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PCR Based Detection of *Phellinus linteus* using Specific Primers Generated from Universal Rice Primer (URP) Derived PCR Polymorphic Band

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This study was carried out to develop specific primers for PCR detection of *Phellinus linteus*. Diverse genomes of 15 *Phellinus* spp. including five *Phellinus linteus* isolates were fingerprinted by Primer Universal rice primer (URP)1F. The URP-PCR pattern differentiated *P. linteus* isolates from other *phellinus* spp. A polymorphic band (2.8 kb), which is unique for *P. linteus* isolates, was isolated and sequenced. Twenty four-oligonucleotide primer pairs were designed based on information of DNA sequence. The primer set (PLSPF2/PLSPR1) amplified single band (2.2 kb) of expected size with genomic DNA from seven *Phellinus linteus*, but not with that of other *Phellinus* species tested. The primers could be used identically in both DNA samples from mycelium and fruit bodies. This specific primers could offer a useful tool for detecting and identifying *P. linteus* rapidly.

KEYWORDS: PCR polymorphic band, *Phellinus linteus*, Specific primers, URP primer

Genus *Phellinus* are taxonomically classified into Aphyllophorales in Hymenochaetcea of Basidiomycota (Larsen and Cobb-Poulle, 1990) and have also been known as a plant pathogen that causes white pocket rot and severe plant diseases such as canker or heart-rot in living trees (Gilbertson, 1980). *P. linteus* was first illustrated and described as *Polyporus linteus* by Berkeley and Curtis (Berkeley and Curtis, 1960). Later, Teng renamed this species as a *Phellinus linteus* (Teng, 1964). *P. linteus* are widely distributed in tropic or subtropic regions of Asia including China, Japan and India. However, there has been considerable confusion in morphological identification of *P. linteus* from other *Phellinus* species due to variable morphological features.

In recent, the interest in *P. linteus* isolates is dramatically increasing since it was reported that polysaccharide components of *P. linteus* play an important role in antitumor, and humoral immunity (Dai and Xu, 1998; Han *et al.*, 1999; Ikekawa *et al.*, 1968; Kim *et al.*, 1999; Lee *et al.*, 1996). Due to their high medicinal value, many fruiting bodies of *Phellinus* isolates regarded as *P. linteus* have recently been introduced into Korea from different countries. *Phellinus* species have been isolated from various geographical regions and it was reported that *P. linteus* occurs mainly on *Morus* and various species of *Quercus* in Korea (Kim *et al.*, 1999). Morphological characters such as basidiospore, 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 characters requires time consuming and labour intensive steps and especially, could be inaccurate due to their morphological variations corresponding to environmental conditions. Moreover, fungal classification based on morphology tends to incline to toward the subjective opinion of an expert in the field.

Molecular techniques, such as DNA hybridization and polymerase chain reaction (PCR) have been extensively employed for confirming genotypes of microorganisms within or between species (Bruns *et al.*, 1991; Caetano-Anolles and Gresshoff, 1997; Gardes and Burns, 1993). Genomic analysis based on molecular techniques can be applied independent of developmental stages of organism and environmental effects. Particularly, 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 (Kang *et al.*, 1998; Wecher *et al.*, 1995). A particular advantage of PCR is that it requires only small amount of biological materials and offers a rapid method in application to extensive sample sizes. Ribosomal DNA analyses were applied to characterise genetic relationships among *Phellinus* spp. (Chung *et al.*, 1999). Specific primers for PCR detection of *P. linteus* have been made from its specific-rDNA sequence regions (Park *et al.*, 2001, 2002).

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URP-PCR methods were developed to fingerprint genomes of diverse organisms including plants, animals and microbes (Kang *et al.*, 2002). The principle of URP-PCR method is similar to randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), except the use of longer primer (20 nucleotides) and relatively high annealing temperature for PCR reaction. The URP-PCR conditions allows to produce reproducible PCR fingerprints compared to RAPD. Recent report showed that URP primers can be used in identification of 13 varieties of three *Pleurotus* species, oyster mushrooms that were bred in Korea (Kang *et al.*, 2001). The species-specific URP-PCR product was successfully used to develop a specific DNA probe that detects *Erwinia carotovora* subsp. *carotovora* that causes soft rot on different plants (Kang *et al.*, 1998).

The objective of this study was to develop a novel molecular detection method of *P. linteus* using URP-PCR. URP primers were used in fingerprinting genomes of *Phellinus* spp. and a URP-PCR derived specific DNA band for *P. linteus* isolates was sequenced. Primers were designed from a nucleotide sequence of the fragment and compared to other *Phellinus* spp. assessed for their specificity to *P. linteus*.

