0	68.4	69.3	55.5	69.6	70.3
18	95.9	95.9	95.6	95.9	95.9	90.3	90.3	100	100	100	93.0	93.1	93.0	99.2	99.7	66.7	99.5	-	71.0	68.5	69.4	55.6	69.7	70.4
19	76.3	75.7	75.8	75.6	75.9	78.5	78.5	77.7	77.7	77.7	79.4	79.4	79.4	76.7	77.2	72.5	77.0	77.1	-	70.2	71.5	61.2	73.3	74.4
20	81.3	78.5	78.5	78.5	78.5	76.7	76.7	78.4	78.4	89.7	76.6	76.6	76.6	77.5	77.0	67.1	76.8	76.9	79.9	-	58.9	53.4	75.1	71.5
21	85.8	83.8	83.8	83.8	83.8	80.8	80.8	89.7	89.7	89.7	81.2	81.2	81.2	88.7	81.9	72.4	81.7	81.8	79.9	72.0	-	67.5	92.5	84.0
22	70.9	67.6	67.6	67.6	67.6	76.7	76.7	67.9	67.9	67.9	70.2	70.2	70.2	71.9	68.2	81.2	68.0	68.1	70.4	69.1	72.1	-	69.4	60.2
23	84.9	84.2	84.2	84.2	84.2	83.1	83.1	83.9	83.9	83.9	84.7	84.7	84.7	84.4	83.5	73.6	81.5	81.6	82.2	77.9	94.8	74.8	-	74.8
24	76.9	81.9	81.9	81.9	81.9	79.8	79.8	78.2	78.2	78.2	79.6	79.6	79.6	77.9	78.7	71.7	78.5	78.6	78.3	83.2	92.8	70.5	82.4	-

The values on the top upper right are percentages of similarity including gaps, and the values on the lower left are percentages of similarity excluding gaps.

1. *P. linteus* ATCC 26710; 2. *P. linteus* IFO 6989; 3. *P. linteus* MPNU 7001; 4. *P. linteus* MPNU 7002; 5. *P. linteus* MPNU 7016; 6. *P. linteus* KACC 500122; 7. *P. linteus* KACC 500411; 8. *P. baumii* MPNU 7004; 9. *P. baumii* MPNU 7005; 10. *P. baumii* MPNU 7006; 11. *Phellinus* sp. MPNU 7003; 12. *Phellinus* sp. MPNU 7008; 13. *Phellinus* sp. MPNU 7009; 14. *Phellinus* sp. MPNU 7007; 15. *Phellinus* sp. MPNU 7010; 16. *Phellinus* sp. MPNU 7011; 17. *Phellinus* sp. MPNU 7012; 18. *P. baumii* MPNU 7013; 19. *P. ribis* f. *ulicis* IMSNU 32011; 20. *P. conchaetus* IMSNU 32017; 21. *P. nigricans* IMSNU 32024; 22. *P. torulosus* IMSNU 32028; 23. *P. tremulae* IMSNU 32029; 24. *P. pini* var. *cariniformans* IMSNU 32031

quences.

Discussion

The nucleotide sequences of ITS regions including the 5.8S rDNA were obtained from 24 strains of the genus *Phellinus*. The nucleotide length of these regions varied from 615 bp to 752 bp depending on the taxa. The investigated taxa were then divided into two large groups based on the ITS length, i. e., a long-ITS group and a short-ITS group. The phylogenetic tree constructed from the nucleotide sequences supported this grouping. This grouping was somewhat similar to that based on morphological characteristics.

Salinas et al. suggested that temperature is an important selection factor for the GC bias in the genomes of plants and warm-blooded animals [21]. For example, the genomes of warm-blooded vertebrates have a higher G+C content than those of cool-blooded vertebrates. Takamatsu et al. suggested that the relatively low G+C content of fungi may reflect their low optimum temperature [25]. In this study, many species isolated in South Korea, exhibited quite a difference in their G+C content ranging from 41.8% to 48.5%. The reason for this low G+C content is unclear because ecological and physiological information on this fungus is currently unavailable. *P. linteus* having 47.5% GC contents supposed to distribute in temperate Asia as well as tropical America and Africa.

