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Development of Species-specific Primers for Rapid Detection of *Phellinus linteus* and *P. baumii*

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Genus *Phellinus* taxonomically belongs to Aphyllophorales and some species of this genus have been used as a medicinal ingredients and Indian folk medicines. Especially, *P. linteus* and morphological-related species are well-known medicinal fungi that have various biological activities such as humoral and cell-mediated, anti-mutagenic, and anti-cancer activities. However, little is known about the rapid detection for complex *Phellinus* species. Therefore, this study was carried out to develop specific primers for the rapid detection of *P. linteus* and other related species. Designing the species-specific primers was done based on internal transcribed spacer sequence data. Each primer set detected specifically *P. linteus* (PL2/PL5R) and *P. baumii* (PB1/PB4R). These primer sets could be useful for the rapid detection of specific-species among unidentified *Phellinus* species. Moreover, restriction fragment length polymorphism analysis of the ITS region with *Hae*III was also useful for clarifying the relationship between each 5 *Phellinus* species.

KEYWORDS: ITS region, *Phellinus*, Phylogeny, RFLP, Species-specific PCR primer

The genus *Phellinus* is taxonomically classified into Aphyllophorales in Hymenochaetceae of Basidiomycota (Larsen and Cobb-Poulle, 1990). These fungi are widely distributed in subtropical and tropical regions of Asia including China, Japan, southeastern USA, Maxico, and India and include both annual and perennial forms (Donk, 1973; Patouillard, 1900). *Phellinus* species are known to cause white pocket rot and severe plant diseases such as root rot, canker, or heart rot in living trees, as well as destroying slash and other woody residues. Larsen and Cobb-Poulle (1990) considered *Quercus* and *Cassia* as major hosts, but Ahmad (1972) added living trees of *Lonicera* species into its host range. The host range of *P. linteus* from Korea seems fairly broad on trees like *Morus*, *Quercus* or *Lonicera* (Kang *et al.*, 2002; Kim *et al.*, 1999).

Some species of these fungi have been used as a medicinal ingredient and Indian folk medicines (Vaidya and Rabba, 1993). Currently, the interest in *P. linteus* isolates is dramatically increasing since it was reported that a complex group of polysaccharides isolated from the basidiomes plays an important role in antitumor, cell-mediated and humoral immunity (Kim *et al.*, 2003a, b).

Despite such great medicinal value, the concept of species and the methods of precise identification is not well studied until now. Morphological characteristics such as basidiocarp, mycelium, and skeletal have been used as taxonomical keys for identifying *Phellinus* species (Dai and Xu, 1998). However, identification of *P. linteus* based on morphological characteristics requires time consuming and labour intensive steps and could be inaccurate due to their morphological variations corresponding to environmental conditions. In order to resolve these problems, relationships between molecular marker of *P. linteus* and morphological-related characteristics of *Phellinus* species should be investigated.

Ribosomal DNA (rDNA) has a mosaic pattern of conserved and variable regions, making them attractive for taxonomic investigations at many levels (Ward and Akrofi, 1994; Ko *et al.*, 1997). The coding regions have evolved slowly and are highly conserved between different species and genera, and therefore can be used for comparing distantly related organisms. Both the internal transcribed spacer (ITS) and intergenic spacer (IGS) have evolved rapidly and can be used for comparison of closely related species and subspecies (Black *et al.*, 1989; Chen *et al.*, 1992; Liu *et al.*, 2002). These spacers are located within the rDNA repeat with ITS 1 between the 18S rDNA and 5.8S and ITS 2 between 5.8S and 28S rDNA.

The methods for identification with species-specific primers and restriction fragment length polymorphism analysis (RFLP) (Fernandez *et al.*, 2001; Pandey *et al.*, 2003; Fisher *et al.*, 1999; Takashi *et al.*, 2003) in the ITS can also be used to identify variation at intraspecific levels and to discriminate between highy relative species (Tymon *et al.*, 2004). Genomic analysis based on molecular techniques can be applied independent of developmental stages of organism and environmental effects. Particu-

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larly, PCR has become an attractive tool for the detection of specific microorganisms in microbial ecology and much efforts have been devoted to develop the species-specific primers (Tymon *et al.*, 2004; Scott *et al.*, 2004; Kang *et al.*, 2002; Gardes and Bruns, 1993; Park *et al.*, 2001). PCR technique may greatly enhance detection sensitivity, simplicity and rapidity and is based on specific amplification of target DNA sequence that is uniquely conserved in a genome.

