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PCR-Based Sensitive Detection of Wood-Decaying Fungus *Phellinus linteus* by Specific Primer from rDNA ITS Regions

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Based on the rDNA ITS sequences data, specific primer set for PCR detection of wood-decaying fungus *Phellinus linteus* was designed. The length of PCR products using designed primer set (SHF and SHR) was about 540 bp. Among 11 species, 17 isolates of *Phellinus* spp. including *Phellinus linteus*, *P. pomaceus*, *P. spiculosus*, *P. baumi*, *P. pini*, *P. igniarius*, *P. gilvus*, *P. biscuspidatus*, *P. weirii*, *P. johnsonianus*, *P. robutus*, and *P. igniarius*, seven isolates of *Phellinus linteus* showed about 540 bp-sized single band. This molecular technique could offer a useful tool for detecting and identifying *Phellinus linteus*.

KEYWORDS: ***Phellinus linteus*, rDNA, Primer, Detection**

Phellinus species are known to cause white pocket rot and severe plant diseases such as canker or heart-rot in living trees. These fungi are widely distributed and have both annual and perennial forms. Some species of them have attracted great attention owing to their high anti-tumor effect and other medicinal value in Korea, China and Japan. *Phellinus linteus* first was illustrated and described as *Polyporus linteus* by Berkeley and Curtis (1860). Later, Teng (1964) renamed this species *P. linteus*. The taxonomic concept of *P. linteus* has not, however, been established due to disagreements among taxonomists.

To solve such problems, DNA markers in molecular methods has been employed for sensitive and rapid identification of fungi (Kim *et al.*, 1999; Oliver and Loria, 1998). Optimal methods for the detection of decay fungi in wood have not, however, been developed. The development of a reliable assay for the early detection of decaying in wood is needed. A specific DNA marker capable of identifying *P. linteus* has remained to be developed. PCR technique may greatly enhance detection sensitivity, simplicity and rapidity compared to probe hybridization (Audy *et al.*, 1994) and is based on specific amplification of target DNA sequence that is uniquely conserved in a genome. The development of the DNA-based polymerase chain reaction (PCR) (Mulliis and Faloona, 1987) and taxon specific primers (Gardes and Bruns, 1993) is making it increasingly feasible to detect and study fungi in their natural substracts.

The objective of this study was to develop PCR primers specific for *P. linteus* from unique ITS sequences of this fungus and to use them for detection of this fungus in plant tissue. We here describe PCR protocol useful for sensitive and specific detection of *P. linteus*.

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Materials and Methods

Fungal isolates. The *Phellinus* species used in this study are listed in Table 1. Seventeen *Phellinus* isolates were obtained from the American Type Culture Collection (ATCC), Centraalbureau voor Schimmelcultures (CBS) and

Table 1. List of *Phellinus* spp. in the study with Genbank Accesion Numbers

Code no.	Isolates	Species	GenBank accession nos
1	ATCC 26710	*Phellinus linteus*	AF153010
2	IFO 6989	*P. linteus*	AF200226
3	SNU 6719	*P. linteus*	
4	P2	*P. linteus*	
5	WD 1222	*P. linteus*	
6	P3	*P. linteus*	
7	P4	*P. linteus*	
8	P5	*P. baumi*	
9	ATCC 60051	*P. johnsonianus*	AF250931
10	SNU 6656	*P. pomaceus*	AF250929
11	ATCC 12240	*P. pini*	AF250930
12	ATCC 26729	*P. gilvus*	AF250932
13	KCTC 6227	*P. igniarius*	AF251437
14	CNU 6017	*P. weirii*	
15	KCTC 6651	*P. biscuspidatus*	
16	SNU 6657	*P. robustus*	
17	SNU 6658	*P. spiculosus*	

* American Type Culture Collection (ATCC): http://www.atcc.org/
* Centraalbureau voor Schimmelcultures (CBS): http://www.cbs.knaw.nl/
* Korean Collection for Type Culture : http://www.kribb.re.kr/
* SNU: Seoul National University, Korea.
* WDI: Tzukuba Forest Institute, Japan.
* P: Applied Microbiology Div., NIAST, Korea.
* CNU: Chungnam National University, Korea.
* Underlined : cited sequences from GenBank.

IFO. The culture media and conditions were recommended in ATCC, CBS, IFO catalogue.

