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Phylogenetic Analysis and Rapid Detection of Genus *Phellinus* using the Nucleotide Sequences of 18S Ribosomal RNA

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Analysis of phylogenetic relationship was performed among *Phellinus* species based on 18S ribosomal subunit sequence data. Twenty-five strains of 19 *Phellinus* species including *P. linteus* were examined in this study. Regions of 18S ribosomal subunit were very conserved, but some variable regions between *Phellinus* species were observed. The species-specific detection primers, modified by 2 or 3 nucleotides in sense primer were designed based on 18S ribosomal DNA (rDNA) sequence data. The 210 bp PCR bands were detected with annealing temperature 48°C. The 18S 2F-18S 4R detection primer set distinguished *P. linteus* from various *Phellinus* species but some species like *P. baumii*, *P. weirianius*, *P. rhabarberinus* and *P. pomaceus* also had weak reactivity on this primer set. The 18S 3F-18S 4R primer set distinguished only *P. linteus* from various *Phellinus* species, although sensitivity with this primer set was lower than that of 18S 2F-18 4R primer set. These primer sets would be useful for the detection of only *P. linteus* among unknown *Phellinus* species rapidly.

KEYWORDS: ***Phellinus*** **and gene probe, Phylogeny**

Hymenochaetaceae is a white-rotter that lacks clamp connections and processes setae (Gilbertson, 1980). At the ultrastructural level, the septal pore is an important criterion for the members of Aphyllophorales (Donk, 1964). These fungi cause wood pocket rot and other fatal plant diseases such as canker and heart-rot in living trees (Hugues *et al.*, 1998). However, they have shown several medical and clinical benefits in the human body. *P. linteus* was known to have immuno-stimulating activity (Lee *et al.*, 1996) and inhibitory effect on tumor growth and metastasis (Han *et al.*, 1999). In Asian countries, especially China, Korea and Japan, *P. linteus* was already used as a medicine to treat stomachache and arthritis (Ying *et al.*, 1987). As the clinical effects were explored, the demand of *Phellinus* species was dramatically increased, and many researchers put their efforts on the development of easy culture method of *P. linteus* in laboratory and the purification of valuable substance from *P. linteus.* However, all *Phellinus* species do not have the same medicinal effects and there are some taxonomical problems in the genus of *Phellinus* (Shon and Nam, 2001). Furthermore, large amounts of *Phellinus* species were imported from China and Russia to meet the demand, but only some species including *P. linteus* were permitted to import in Korea. Therefore, the correct identification of species is very important for various reasons.

In our previous studies, phylogenetic relationship of *Phellinus* species was evaluated in the Internal Transcribed Spacer (ITS) and 28S rDNA regions (Kim *et al.*, 2001; Park *et al.*, 2002). Add to the previous results, analysis of 18S rDNA regions would help to accomplish more accurate systematics of *Phellinus* species. In this study, we used partial 18S universal primers (Ito and Hirano, 1996; Ito and Hirano, 1997) for more reliable classification of *Phellinus* species systematics. And we designed species-specific primers (18S 2F, 3F and 4R) based on 18S partial sequence data, for the specific and easy detection of *P. linteus* on PCR level.

Materials and Methods

Fungal isolates. The *Phellinus* species used in this study are listed in Table 1. Twenty-five strains of *Phellinus* species were obtained from the American Type Culture Collection (ATCC; Manassas, U.S.A.), Centraalbureau voor Schimmelcultures (CBS; Utrecht, Netherlands), the Institute for Fermentation (IFO; Osaka, Japan) and Korea Collection for Type Cultures (KCTC; Dae-Jeon, Korea). Two strains of *P. linteus* (MPNU7001 and MPNU7002) and two strains of *P. baumii* (MPNU7004 and MPNU7005) were isolated from South Korea. The culture media and conditions were followed by the recommendation in ATCC, CBS, IFO and KCTC catalogues.