Based on a survey of the world taxa, Larsen and Cobb-Poule divided the genus *phellinus* into five large groups according to the context, tramal setal hyphae, tramal setate, basidiospores, and basidiocarps [14]. Those with the characteristics of hymenial setae presence, eventual basidiospores pigmentation, and septal hyphae absence included *P. linteus*, *P. baumii*, *P. chrysoloma*, *P. lavigatus*, *P. pini*, *P. robustus*, and *P. weirianus* etc. These species included Larsen and Cobb-Poulle's group III. Some of these investigated species, *P. linteus*, *P. baumii*, *Phellinus* sp. MPNU 7003, 7007, 7010, 7012, and 7013, had a long ITS-length. These species coincided with the grouping based on the ITS length as well as the morphological characteristics. However, *P. chrysoloma*, *P. lavigatus*, *P. pini*, and *P. robustus* included in group III by Larsen and Cobb-Poule also had an exceptionally short ITS-length. Accordingly, this result does not agree with grouping according to ITS length. In contrast the rest of groups determined by Laser and Cobb-Poulle included *P. nigricans* IMSNU 32024, *P. torulosus* IMSNU 32028, *P. tremulae* MPNU 32029, *P. ribis* f. *ulicis* IMSNU 32011, and *Phellinus*

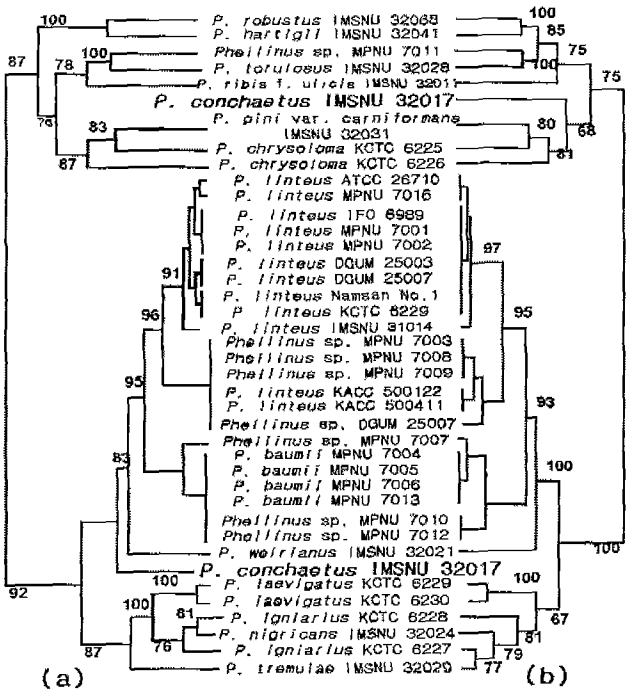

Fig. 4. Phylogenetic relationships of *Phellinus* species inferred from the nucleotide sequences of internal transcribed space regions ITS1 (a) and ITS2 (b). The phylograms were generated from 1,000 bootstrap replications from the data recorded, as described in Materials and Methods. The percentages above the branches are the confidence levels of the bootstrap replications.

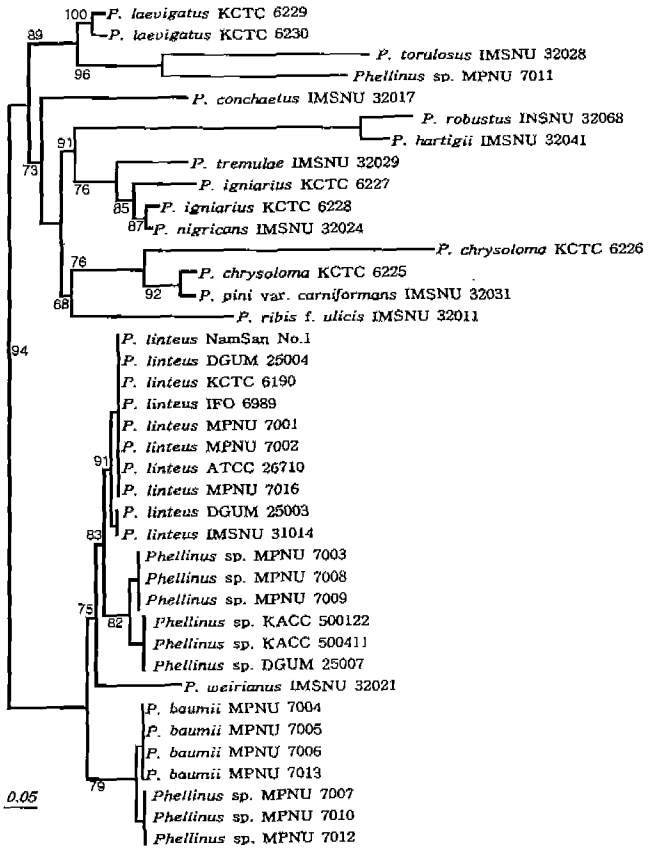

Fig. 5. Phylogenetic tree showing the relationships of the genus *Phellinus*. The bar represents 5 nucleotide substitutions per 100 nucleotides in the ITS1 and ITS2, and 5.8S rDNA sequences. The bootstrap probabilities are indicated at the branch points.