DNA extraction. For the preparation of total genomic DNA from *Phellinus* isolates, colonies were transferred to flasks containing malt-yeast broth (2% malt extract, 0.2% yeast extract). After 10 days culturing, mycelia were filtered and dehydrated. Total DNA from the fungi was prepared as described by Graham *et al.* (1994). The 200 mg mycelia were transferred to 1.5 ml test tube and of 400 μl lysis buffer (200 mM Tris-HCl [pH 8.0], 100 mM NaCl, 25 mM EDTA, 0.5% SDS) containing proteinase K (50 ug) was added in the tube. After incubation at 37°C for 1 h, 2 × CTAB buffer (2% CTAB [w/v], 100 mM Tris [pH 8.0], 1.4 M NaCl, 1% polyvinylpyrrolidone Mr 40,000) was added in the tube, placed in 65°C water bath for 15 min and then extracted with chloroform-isoamyl alcohol (24 : 1, v/v) and centrifuged at 12,000 rpm for 10 min. The upper phase was transferred to new tube and DNA was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol, dried, and resuspended in 50 μl of TE buffer. Five microliter of DNA sample was used for PCR amplification. DNA pellets were air-dried and resuspended in TE buffer (10 mM Tris-HCI, 1 M EDTA, pH 8.0). Concentrations of DNA were estimated by comparing the intensity of DNA band in 1% agarose gels with a series of DNA dilutions (viewing under UV light after staining with ethidium bromide).

rDNA ITS region sequencing and accession number assigned. The clones containing rDNA ITS region were sequenced by automatic sequencer (ABI PRISM™ 377 DNA sequencer) using DNA sequencing Kit with BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems Co.) according to the supplier's instructions. Sequences were aligned using the DNASTAR software package (DNASTAR Inc.) and subjected to a database search (GenBank, release 98.0), using both the BLAST and FASTA programs. The sequences were deposited in the GenBank database under National Center for Biotechnology Information (NCBI) and the accession numbers were assigned (Table 1).

Primer design and PCR amplification. Oligonucleotide primers were designed (Table 2). Primer pair SHF-SHR (Synthesized by TAKARA Co.) were tested for specificity with total genomic DNA from 17 isolates of *Phellinus* spp. in PCR amplifications. PCR reaction was performed in a 50 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each of dNTP, 100 ng primer, 2.5 unit of *Taq* polymerase (Promega Co.). PCR amplification was carried out in a PTC-100TM (MJ Research, Inc) using the following profile: one cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; one cycle of a final extension for 7 min at 72°C. PCR products were separated on a 1.5% agarose gel in 1X TAE buffer and visualized by staining with ethidium bromide.

Table 2. Primer sequences used in this study

Primer	Sequence(5'-3')
SHF	5'-GAAGTTAGTAGCCTGAGGTCTT-3'
SHR	5'-AGGTGTCTTGTTGGAGGTGA-3'

Results and Discussion

rDNA ITS regions of 11 species, seven isolates including two ITS sequences of *P. linteus* from GenBank were sequenced and aligned. In this multiple alignment, the sequence array of *P. johnsonianus* and *Phellinus baumi* were very similar to that of *P. linteus* (Fig. 2). It was the difficult point to design for specific detection of *P. linteus.* The selected spans for specific primer were 139~173 bp and 724~752 bp, respectively (Fig. 2). For the more sensitive detection of *P. linteus*, the oligomers of SHR were modified, i.e. 8th and 9th nucleotide "AA" into "TT", 11th and 12th "AA" into "TT". Primer pair SHF-SHR amplified a single product (540 bp) from DNA of all isolates of *P. linteus* (Fig. 1).

In recent, many fruiting-bodys of *Phellinus* group have been imported from foreign countries every year because they are recognized to have high anti-tumor effect. They named "Sang-Hwang" are being sold at market places in too much high price. Generally, not officially, *P. linteus* or *P. igniarius* is called as "Sang-Hwang", but *P. linteus* is preferred to *P. igniarius*.

