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Molecular Detection of *Phellinus linteus* and *P. baumii* by PCR Specific Primer

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Specific primer sets based on ribosomal DNA (rDNA) internal transcribed specer (ITS) sequences were designed for rapid detection of *Phellinus linteus* and *P. baumii*. Polymerase chain reaction (PCR) with these primers produced unique bands for each *Phellinus* species. The annealing temperature range is from 40°C to 55°C. The length of PCR products (*P. linteus* and *P. baumii*) using designed combinative primer sets of PL1F, PL2R, PB1F, PB2R, ITS5F and ITS4R, were from 520 bp to 730 bp. Fifteen strains of *Phellinus* species including *P. linteus*, *P. baumii*, *P. weirianus*, *P. johnsonianus*, *P. rhabarberinus*, *P. pini*, *P. gilvus*, *P. igniarius*, *P. nigricans and P. laevigatus* were examined in this study. Five strains, including two isolated strains of *P. linteus* (MPNU 7001 and MPNU 7002), and two isolated strains of *P. baumii* (MPNU 7004 and MPNU 7005) were shown to have about 520 bp (PL1F-PL2R), 700 bp (ITS5F-PL2R) and 600 bp (PB1F-ITS4R) -sized PCR single bands respectively. This molecular genetic technique provided a useful method for rapid detection and identification of *P. linteus* and *P. baumii*.

KEYWORDS: ***Phellinus* species, Rapid detection, Specific primer**

Genus *Phellinus* are known to have the ability to decompose organic substance by breaking down decaying wood and plant material. Also, these fungi cause wood pocket rot and other plant diseases such as canker and heart-rot in living trees (Hugues *et al.*, 1998). In recent, many reports demonstrated that *Phellinus* species contained medicinally valuable substances. In Asian countries such as China, Korea, and Japan, *Phellinus* species has been considered to cure stomachache and arthritis as an oriental medicine (Ying *et al.*, 1987). It was reported that polysaccharides from *P. linteus* showed immuno-stimulating activity (Lee *et al.*, 1996) as well as an inhibitory effect on tumor growth and metastasis (Han *et al.*, 1999). Song *et al.* (1995) revealed that these polysaccharides were able to stimulate the activity of B-lymphocyte, T-lymphocyte, and macrophages and increase antibody production. Thus, there is growing interest in medicinal value of this material for anti-tumor and immuno-stimulating properties. However, correct identification of *Phellinus* species with medicinal properties is critical to develop medicine, since all *Phellinus* species do not have the same medicinal effects (Shon and Nam, 2001). Moreover, *P. linteus* was phenotypically similar to *P. igniarius*, *P. nigricans*, *P. laevigatus*, *P. robustus*, *P. hartigii*, and especially *P. baumii* (Kim *et al.*, 2001).

The taxonomy of the genus *Phellinus* has been based on the morphological property of its fruiting body, pores of its fruiting body, development of the basidiocarps, and to a lesser degree on host plant association. Besides these morphological traits other taxonomic characteristics have also been investigated for the system of the genus *Phellinus*. The characteristics of fungal culture were also used to classify the genus *Phellinus* (Chi *et al.*, 1996, 1998; Choi, 1999; Song *et al.*, 1997). Restriction Fragment Length Polymorphism (RFLP) analyses provided molecular taxonomic tools at the species level (Nei and Li, 1979). However, these methods might be incorrect and variable in the band patterns when using lower taxonomic levels.

In our previous work, we analyzed 5.8S and internal transcribed spacers (ITS) region of ribosomal DNA to investigate their applicability in the systematics of the genus *Phellinus* (Kim *et al.*, 2001). The analyses of ribosomal DNA (rDNA) sequences have been used for fungal molecular taxonomy since they consist of a mosaic pattern of conserved and variable DNA regions (Bruns *et al.*, 1991; Hibbett, 1992). Taylor *et al.* (1990) showed that the levels of the sequence variability in a given region are quite different in various fungal taxa and no unique region can be used to identify or to address phylogenetic relationships among all fungi. The analysis of the variable region of fungal rDNA such as intergenic spacer (IGS), ITS and the divergent domain of 28S have been applied for the systematics of low taxonomic level. In this study, oligonucleotide primers based on ITS sequences of rDNA of *P. linteus* were designed (Kim *et al.*, 2001; Jung *et al.*, 1999). These primers specifically amplified the ITS sequence of rDNA of *P. linteus*. The development of these polymerase chain reaction (PCR) primers made it easier to detect *P. linteus* and *P. baumii*.