DNA extraction. Fungi colonies, grown on Potato-Dextrose Agar (PDA; 0.4% potato extract, 2% dextrose, 1.5% agar) medium were transferred to flasks containing Potato-Dextrose Broth (PDB; 0.4% potato starch, 2% dextrose) medium. After 2 to 3 weeks of culture, mycelia were filtered, dehydrated and collected in new tubes for the pres-

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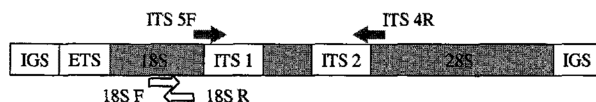

Fig. 1. Location and orientation of primers for ITS and 18S partial site. The gray arrows are sense and anti-sense ITS universal primers. The white arrows are partial 18S sense and anti-sense universal primers. (18S: 1700~1800 bp, ITS1, 5.8s and ITS2: 600~700 bp and 28s: 3400~3700 bp).

ervation. For the preparation of total genomic DNA from *Phellinus* species, we used DNA extraction method using benzyl chloride (Zhu *et al.*, 1993; Gragam *et al.*, 1994) or extraction kit (Bioneer Co.). In the former method, cells were lysed with extraction buffer [100 mM Tris-HCl (pH 9.0), 40 mM EDTA], benzyl chloride and 10% sodium dodecyl sulphate (SDS). Cell lysate was treated with ribonuclease to remove RNA. Genomic DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and concentrated by ethanol precipitation. In the latter method, we followed the instruction manual of manufacturer (Bioneer Co.).

Method using benzyl chloride (Zhu *et al.*, 1993; Gragam *et al.*, 1994) yielded higher density of genomic DNA, but had a serious problem. Because most *Phellinus* species pigmented as mycelia grow and the pigment would prevent primers from binding chromosomal DNA, PCR products might not be obtained. Another method using extraction kit (Bioneer Co.) did not yield high density of genomic DNA, but isolated much more pure DNA. As this method removed pigment through the column, the problem of interrupting primer binding was solved.

Primers and PCR amplification. The partial 18S rDNA universal primers (Ito and Hirano, 1997), derived from the 18S conserved regions of ribosomal DNA in *Saccharomyces cerevisiae* at positions 1284-1306 and 1766-1785 (Fig. 1) were tested for reactivity with total genomic DNA from 25 strains of *Phellinus* spp. The sequences of primers (synthesized by Bioneer Co.) were as follows.

18S Sense universal primer; 5'-gttggtggagtgatttgtctgc-3' (22mer)

18S Anti-sense universal primer; 5'-taatgatccttccgcaggtt-3' (20mer).

PCR reaction (Gene Amp PCR System 2400 Perkin-Elmer) was performed in 20 μl of PCR PreMix (Bioneer Co.; 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 1 unit of Taq polymerase, stabilizer and tracking dye) and 1 μl of 20 pM primers. PCR conditions were as follows: Pre-denaturing at 94°C for 5 min, denaturing at 94°C for 1 min, annealing from 43°C to 55°C for 1 min and extension at 72°C for 1 min, 30 cycles. PCR products were subjected to electrophoresis in a 2.0% agarose gel. The gel bands were excised and purified by using QIAGEN gel elution kit (Qiagen, Wartworth CA).

DNA sequencing. DNA sequencing reactions were performed by use of Bigdye Terminator Cycle DNA sequencing v2.0 Kit (PE Applied Biosystems, Roche Molecular Systems, Inc., Branchbrug, New Jersey, U.S.A.). The analysis of nucleotide sequence was carried out by using ABI 377 fluorescent DNA sequencer (PE Applied Biosystems, Roche Molecular Systems, Ine., Branchbrug, New Jersey, U.S.A.) or ABI 310 auto DNA sequencer (PE Applied Biosystems, Roche Molecular Systems, Ine., Branchbrug, New Jersey, U.S.A).