Materials and Methods

Isolates of *Phelinus* spp. The *Phellinus* species used in this study are listed in Table 1. Twenty seven *Phellinus* isolates were obtained from the American Type Culture Collection (ATCC), Centraalbureau voor Schimmelcultures (CBS), Korea Collection for Type Culture (KCTC), IFO (Institute for Fermentation, Osaka, Japan) and Dr. In-Pyo, Hong of National Institute of Agricultural Science & Technology, RDA, Suwon, Korea.

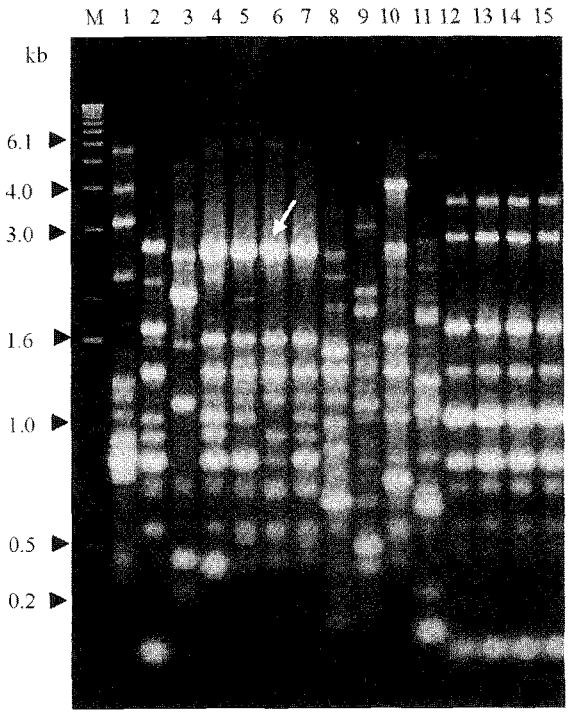

Fig. 1. PCR fingerprinting of *Phellinus* species using primer URP1F. White arrowhead on 2.8 kb-polymorphic band 1 indicates specific band that share *Phellinus linteus* isolates. 1 kb ladder (GIBCO BRL) (lane M), *P. gilvus* (ATCC26729) (lane 1), *P. jhonsonianus* (KCTC 6654) (lane 2), *P. tuberculosis* (KCTC 6656) (lane 3), *P. linteus* (PL-2) (lane 4), *P. linteus* (PL-5) (lane 5), *P. linteus* (P5) (lane 6), *P. linteus* (WD1222) (lane 7), *P. igniarius* pv *igniarius* (KCTC6227) (lane 8), *P. rigricans* (CBS 213.48) (lane 9), *P. linteus* (ATCC26710) (lane 10), *P. werii* (CNU6017) (lane 11), *Phellinus* sp. (Andong/Korea) (lane 12), *Phellinus* sp. (Bupyeong/Korea) (lane 13), *Phellinus* sp. (Heunglim/Korea) (lane 14), *Phellinus* sp. (Nonggong/Korea) (lane 15).

DNA extraction. *Phellinus* isolates were grown in a shaking culture of malt-yeast broth (2% malt extract, 0.2% yeast extract) for 20 days at 30°C. The mycelia were then harvested and freeze-dried. For the DNA extraction, the mycelium was placed in a microcentrifuge tube (1.5 m*l*) and ground with a toothpick and 400 μ*l* of extraction buffer (200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA) and proteinase K (50 μg) was added to the tube. The tube was incubated at 37°C for 1 hour and 2% CTAB (cetyltrimethylammonium bromide) solution of the same volume was added to the tube and then incubated at 65°C for 15 min. The mixture was gently extracted with chloroform : isoamylalcohol (24 : 1) containing 5% phenol and centrifuged at 12,000 rpm at room temperature for 5 min and then the supernatant was transferred to a new tube and CTAB treatment was repeated by same steps mentioned above. Isopropanol with 0.6 volume was added in the solution and centrifuged for pelleting DNA for 5 min at 12,000 rpm. The pellet was washed with 70% ethanol and then dissolved in 100 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA in the sample was removed by adding RNase. DNA was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol, dried, and resuspended in 50 μl of TE buffer. 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).