Materials and Methods

Fungal isolates. The *Phellinus* species used in this study are listed in Table 1. Eleven strains of *Phellinus*

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Table 1. List of *Phellinus* species used in this study with GenBank accesion Numbers

Code no.	Isolates[a]	*Phellinus* spp.	GenBank accession no.[b]
1	ATCC 26710	*P. linteus*	AF153010
2	IFO 6989	*P. linteus*	AF200226
3	CBS 454.76	*P. linteus*	AF082101
4	MPNU 7002	*P. linteus*	AF200228
5	MPNU 7001	*P. linteus*	AF200227
6	MPNU 7004	*P. baumii*	AF200229
7	MPNU 7005	*P. baumii*	AF200230
8	CBS 618.89	*P. weirianus*	AF110989
9	ATCC 60051	*P. johnsonianus*	AF250931
10	ATCC 26713	*P. rhabarberinus*	AY189698
11	ATCC 12240	*P. pini*	AF250930
12	ATCC 26729	*P. gilvus*	AF250932
13	KCTC 6228	*P. igniarius*	AF056192
14	CBS 213.48	*P. nigricans*	AF200239
15	KCTC 6229	*P. laevigatus*	AF053226

[a](ATCC) American Type Culture Collection, Manassas, USA: http://www.atcc.org, (IFO) Institute for Fermentation, Osaka, Japan, (CBS) Centraalbureau voor Schimmelcultrues : http://www.cbs.knaw.nl/, (KCTC) Korean Collection for Type Culture: http://www.kribb.re.kr/, (MPNU) Microbiological lab. of Pusan National University.
[b]These accession no. were registrated in our previous study.

were obtained from the ATCC (American Type Culture Collection, Manassas, USA), CBS (Centraalbureau voor Schimmelcultures) and IFO (the Institute for Fermentation), Osaka, Japan. Four strains of *P. linteus* (MPNU7001 and MPNU7002) and *P. baumii* (MPNU7004 and MPNU7005) were isolated from South Korea.

Table 2. Primer sequences used in this study

Primer	Sequence (5'-3')
PLF	5'-TCTTgTAAgTAATTAgTAg-3'(19mer)
PLR	5'-TgTCAAgAAggAggTgACTC-3'(20mer)
PBF	5'-AAgTAATTgTTAgTTAgTAg-3'(20mer)
PBR	5'-AAAggAggTAACAACAACTC-3'(20mer)

DNA extraction. For the preparation of total genomic DNA from *Phellinus* species, isolates were grown on a potato dextrose agar (PDA) medium. After sufficient growth, the fungi were transferred to flasks containing a potato dextrose broth (PDB) medium. After 2 to 3 weeks incubation, mycelia were harvested for DNA extraction.

The DNA extraction was performed by modifed Zhu and Graham's method (Zhu *et al.*, 1993; Gragam *et al.*, 1994). The 0.1 g of mycelia were chopped on the petri dish and transferred to a 1.5 m*l* tube. Then 500 μl of extraction buffer [100 mM Tris-HCl (pH 9.0), 40 mM EDTA], 300 μl of benzyl chloride and 150 μl of 10% sodium dodecyl sulphate (SDS) were added and incubated at 50°C for 30 min, 300 μl of 3 M sodium acetate (pH 5.0) was added, and then tubes were placed in a 4°C for 15 min. The test tubes were centrifuged at 12,000 rpm for 10 min. The upper phase was transferred to a new tube and extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and centrifuged at 12,000 rpm for 15 min.