Data analysis. Gene bank accession numbers of the sequenced data were shown in Table 1. Nucleotide alignments and distance matrix of the related genera were done. And based on it, phylogenetic tree was drawn using the neighbor-joining method program (Thomson *et al.*, 1994; Saitou and Nei, 1987). Phylogenetic relationship between determined sequencing data and the length of internal branched trees were calculated by bootstrap analysis method based on 1,000 bootstrap replications (Felsen-

Table 1. List of *Phellinus* species used in the nucleotide sequence analyses

Strains[a]	Species	Accession no.
ATCC 26710	*P. linteus*	AY178007
IFO 6989	*P. linteus*	AY178008
CBS 454.76	*P. linteus*	AY178009
MPNU 7001	*P. linteus*	AY178010
MPNU 7002	*P. linteus*	AY178011
MPNU 7004	*P. baumii*	AY178012
MPNU 7005	*P. baumii*	AY178013
CBS 618.89	*P. weirianus*	AY178014
ATCC 60051	*P. johnsonianus*	AY178015
ATCC 26713	*P. rhabarberinus*	AY178016
ATCC 12240	*P. pini*	AY178017
ATCC 26729	*P. gilvus*	AY178018
KCTC 6228	*P. igniarius*	AY178019
KCTC 6229	*P. laevigatus*	AY178020
CBS 213.48	*P. nigricans*	AY178021
KCTC 6651	*P. biscuspidatus*	AY178022
KCTC 6652	*P. ferruginosus*	AY178023
KCTC 6657	*P. robustus*	AY178024
KCTC 6658	*P. spiculosus*	AY178025
KCTC 6659	*P. tremulus*	AY178026
KCTC 16881	*P. pomaceus*	AY178027
KCTC 16883	*P. pectinatus*	AY178028
KCTC 16884	*P. chrysoloma*	AY178029
KCTC 16888	*P. ribis f.ulicis*	AY178030
KCTC 16890	*P. igniarius*	AY178031

[a]American Type Culture Collection, Manassas, USA (ATCC), Institute for Fermentation, Osaka, Japan (IFO), Korean Collection for Type Culture (KCTC), Centraalbureau voor Schimmelcultrues (CBS), Micro. lab. of Pusan National Uni. (MPNU).

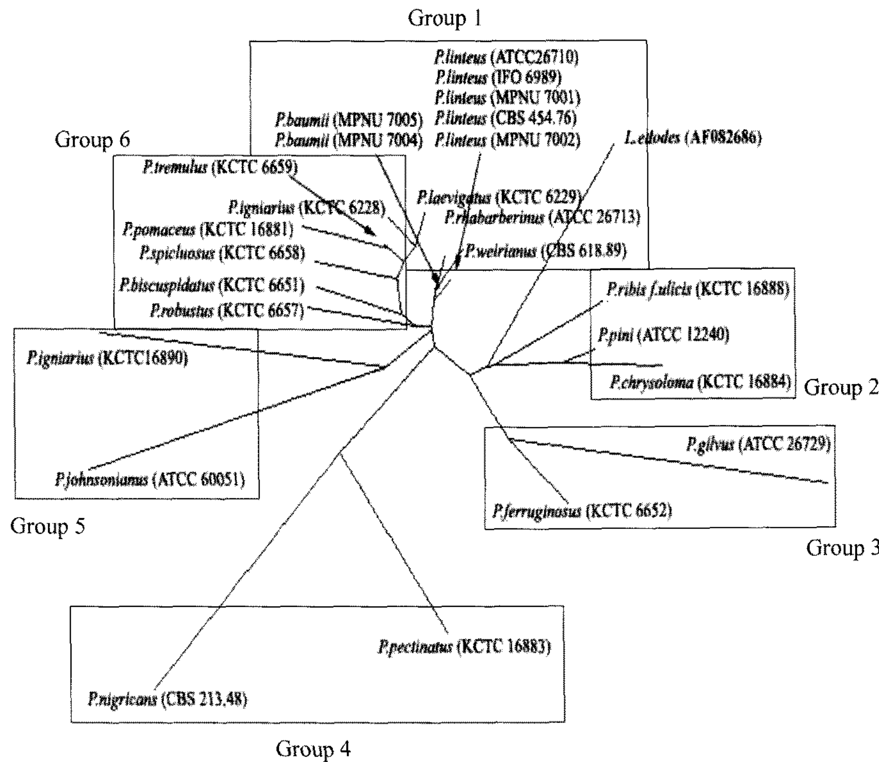

Fig. 2. Unrooted neighbor-joining tree basis on the nucleotide sequence analyses of 18S partial region. The scale bar indicates the distance of 0.01 units. Group 1: *P. linteus* and *P. baumii* group, Group 2: *P. pini* group, Group 3: *P. gilvus* group, Group 4: *P. nigricans* group, Group 5: *P. johnsonianus* group, Group 6: *P. igniarius* group.

stein, 1985). Also, Njdist from the PHYLIP 3.5 (Felsenstein, 1989) software package was used for the reconstruction of distance matrix.