In previous studies, there is no comparison between *P. linteus* and *P. baumii* directly although differentiation of *Phellinus* species have been performed using various molecular detection methods. *P. linteus* and *P. baumii*, are commonly referred to as 'Sangwhang' in Korea. Therefore, the aim of this study was to develop species-specific markers that could be used to distinguish *P. linteus* from other related *Phellinus* speices including *P. baumii.*

Materials and Methods

Fungal isolates. List of *Phellinus* species used in this study are shown at Table 1.

DNA extraction. For the preparation of genomic DNA from *Phellinus* isolates, colonies from potato dextrose agar (PDA: Gibco BRL, CA) medium were transferred to flasks containing potato dextrose broth (PDB: Gibco BLR) medium. The flask cultures were incubated in a stationary state at 25°C for 2 or 3 weeks, then mycelia were harvested and freeze-dried. DNA extraction was performed with the DNeasy Plant Mini Kit (QIAGEN, CA) according to the manufacturer's protocol.

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

Isolates*	Species	GenBank accession no.
ATCC 26710	*P. linteus*	AF153010
IFO 6980	*P. linteus*	AF200226
MPNU 7001	*P. linteus*	AF200227
MPNU 7002	*P. linteus*	AF200226
MPNU 7016	*P. linteus*	AF153009
MPNU 7004	*P. baumii*	AF200229
MPNU 7005	*P. baumii*	AF200230
MPNU 7006	*P. baumii*	AF200231
KCTC 6227	*P. igniarius*	AF110991
KCTC 6228	*P. igniarius*	AF056192
ATCC 26729	*P. gilvus*	AF250932
ATCC 12240	*P. pini*	AF250930
KFDA P019	*P. pini*	AF436619
KFDA P020	*P. pini*	AF436621
KFDA P023	*P. pini*	AF436622

*ATTC : American Type Culture Collection, Manassas, USA, KCTC : Korean Collection for Type Culture. KFDA : Korea Food & Drug Administration, MPNU: Micro. lab. of Pusan National University, IFO : Institute for Fermentation, Osaka, Japan.

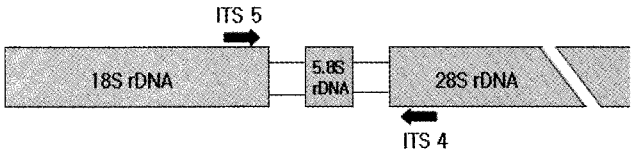

Fig. 1. A diagram of rDNA cluster of higher fungi and PCR primers for ITS amplification and sequencing of the amplified ITS region.

Primer design. The primers to specifically detect *Phellinus* species were developed through visual comparison of the alignment of all sequences used in phylogenetic analysis. The specificity of potential primers was further tested by BLAST (Altschul *et al.*, 1997) searching through the GenBank database for compatible sequences. Following the identification of suitable target regions in the *Phellinus* species sequence, the primers were tested in against all the tested isolates.

PCR amplification. DNA was usually diluted 1 : 10 with distilled water. Approximately 650 bp of the ITS region in the rDNA fragments were amplified using universal primers ITS5 and ITS4 that target conserved regions in the 18S and 28S rDNA genes (Fig. 1; White *et al.*, 1990). PCR reaction was carried out in a 20 μl PCR PreMix (Bioneer Co. Daejon, Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgC, 250 μM dNTP, 1 unit of *Taq* polymerase (all from Takara, Japan). Amplifications were carried out in a PCR thermal cycler (Bioneer Co., MyGenie 96 Thermal Block) using an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C and extension for 1 min at 72°C. This was concluded with a final extension for 5 min at 72°C. Amplicons were separated in 1% agarose gels in 0.5 × TBE buffer at 50 V for 40 min, stained with ethidium bromide and visualised under UV light. PCR products were purified with Gel Extraction Kit (Core-One™).