Our laboratory has focused on molecular assay for

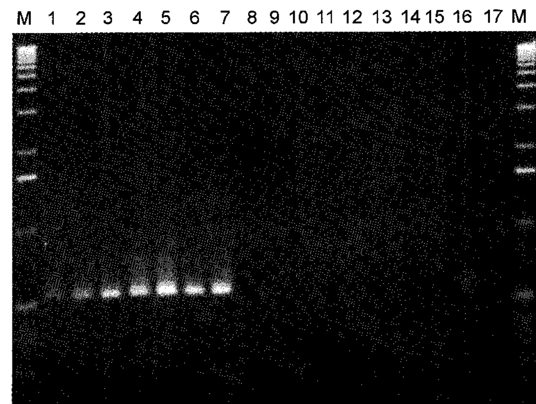

Fig. 1. PCR products of *Phellinus* spp. by specific primers for detection of *P. linteus.*
1-7) *P. linteus*, 8) *P. baumi*, 9) *P. johnsonianus*, 10) *P. pomaceus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. weirii*, 15) *P. biscuspidatus*, 16) *P. robustus*, 17) *P. spiculosus* M) 1 Kb ladder marker.

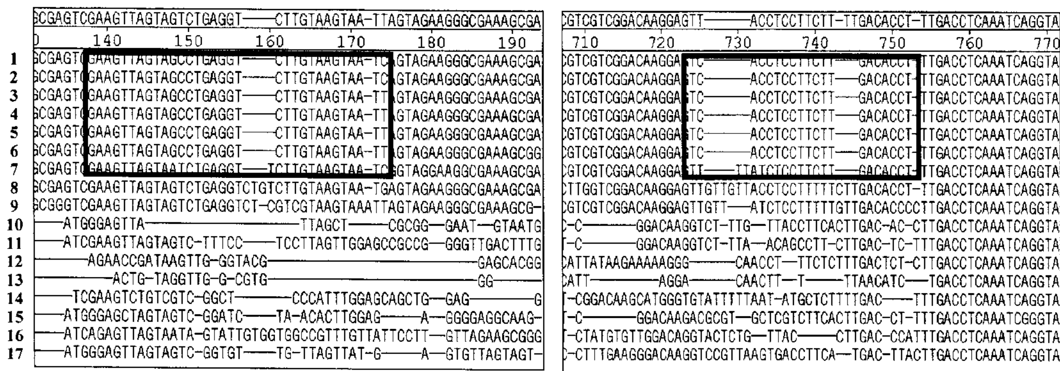

Fig. 2. *Phellinus linteus* specific detection primer design based on the hypervariable regions of rDNA ITS region between isolates of *Phellinus* spp.
1-7) *P. linteus*, 8) *P. baumi*, 9) *P. johnsonianus*, 10) *P. pomaceus*, 11) *P. pini*, 12) *P. gilvus*, 13) *P. igniarius*, 14) *P. weirii*, 15) *P. biscuspidatus*, 16) *P. robustus*, 17) *P. spiculosus*.

detection and identification of *P. linteus* for quarantine. Specific DNA markers for confirming plant pathogen at levels of subspecies was well documented by many reports (Audy *et al.*, 1994; Mill *et al.*, 1997; Minsavage *et al.*, 1994). There have been several reports for specific detection of bacteria using serological and DNA hybridization methods (Klopmeyer *et al.*, 1988; De Boer *et al.*, 1987). However, DNA detection of plant pathogenic fungi has largely been focused at level of species. Recently, for specific detection of *P. infestans*, *P. erythroseptica* and *P. nicotianae* which are already notorious for major pathogen of potato and tobacco in Korea and other countries, primer sets based on ribosomal DNA ITS sequence data have been developed (US patent No. US 5,874,221). Detection with species-specific primers from nuclear ribosomal ITS regions has several advantages. The presence of multiple copies of ITS regions in the genome allows easier amplification from preparations containing very low concentration of the target DNA, but large amounts of host DNA, e.g. plant tissue.

Recently, design of PCR primers specific for the β-tublin gene of plant pathogenic fungus, *Helminthosporium solani*, was reported. That study was to identify thiabendazole-resistant isolates of *H. solani*. But, β-tublin gene is only present in very few copies in the genome. Primers based on the sequence of this gene would be less sensitive in detection of fungus than those based on the multi-copy ribosomal gene cluster.

In our study, ITS regions and 5.8S rDNA from *P. linteus* originating from North America, India, Europe, Japan and Korea had almost identical nucleotide sequences, indicating that these strains are a homogeneous phylogenetic group and that primers designed from ITS regions should be useful for detection of the fungus regardless of the source of the strain.

Therefore, these PCR primers can be used to detect and diagnose the fungus on wood at any stage of disease development.

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