Detection primer design for *P. linteus* by 18S partial nucleotides sequence analysis. The detection primers were designed on the basis of 18S rDNA partial sequence data (Figs. 4 and 5). The 18S 2F sense detection primer [5'-CTTAATGCCGATTACGAA-3' (18 mer)] by changing 2 nucleotides (C→G, A→T), 18S 3F sense detection primer [5'-CTTAATGCCCATTACGAA-3' (18mer)] by changing 3 nucleotides (C→G, G→C, A→T) and 18S 4R anti-sense detection primers [5'-GCCGGTCCAGGAGAAAGT-3' (18 mer)] were designed.

Results

Sequence alignments and phylogenetic analysis. Nucleotide alignments and distance matrix of the related genera were done (data not shown). And based on it, we described phylogenetic relationship of nineteen *Phellinus* species of 25 strains and one species of out-group [*Lentinula edodes* AF082686 from National Center for Biotechnology Information (NCBI)] by unrooted neighbor-joining tree program and bootstrap N-J methods (Fig. 2 and Fig. 3). Five strains of *P. linteus* revealed almost

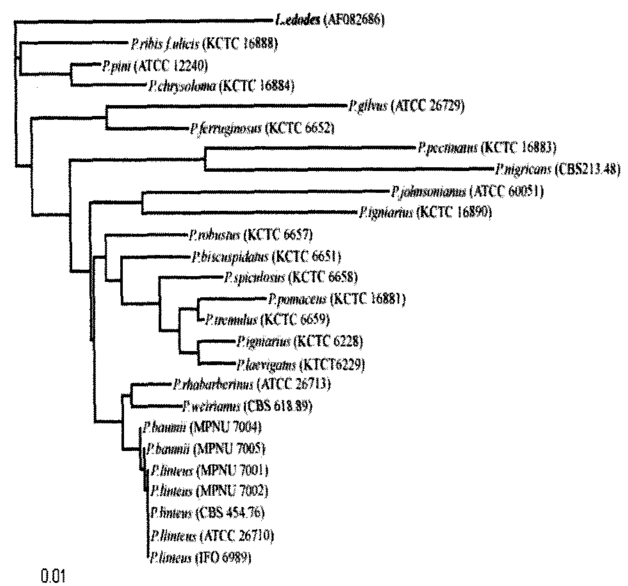

Fig. 3. Phylogenetic relationship of 18S ribosomal DNA in *Phellinus* species.

same nucleotide sequences. The nucleotide sequences of 18S ribosomal DNA partial region of *P. linteus* were very similar with *P. rhabarberinus*, *P. weirianus* and especially *P. baumii*, which were bounded as same group. *P. pini*, *P. ribis* f. *ulicis*, *P. chrysoloma* were clustered as Group 2, *P. gilvus* and *P. ferruginosus* as Group 3, *P. nigricans* with *P. pectinatus* as Group 4, *P. igniarius* (KCTC 16890) and *P. johnsonianus* as Group 5, *P. laevigatus*, *P. igniarius* (KCTC 6228), *P. tremulus*, *P. pomaceus*, *P. spiculosus*, *P. biscuspidatus* and *P. robustus* as Group 6.