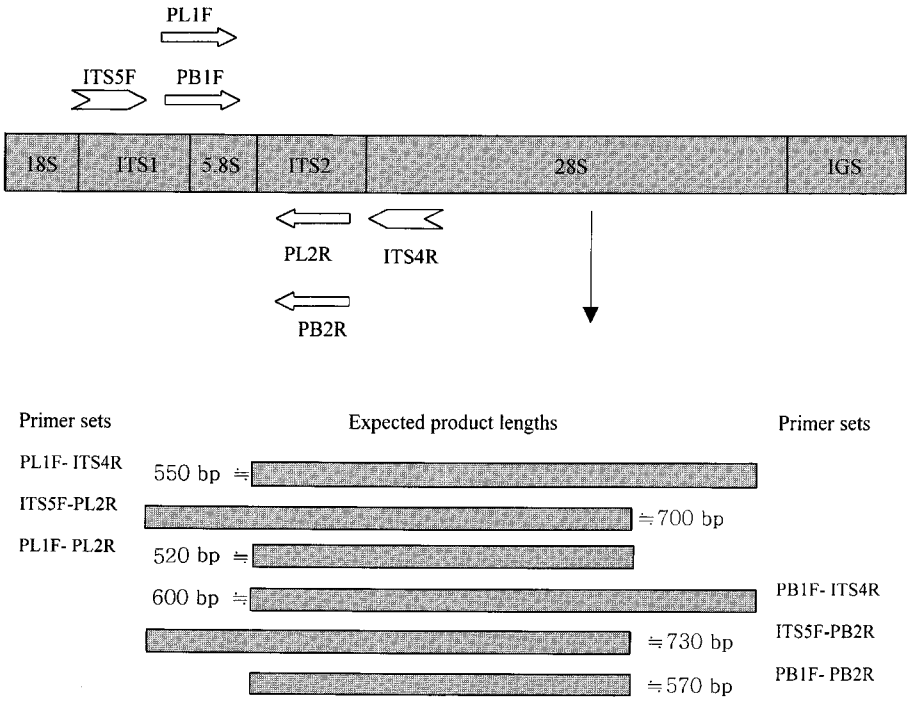

Fig. 1. Schematics of species-specific primer sites and the length of PCR product using specific primer sets about *Phellinus linteus* and *P. baumii*.

The upper phase was transferred to a new tube. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol. The pellet was washed with 2 volumes of 70% ethanol. The DNA pellets were vacuum dried and resuspended in 20 μl distilled water. The concentrated DNA were loaded in 1% agarose gel with ethidium bromide in 0.5× TBE buffer. The intensity of estimated the DNA bands were compared under UV light.

Primer design and PCR amplification. Oligonucleotide primers based on ITS sequences were designed (Table 2) in our previous study (Kim *et al.*, 2001). Primer pairs PL1F-PL2R and PB1F-PB2R (Synthesized by Bioneer Co.) were tested with total genomic DNA from 15 strains of *Phellinus* species. for specificity in PCR. Also, the ITS5F-ITS4R primer set was used as the universal primer for the amplification of ITS regions. The PCR reaction was carried out in a 20 μl PCR PreMix (Bioneer Co.) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 1 unit of *Taq* polymerase. PCR amplification was performed in a Perkin-Elmer GeneAmp PCR System 2400 under the following cycle conditions: Predenaturating at 94°C for 5 min, denaturating at 94°C for 1 min, annealing from 43°C to 55°C for 1 min, and extension at 72°C for 1 min for 30 cycles and post-run of extension at 72°C for 5 min. Annealing temperature was modulated from 40°C to 58°C for optimal band production. Also, if needed, annealing time and PCR cycles were manually controlled. Amplification products were separated by electrophoresis in 2% agarose gel of visualized by ethidium bromide staining under UV light.

Results and Discussion

For rapid detection and identification of *P. linteus* and *P. baumii,* PL1F-PL2R and PB1F-PB2R specific-primer sets were used in this study. These primer sets were designed on the basis of ITS sequences (Table 2). Schematics of species-specific primer sites and the length of PCR product using specific primer sets about *P. linteus* and *P. baumii* were shown Fig. 1. The primer PL1F-PL2R amplified 520 bp-sized band for *P. linteus* species (Fig. 2A). ITS5F-PL2R primer sets also produced 700 bp-sized specific band for *P. linteus* (Fig. 2B). However, PL1F-ITS4R primer sets also amplified about 550 bp-sized single bands of *P. baumii* (Fig. 2C lanes 6, 7), *P. johnsonianus* (lane 9) and *P. rhabarberinus* (lane 10), respectively. PL2R primer with ITS5F or PL1F could be used for detecting only *P. linteus*, specially.