PCR-RFLP analysis. Usually 100 ng of the ITS-PCR products were digested with the restriction enzyme *Hae*III at 37°C for 24 h. The restriction products and a molecular size maker (Sigma: St. Louise, MO) were separated on 3% agarose gels for 4 h at 25 V. Results were recorded by photographing gels over a UV transilluminator.

Results

Sequence alignments and phylogenetic analysis. To conduct phylogenetic analysis, we examined the alignment of sequences initially accomplished by Clustal G (version 1. 4). All of ITS sequences used in this study refered to GenBank database and accession numbers were shown at Table 1. According to the sequence alignment

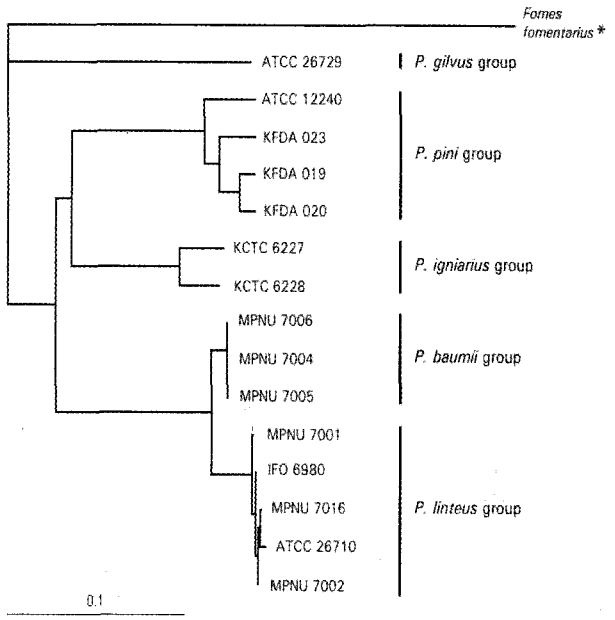

Fig. 2. Phylogenetic relationship among *Phellinus* species assessed by the bootstrap NJ method using sequence data of ITS regions. The asterisk represents outgroup.

analysis, ITS 1 region is more variable than ITS 2. Phylogenetic relationship among *Phellinus* species was assessed by the bootstrap neighbor-joining method (Fig. 2). We described phylogenetic relationship of five *Phellinus* species of 15 strains and one species of outgroup (*Fomes fomentarius*: Accession No. AY354213). The phylogram generated from ITS sequences produced five discrete clusters, one each for *P. linteus*, *P. baumii*, *P. igniarius*, *P. gilvus*, and *P. pini*. The topologies of phylogenetic trees produced by distance and maximum parsimony methods were almost identical.

Primer design. To develop the species-specific primer, PCR was first conducted using ITS-PCR products as template that were obtained using ITS5/4 primer set (White *et al.*, 1990). The size of the amplified ITS region for five species was more or less uniform for most taxa, and ranged between 650 and 700 bp. From above data, we designed the specific primer for detecting the differences between *P. linteus* and *P. baumii*, and the sequences and length were described at Table 2. As shown in Fig. 3, the primer set PL2/PL5R produced a single DNA band approximately 420 bp in size for *P. linteus* (reference strain

Table 2. Species-specific primers designed in ITS regions

Species	Primer	Sequence (5'-3')
P. linteus	PL2	5'-GTCTGTCTTGTAAGTAATGAG-3'
	PL5R	5'-CGACCCCTCGAAAGGCGC-3'
P. baumii	PB1	5'-GCGAATGAATTTTTGCGCATG-3'
	PB4R	5'-CCCTTCGAAAGGCAAACAG-3'