Primers and PCR amplification. Twenty URP primers (Kang *et al.*, 2002) were applied for searching their usefulness for producing PCR polymorphism of *Phellinus* spp. with PCR. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify rDNA ITS region containing 5.8S. PCR reactions were performed in a 50 μl PCR mix

Table 1. List of *Phellinus* spp. used in the study

Phellinus spp.	Isolates/sources	*Phellinus* spp.	Isolates/sources
Phellinus linteus	ATCC 26710/USA	*P. robustus*	KCTC 6657
	IFO 6989/Japan	*P. spiculosus*	KCTC 6658
	PL-2/Korea	*P. tuberculosis*	KCTC 6656
	PL-5/Korea	*P. ferreus*	CBS 444.48
	P5/Korea	*P.nigricans*	CBS 213.48
	P2/Korea	*P. torulosus*	CBA 100117
	WDI 1222/Japan	*P. fragrans*	CBS 209.90
P. johnsonianus	ATCC 60051	*P. ribs*	CBS 175.29
P. pomaceus	KCTC 6656	*P. weirianus*	CBS 618.89
P. pini	ATCC 12240	*P. cinereus*	CBS 10016
P. gilvus	ATCC 26729	*Phellinus* sp.	Nonggong/Korea
P. igniarius	KCTC 6227	*Phellinus* sp.	Heunglim/Korea
P. weirii	CNU 6017	*Phellinus* sp.	Bupyeong/Korea
P. biscuspidatus	KCTC 6651	*Phellinus* sp.	Andong/Korea

*American Type Culture Collection (ATCC): http://www.atcc.org/
*Centraalbureau voor Schimmelcultures (CBS): http://www.cbs.knaw.nl/
*Korean Collection for Type Culture (KCTC) : http://www.kribb.re.kr/
*WDI : Tzukuba Forest Institute, Japan
*P : Applied Microbiology Div., NIAST, Korea
*P-L : Ja-Kwang Research Institute, Hankook SinYak Pharm. Co., LTD.
* CNU : Chungnam National University, Korea
*Isolates of *Phellinus* sp. were obtained from mushroom farms in Korea, Nonggong, Heunglim, Bupyeong and Andong that cultivate them.

ture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 mM of dNTP, each 200 ng for each of primers, and 2.5 unit *Taq* polymerase (Promega, Madison, WI, USA). The total amount of genomic DNA from various organisms added to the PCR mixture was approximately 50 ng. PCR amplification was carried out in a PTC-200TM Gradient cycler (MJ Research, Inc., Waltham, MS, USA) using the following profile: one cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; one cycle of a final extension for 7 min at 72°C. PCR products were electrophoresed in 1.8% agarose gel at 6 voltage/cm with TAE buffer for 4 hours. DNA fragments in the gel were visualized by staining with ethidium bromide and photographed under UV transilluminator.

Molecular cloning and Sequencing analysis. A URP-PCR derived band (2.8 kb) that is shared in *P. linteus* isolates was eluted from agarose gel with a gel extraction tube (SUPREC™, TAKARA), ligated into TA-cloning vector (Promega) by the suggestions of the manufactures manuals and transformed into *Escherichia coli* strain (DH5α). The clones were sequenced by automatic sequencer (ABI PRISMTM 377 DNA sequencer) using a DNA sequencing Kit with BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems Co.) according to the supplier's instructions. rDNA and sequences were aligned using the DNASTAR software package (DNASTAR Inc.) and homology search of the sequences was performed by the Blast program of National Center for Biotechnology Information database (NCBI). The sequence of URP-PCR derived band (2.8 kb) has been deposited in the GenBank database under the accession number AF433654. Primer set with PLSPF2 (5'-ACTTAT-TCCATCGCAGGTTA-3') and PLSPR1 (5'-CTCGTAC-CTCGTCATCAAGT-3') was designed from both ends of the sequences and was used as specific primers for detecting *P. linteus* isolates.

Results and Discussion

URP-PCR fingerprinting. This report is aimed at providing a novel molecular method for identification of *P. linteus*. Recently, PCR fingerprinting methods such as RAPD have been used for developing specific molecular markers at the level of species or strain (Oliver and Loria, 1998; Wecher *et al.*, 1995; Yoder and Christianson, 1998; Zaremski *et al.*, 1998). In this study, the polymorphic band produced by URP-PCR fingerprinting was used. Twelve URP primers (Kang *et al.*, 2002) were screened to select primer which was able to amplify genomes of *Phellinus* spp. and produced DNA polymorphic bands (data not shown). Of them, URP1F (5'-ATCCAAGGTC-CGAGACAACC-3') amplified PCR bands for varying in size from 100 bp to 4,000 bp, and produced specific band patterns for *P. linteus* isolates. The primer produced polymorphic patterns around 2.8, 1.0 and 0.7 kb which are dependent on *P. linteus* isolates. For instance, *P. linteus* isolate (ATCC 26710) has intense bands around 4.0 and 0.7 kb that are absent in other *P. linteus* isolates. These results suggest that URP-PCR profiles can possibly be used in identifying *Phellinus* isolates at intra and interspe-

cies level.