The PCR reaction for detection of *P. linteus* was carried out under at a 43°C annealing temperature for 1 min, otherwise the PCR reaction temperature for *P. baumii*, was modulated from 40°C to 55°C for optimal band production. The primer sets were combined with ITS5F and ITS4R primers for detecting *P. baumii* (PB1F - PB2R, ITS5F - PB2R, PB1F - ITS4R). Expected PCR product length for *P. baumii* was also shown in Fig. 1. PB1F-PB2R and ITS5F-PB2R primer sets didnt amplification for detecting *P. baumii* (Fig. 3A, B). ITS5F PB2R primer set only amplified *P. johnsonianus* and *P. rhabarberinus* (Fig. 3B, lanes 9, 10). But, PB1F ITS4R primer produced specific single band for *P. baumii* at 55°C annealing temperature (Fig. 3C). For obtaining more clear band, annealing temperature was regulated from 40°C to 52°C. Expected 600 bp-size single band was not only amplified

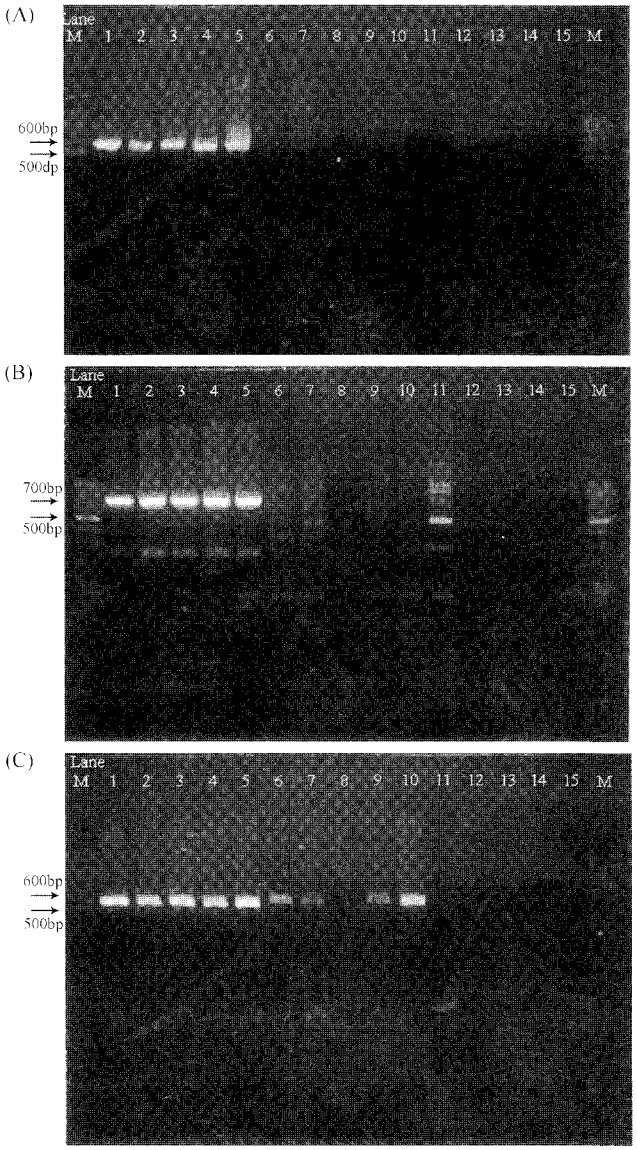

Fig. 2. PCR products of *Phellinus* species by specific primers for detection of *P. linteus.* Primer sets: (A) PL1F-PL2R (B) ITS5F-PL2R (C) PL1F-ITS4R.
Strains: 1-5) *P. linteus*, 6-7) *P. baumii*, 8) *P. weirianus*, 9) *P. johnsonianus*, 10) *P. rhabarberinus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. nigricans*, 15) *P. laevigatus*, M) 1.5 kb ladder marker.