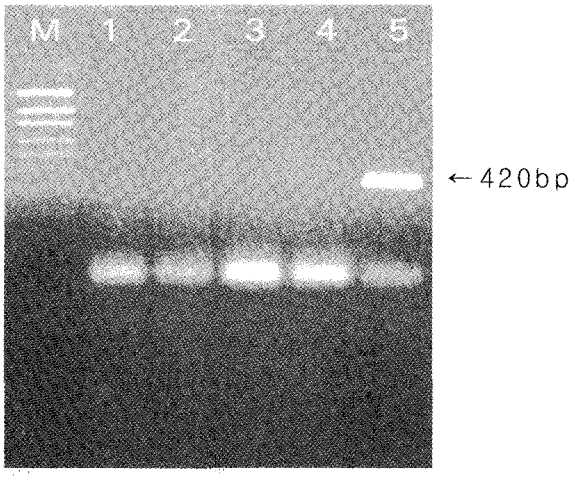

Fig. 3. PCR products for detecting *Phellinus linteus*. PL2-PL5R primer set was used. M; size marker, Lane 1; *Phellinus baumii* MPNU 7005, Lane 2; *P. gilvus* ATCC 26729, Lane 3; *P. igniarius* KCTC 6228, Lane 4; *P. pini* ATCC 12240 and Lane 5; *P. linteus* ATCC 26710.

ATCC 26710). The primer set PB1/PB4R produced a single band about 500 bp in size specifically *P. baumii* (reference strain MPNU 7005; Fig. 4). Therefore, these primers seem to be specific for *P. linteus* and *P. baumii*, respectively.

PCR-RFLP analysis. We additionally examined the PCR-RFLP analysis because the genus *Phellinus* contains many

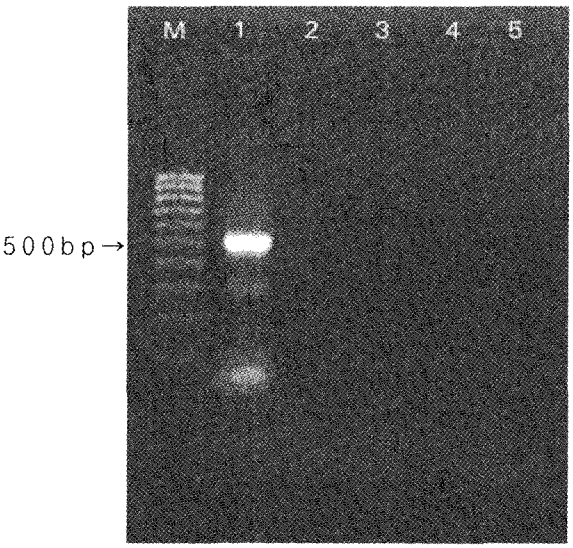

Fig. 4. PCR products for detecting *Phellinus baumii*. PB1-PB4R primer set was used. M; size marker, Lane 1; *Phellinus baumii* MPNU 7005, Lane 2; *P. gilvus* ATCC 26729, Lane 3; *P. igniarius* KCTC 6228, Lane 4; *P. pini* ATCC 12240 and Lane 5; *P. linteus* ATCC 26710.

species with morphologically similar characteristics. In the assay, each five reference species was digested with *Hae*III. For five taxa, the *Hae*III fragments are shown in Fig. 5 and the expected sizes of the restriction fragments are given at Table 3. Digestion of the ITS-PCR products with restriction endonuclease *Hae*III showed that band sizes obtained by electrophoresis in 3% agarose gels were in agreement with the expected sizes of the restriction fragments inferred from sequence analysis. Using *Hae*III, all the species showed one to three bands between 80 and 706 bp (Fig. 5). Five patterns were unique to one taxon only. The banding profiles, evaluated from the sequence data, seemed to be indistinguishable on the gel. Thus, digestion of the ITS-PCR products with endonuclease *Hae*III resulted in the identification of 5 *Phellinus* species.