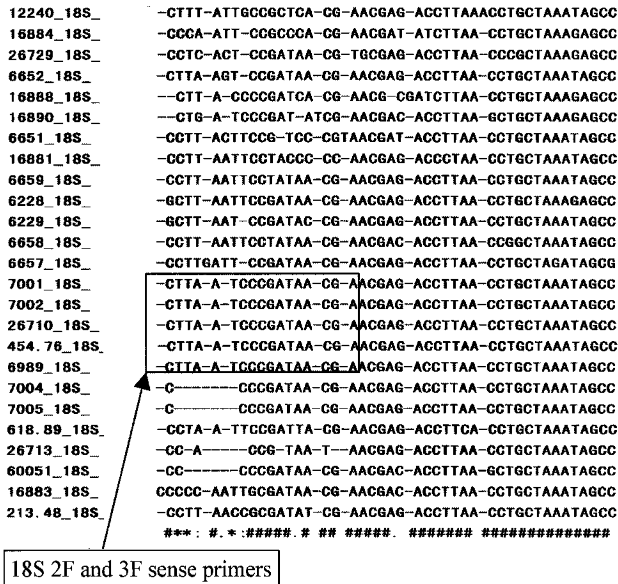

Fig. 4. Design of 18S 2F and 18S 3F sense detection primers in 18S partial sequences.

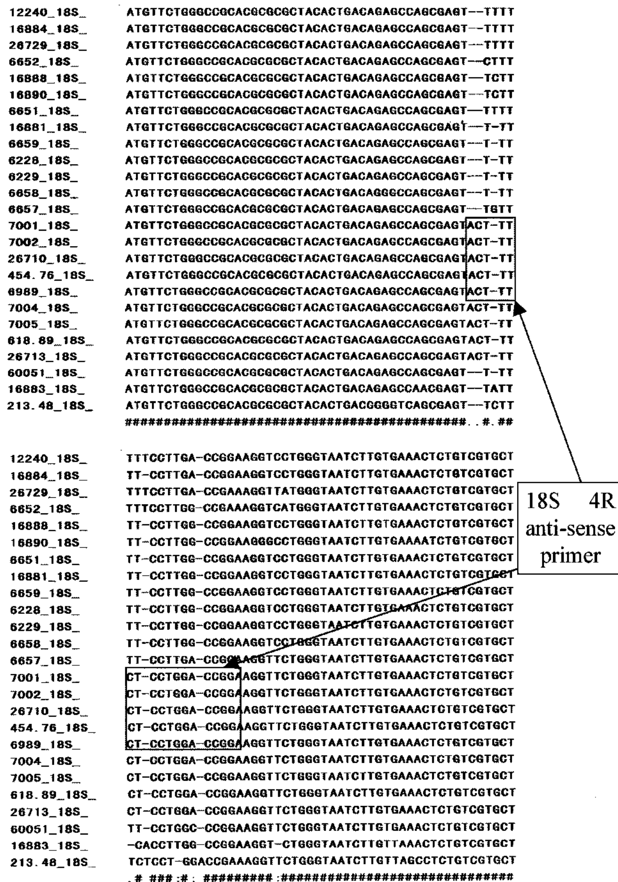

Fig. 5. Design of 18S 4R anti-sense detection primer in 18S partial sequences.

Detection of *P. linteus* by designed primers. Although 18S rDNA partial regions were conserved in genus *Phellinus*, there were some variables between species. We designed the detection primers (18S 2F sense, 18S 3F sense and 18S 4R anti-sense primers) on the basis of 18S rDNA partial sequence data (Figs. 4 and 5). The primer set of 18S 2F - 18S 4R amplified 210 bp single band (Fig. 6). The lane 1~5 of *P. linteus* showed obvious PCR products, but there were also weak bands in land 6, 7 of *P. baumii* (MPNU 7004 and MPNU 7005), lane 8 of *P. weirianus*, lane 10 of *P. rhabarberinus* and lane 21 of *P.*