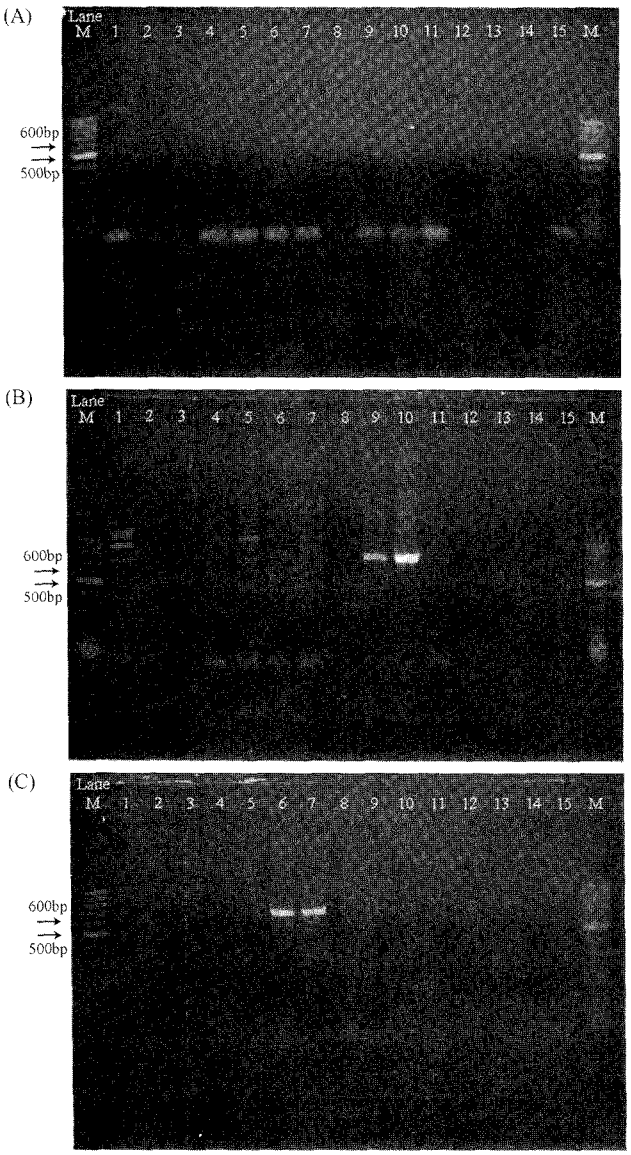

Fig. 3. PCR products of *Phellinus* species by specific primers for detection of *P. baumii*. Primer sets (A) PB1F-PB2R (B) ITS5F-PB2R (C) PB1F-ITS4R.
Strains: 1-5) *P. linteus*, 6-7) *P. baumii*, 8) *P. weirianus*, 9) *P. johnsonianus*, 10) *P. rhabarberinus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. nigricans*, 15) *P. laevigatus*, M) 1.5 kb ladder marker.

for *P. baumii* but also other *Phellinus* species, *P. weirianus*, *P. johnsonianus* and *P. rhabarberinus*, amplified same size bands (data not shown). Continually, PCR reaction was reacted at a 58°C of annealing temperature, but it might be over temperature for attaching the primer sets on DNA (data not shown). As a result, PB1F-ITS4R primer set could detect *P. baumii*, specially. Two-primer sets of ITS5F-PL2R and PB1F-ITS4R on basis ITS sequences would detect *P. linteus* and *P. baumii* between *Phellinus* species without ITS sequencing. In this study, fifteen strain, ten species, of genus *Phellinus* were examined for rapid detection, specific primer sets need to apply more many *Phellinus* species.

Until now, the taxonomy of the genus *Phellinus* has been based on the morphological property of its fruiting body, pores of its fruiting body, development of the basidiocarps. But it was impossible that the morphological variation and genetic modification of long time were applied to classify all species. And these might be required introduction of new molecular techniques for identification and detection. Phylogenetic analysis basis on molecular detection would complement morphological view to explain interspecies relationships. Especially, *P. linteus* and *P. baumii* have trait of similar shape, color and pores of fruit body, and these similar traits has occurred problem what is the original thing with containing valuable substances, medicinally. *P. linteus* has been considered to cure stomachaches and arthritis as an oriental medicine (Ying *et al.*, 1987), and it has inhibitory effect on tumor growth and metastasis (Han *et al.*, 1999). But *P. baumii* might have evolved from *P. linteus* on the basis phylogenetic similarity and divergence (Kim *et al.*, 2001), was not found out containing medicinally valuable substances yet. This is reason why it is important to develop a gene probe for detecting valuable species, rapidly and exactly. Also, it should be useful for detecting relative genus and valuable species.

In this study, to solve these problems, *P. linteus* or *P. baumii* focused on developing of gene probes with the specificity to bind it only. Continually, development of a gene probe using sequencing of more many regions like 18S or 28S rDNA, would be helped to review phylogenetic relationships of interspecies.

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