sp MPNU 7011. Regardless of Larsen and Cobb-Poulle's opinion, all these species exhibited a short ITS length, and were scattered in between the four groups, except for group III. As a result, the grouping by ITS length partially corresponded to the grouping based on the morphological characteristics of hymenial setae presence, basidiospores pigmentation, basidiospores haline, and setal hyphae absence. The variable spacer regions of rDNA have been considered to be useful for the phylogenetic analysis of closely related genera, interspecies or intraspecies. This is also true for the genus *Phellinus*, although the obtained sequences were sometimes difficult to align between distantly related taxa. However, some conserved sequences were found in the spacer regions, thereby allowing the phylogenetic analysis of the genus *Phellinus* using the conserved sequences of the spacers and the coding regions. Separate analyses based on ITS1 and ITS2 data set produced very similar trees topologically. The main difference between the two analyses was the placement of *P. conchaetus* IMSNU 32017. It was found that 2 strains of *P. linteus* (MPNU 7001 and MPNU 7002) isolated from South Korea had almost identical sequences with *P. linteus* ATCC 26710. Accordingly, it would appear that *P. linteus* is distributed in the South Korean mountains whereas most of the artificially cultivated mushrooms or farms is *P. baumii* which has been misnamed as *P. linteus*. The sequences of two strains received from the Korean Agricultural Culture Collection (KACC), *P. linteus* KACC 500122 and KACC 500411, were different from the sequences of other *P. linteus*, and their similarity was above 96.8% excluding gaps and above 90.0% including gaps. These two strains had a high similarity of about 98.1% compared with the sequences of *Phellinus* sp. MPNU 7003 purchased from Korean farms and *Phellinus* sp. MPNU 7008 and MPNU 7009 collected from the mountain. Therefore, these two strains have been misnamed as *P. linteus*, and should be re-identified. Dai and Xu indicated that some species from South Korea, China, Japan, and Far Eastern Russia had deviated from the American *P. linteus* in that they had significantly smaller spores and narrower tremal skeletal hyphae, plus, hyphae their remained unchanged in KOH. Those from Vladivostok in Far Eastern Russia were all identified as *P. baumii* [19]. *P. linteus* has been repeatedly used to refer to the species found in South Korea and other Asian countries [15]. Recently Teng merged *P. baumii* with *P. linteus*, however did not discuss the critical differences between *P. linteus* and *P. baumii*. It has also been reported that *P. baumii* has been successfully cultivated in South Korea and recognized as the identical species with *P. linteus*. However, based on sequence alignment, this study revealed that most of the culti-

vated *P. linteus* in South Korea have been misnamed and misidentified as a *P. baumii* and *Phellinus* sp. MPNU 7003. In contrast to *P. lintues*, the sequences of *P. baumii* and *Phellinus* sp. MPNU 7003 have many insertion sites and some mutation in ITS regions. These results agree with Dai's opinion that most of the artificially cultivated mushrooms in South Korea are *P. baumii*, and *P. linteus* is clearly different from *P. baumii* in the sequence alignment. Some mycologists insist that *P. linteus* does not exist in South Korea, and that it is only distributed along the Gulf Coast Region of the USA and Mexico, Costa Rica, Panama, Cuba, Bahama, Island, Puerto Rico, Trinidad and Brazil [16]. However, this study identified 2 strains of *P. linteus* (MPNU 7001 and MPNU 7002) in South Korea which had almost identical sequences with *P. linteus* ATCC 26710. It would appear that *P. linteus* is distributed in the South Korean mountains whereas most of the artificially cultivated mushrooms are actually *P. baumii* yet have been misnamed as *P. linteus*. *P. linteus* and *P. baumii* had similar morphological characteristics exhibiting a nucleotide divergence of about 4.0% (using NucML from the PHYLIP 3.5 software package) from each other. *P. linteus* was approximately 3.6% divergent from *Phellinus* sp. MPNU 7003, while *P. baumii* was 6.9% divergent. As a result, *P. linteus* is postulated to be the earliest lineage to diverge within the complex species of *P. linteus*, while the remaining *P. baumii* and *Phellinus* sp. MPNU 7003 species are suggested to have evolved more recently.

Anowledgement

This research was funded by the SGRP/HTDP (High-Technology Development Project for Agriculture and Forestry) in Korea (2000).

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