Four isolates of *Phellinus* sp. used in this study were collected from the mushroom farms in Korea and have not been identified at species level. On the basis of morphological characters such as basidiospore, skeletal and basidiocarp, Dai and Xu reported that *P. baumii* was successfully cultivated in Korea (Dai and Xu, 1998). Four unidentified *Phellinus* sp. listed in Table 1. have artificially been cultivated in Korea. Thus, there was much interest in the URP-PCR result of the four *Phellinus* sp. URP-PCR band patterns of the *Phellinus* sp. was identical, but differed from those of *P. linteus* isolates and other *Phellinus* species. However, polymorphic bands of *P. linteus* isolates partially shared with those of *P. johnsonianus* and the *Phellinus* sp. banding sizes around 0.7~1.6 kb, suggesting that the genetic background may be related among them. The nuclear ribosomal DNA (rDNA) has been used to analyse major evolutionary events, and especially the internal transcribed space (ITS) region has been established as an useful tool for identifying fungi at the species level (White *et al.*, 1990). Thus, rDNA ITS regions containing 5.8 rDNA were sequenced to investigate genetic relatedness among different *Phellinus* spp. including *P. johnsonianus*, *Phellinus* sp. and *P. linteus* isolates. In this multiple alignment, the sequence analysis among *P. johnsonianus*, *Phellinus* sp. and *P. linteus* showed high genetic similarity (Fig. 2). Interestingly, the rDNA sequence of *Phellinus* sp. showed 98.0% similarity to that of *P. baumii* reported in Blast GenBank, suggesting that the isolates of *Phellinus* sp. can potentially be regarded as *P. baumii*. In view of such fact, URP-PCR fingerprinting pattern showed good agreement with rDNA ITS sequencing data in genetic relationship among *P. johnsonianus, Phellinus* sp. and *P. linteus*. Recently, 28S rDNA sequences of *Phellinus* spp. were used to analyse the genetic relationship among them (Park *et al.*, 2002). The sequencing data showed that *P. linteus* and *P. baumii* belong to the same group, but they were not the same species. While, *P. johnsonianus* belong to different group.

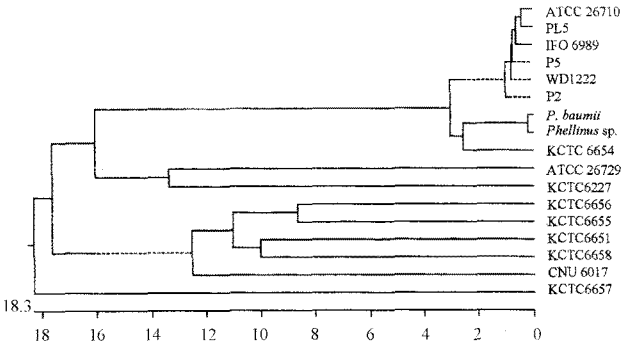

Fig. 2. Genetic relationship of *Phellinus* spp. based on rDNA ITS sequences.

***P. linteus*-specific primers.** It was observed that all *P. linteus* isolates shared a major band of approximately 2.8 kb in length. The 2.8 kb-DNA fragment was eluted from agarose gel and was cloned into TA-vector (Promega), yielding plasmid pPLS1F. The insert DNA of pPLS1F was partially sequenced from both its ends to generate potential specific primers for the detection of *P. linteus* isolates. Nucleotide sequences of 376 and 440 bp-long