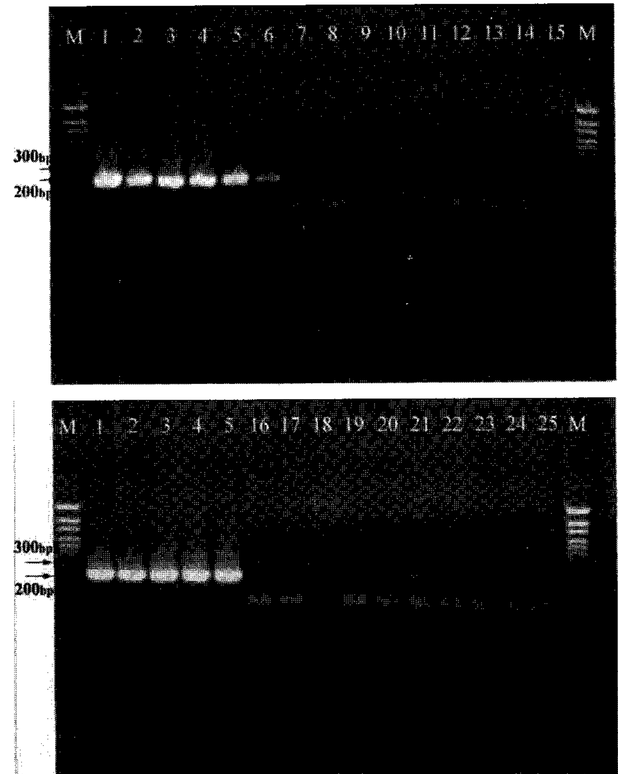

Fig. 6. PCR products of *Phellinus* species by specific primer set (18S 2F - 18S 4R primer set) for detecting of *P. linteus*. Strains: 1~5) *P. linteus*, 6~7) *P. baumii*, 8) *P. weirianus*, 9) *P. johnsonianus*, 10) *P. rhabarberinus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. nigricans*, 15) *P. laevigatus*, 16) *P. biscuspidatus*, 17) *P. ferruginosus*, 18) *P. robustus*, 19) *P. spiculosus*, 20) *P. tremulus*, 21) *P. pomaceus*, 22) *P. pectinatus*, 23) *P. chrysoloma*, 24) *P. ribis* f. *ulicis*, 25) *P. igniarius*, M) 1.5 kb ladder marker.

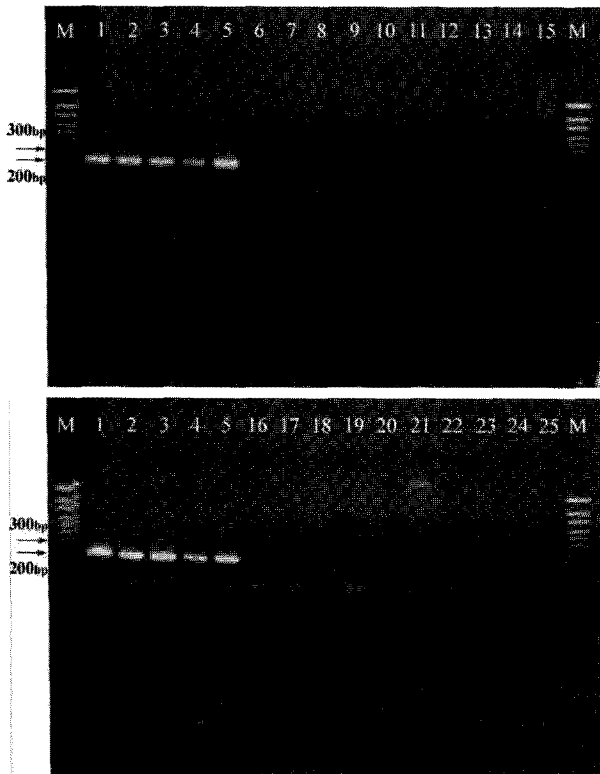

Fig. 7. PCR products of *Phellinus* species by specific primer set (18S 3F - 18S 4R primer set) for detecting of *P. linteus*. Strains: 1~5) *P. linteus*, 6~7) *P. baumii*, 8) *P. weirianus*, 9) *P. johnsonianus*, 10) *P. rhabarberinus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. nigricans*, 15) *P. laevigatus*, 16) *P. biscuspidatus*, 17) *P. ferruginosus*, 18) *P. robustus*, 19) *P. spiculosus*, 20) *P. tremulus*, 21) *P. pomaceus*, 22) *P. pectinatus*, 23) *P. chrysoloma*, 24) *P. ribis* f. *ulicis*, 25) *P. igniarius*, M) 1.5 kb ladder marker.

pomaceus. The use of 18S 3F sense primer instead of 18S 2F enhanced the specificity for *P. linteus*. Although this primer set of 18S 3F - 4R had low sensitivity than 18S 2F - 4R primer set for *Phellinus* species, it reacted with only land 1~5 of *P. linteus* (Fig. 7).