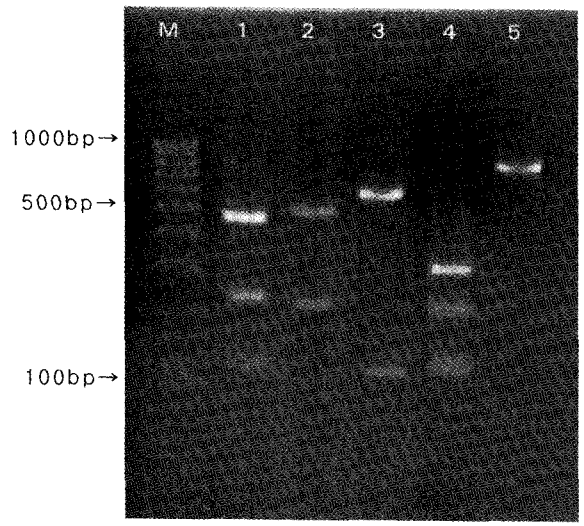

Fig. 5. RFLP analysis of complete ITS regions amplified with primers ITS5/4. PCR amplicons were digested with *Hae*III. M; size marker, Lane 1; *Phellinus baumii* MPNU 7005, Lane 2; *P. gilvus* ATCC 26729, Lane 3; *P. igniarius* KCTC 6228, Lane 4; *P. pini* ATCC 12240 and Lane 5; *P. linteus* ATCC 26710.

Table 3. Sizes of restriction fragments estimated in some *Phellinus* species

Species (total length analyzed) Strain (reference) (accession no.)	Enzyme used/ size of fragments (bp)
Phellinus baumii (747 bp) MPNU 7005 (AY178013)	*Hae*III; 479, 195, 73
P. gilvus (673 bp) ATCC 26729 (AY178018)	*Hae*III; 460, 213
P. igniarius (654 bp) KCTC 6228 (AY178019)	*Hae*III; 574, 80
P. pini (695 bp) ATCC 12240 (AY178017)	*Hae*III; 307, 202, 120, 66
P. linteus (706 bp) ATCC 26710 (AY178007)	*Hae*III; 706

Discussion

P. linteus and the related species have been well known as plant pathogens that cause a white pocket rot on living tree in tropical or subtropical regions worldwide (Gilbertson, 1980). On the other hand, they are also known for their medicinal uses in the Orient. Recently, the interest in *P. linteus* isolates is dramatically increasing since it was reported that the polysaccharide components have the ability to enhance and stimulate the immune system of humans. Despite such great medicinal value, the species-concept and discrimination of these fungus is not well studied (Kim *et al.*, 1999).

Morphological characters such as basidiocarp and mycelium have been used as taxonomical criteria for identifying *Phellinus* species (Dai and Xu, 1998). However, we need more objective criteria about taxonomy of *Phellinus* species since morphological characters are changeable according to their environmental conditions.

ITS region contains two variable non-coding regions that are nested within the rDNA repeat between the highly conserved small subunit, 5.8S and large subunit rRNA genes. Several features make a convenient target region for molecular identification of fungi: (i) in fungi, the entire ITS region is often between 600 and 800 bp and can be readily amplified with 'universal primers' that are complementary to sequences within the rDNA (White *et al.*, 1990), (ii) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute, or highly degraded DNA samples, and (iii) several studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species (Fernandez *et al.*, 2001; Gardes and Bruns, 1993; Shin *et al.*, 2001; Skouboe *et al.*, 1989; Pandey *et al.*, 2003).

Until now, a number of studies on the phylogenetic analysis in *Phellinus* and its related species have been conducted in partial regions of rDNA such as D1-D2 region in 28S rDNA, 18S rDNA, and ITS region (Jin *et al.*, 2003; Nam *et al.*, 2002, 2003; Park *et al.*, 2002). In this study, we focused on developing detectable primers and differentiation of *P. linteus* and *P. baumii* from other related species.

In this study, *P. linteus* and *P. baumii* were targeted to develop species-specific diagnostic PCR gene probes. These probes developed in this study, PL2/PL5R and PB1/PB4R, could be useful for detecting relative genus and valuable species. In addition, PCR-RFLP analysis in ITS region would be an efficient diagnostic method potentially. Also these methods could potentially be applied in monitoring the two species in infected materials such as plant tissues for epidemiological and ecological study.

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