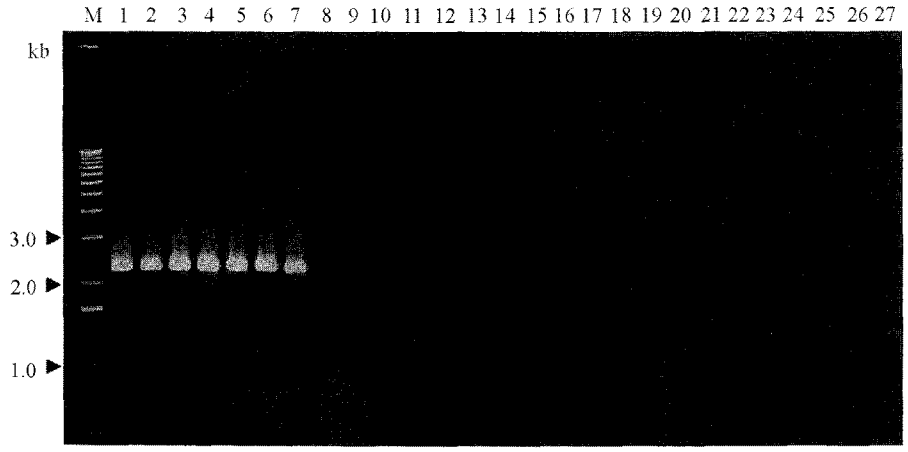

Fig. 3. Specific detection of *Phellinus linteus* isolates by PLSPR/ PLSPF primer pair. 1 kb ladder (GIBCO BRL) (lane M), *P. linteus* isolates (ATCC26710, IFO 6989, PL-2, PL-5, WD1222, P2, P5) (lane 1-7), *Phellinus* sp. (Andong/ Korea) (lane 8), *Phellinus* sp. (Bupyeong/ Korea) (lane 9), *Phellinus* sp. (Nonggong/Korea) (lane 10), *Phellinus* sp. (Heunglim/Korea) (lane, 11) *P. gilvus* (ATCC26729) (lane 12), *P. jhonsonianus* (KCTC 6654) (lane 13), *P. pini* (KCTC 6655) (lane 14), *P. nigricans* (CBS 213.48) (lane 15), *P. chrysoloma* (KCTC 6225) (lane 16), *P. spiculosus* (KCTC 6658) (lane 17), *P. tuberculosis* (KCTC 6656) (lane 18), *P. werii* (CNU6017) (lane 19), *P. biscuspidatus* (KCTC 6651) (lane 20), *P. igniarius* var *igniarius* (KCTC 6227) (lane 21), *P. ferreus* (CBS 444.48) (lane 22), *P. torulosus* (CBS 100117) (lane 23), *P. fragrans* (CBS 202.90) (lane 24), *P. ribs* (CBS 175.29) (lane 25), *P. weirianus* (CBS 618.89) (lane 26), and *P. cinereus* (CBS10016) (lane 27).

were partially determined from both ends of the DNA fragment of pPLS1F. The sequences were not significantly homologous to genes on the Blast GenBank database of NCBI, suggesting they are novel nucleotide sequences.

Primers consisting of twenty oligonucleotides were designed from the sequence regions of the both ends and were screened to test PCR specificity to *P. linteus*. Of tested primer pairs, PLSPF2 (5'-ACTTATTCCATCGCAGGTTA-3') and PLSPR1 (5'-CTCGTACCTCGTCATCAAGT-3') primer pair which was designed in internal sequence regions downstream 600 bp from both end sequences amplified a single band with the expected size of 2.2 kb only on genomic DNA from *P. linteus* isolates (Fig. 3), whereas no band was observed in other *Phellinus* spp. Moreover, the specific primers did not detect *P. johnsonianus* and *Phellinus* sp. that showed high genetic similarity to *P. linteus*. These results indicate that primers PLSPF2 and PLSPR1 are specific for *P. linteu* and could be used to differentiate this fungal species from other closely related *Phellinus* spp.

Previously, PCR primers designed from rDNA-ITS sequences were applied for confirming *P. linteus* isolates (Kim *et al.*, 1999). Specific primers for detection of *P. linteus* were also designed by using *P. linteus*-rDNA ITS sequences (Park *et al.*, 2001). However, the primers also detected *P. johnsonianus* and *Phellinus* sp. (*P. baumii*) under certain PCR condition since DNA sequences of primers based on the rDNA sequence of *P. linteu* are relatively overlapped with that of *P. johnsonianus* and *Phellinus baumii*. In contrast, primer set (PLSPF2/PLSPR1) developed in this study showed high specificity to *P. linteus* isolates, regardless of their geographic origins. Moreover, the primers did not amplify the genomic DNA of *P. johnsonianus* and *Phellinus* sp. that showed high genetic similarity to *P. linteus*. In comparison to complex polymorphisms associated with PCR fingerprinting and rDNA sequencing analysis, the single target PCR detection described here is relatively easy to interpret, because it is based on the presence or absence of a single DNA fragment.

On the other hand, *P. linteus* has been well known as phytopathogen that causes a white pocket rot on living tree in tropical or subtropical regions worldwide (Gilbertson, 1980). Our primers could potentially be applied in monitoring it in infected materials such as plant tissues for epidemiological and ecological study. In conclusion, URP-PCR fingerprinting and *P. linteus*-specific primers described in this study will be utilized as diagnostic tool rapid identification of *P. linteus*.

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