Discussion

Polysaccharides isolated from *Phellinus linteus* were known to stimulate the immune system - activation of B lymphocyte, T lymphocyte and macrophages, and increase of antibody production (Lee *et al.*, 1996; Song *et al.*, 1995). These polysaccharides were also reported to inhibit the tumor growth and metastasis (Han *et al.*, 1999), which many studies have focused on to develop new anti-tumor drug. Therefore, It has clinical significance to identify *P. linteus* exactly, but correct identification of *P. linteus* is not simple.

Generally, the taxonomy of the genus *Phellinus* has been based on the morphology of fruiting body, the pores of fruiting body, the development of basidiocarps and the association with host plant. In these contexts, *P. linteus* showed similar phenotype with *P. igniarius*, *P. nigricans*, *P. laevigatus*, *P. robustus*, *P. hartigii* and *P. baumii* (Teng, 1996; Kim *et al.*, 2001). Other taxonomic characteristics also have been investigated for the classification of genus *Phellinus*. Many researchers revealed new distinctions to the species level (Chi *et al.*, 1996, 1998; Choi, 1999; Song *et al.*, 1997), using Restriction Fragment Length Polymorphism (RFLP) analysis (Nei and Li, 1979). However, this method showed some variables in band pattern when applied in lower taxonomic levels (Donk, 1971). The nucleotide sequences of ribosomal RNA have been frequently used in molecular systematics. They include both highly conserved (18S) and highly variable sequences (Non-transcribed Spacers and Internal Transcribed Spacer) (Roderic and Edward, 1998), which make them attractive for taxonomic analysis at many levels (Bruns *et al.*, 1991; Hibbett, 1992). We previously studied divergent domain of a large ribosomal subunit to determine their applicability in the systematic of the genus *Phellinus*; 5.8S ribosomal RNA coding genes (rDNA) (Kim *et al.*, 2001) and 28S rDNA (Park *et al.*, 2002). Species-specific primers were designed from our previous studies (Kim *et al.*, 2001; Jung *et al.*, 1999), of which PCR products were unique to *P. linteus* (Park *et al.*, 2001; Nam *et al.*, 2002). ITS and 28S nucleotide sequence analysis (Kim *et al.*, 2001; Park *et al.*, 2002) constructed base of phylogenetic relationship between *Phellinus* species. However, they are not sufficient to detect *P. linteus* efficiently among over 200 *Phellinus* species. Therefore, 18S partial nucleotide sequence analyses of this study supplemented more information to the previous systematics construction using ITS and 28S nucleotide sequence analysis. For example, while phylogenetic position of *P. igniarius* in *P. laevigatus* complex group was ambiguous in the previous study (Kim *et al.*, 2001), 18S partial sequence analysis of this study clearly defined it.

Although 18S rDNA regions are conserved, some variable regions are observed between *Phellinus* species. The species-specific detection primers was designed on the basis those results. The primer set of 18S 3F 18S 4R distinguished only *P. linteus* from various *Phellinus* species, although the sensitivity of this primer set was lower than that of 18S 2F - 18 4R primer set. These primer sets designed in this study can be used for rapid certification of *P. linteus* among unknown *Phellinus* species on PCR level. As we add 18S sequence analysis to previous ITS and 28S, we construct phylogenetic relationship of total ribosomal RNA gene. Design of specific primers set by using combination of three ribosomal RNA region genes can provide more rapid and accurate detection tool for the discrimination of *Phellinus* species.

The studies for the development of species-specific

detection primers would be continued to identify similar species like *P. baumii*, *P. igniarius*, *P. pini* and *P. gilvus* and related genus like *Inonotus* and *Fomes*.

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