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Identification of *Phellinus linteus* by Morphological Characteristics and Molecular Analysis

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형태적 · 분자생물학적 방법에 의한 *Phellinus linteus*의 동정에 관한 연구

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ABSTRACTS: The context and upper surface of *Phellinus* basidiocarp become blackened, rimose and woody. The basidiocarp is sessile, dimidiate and elongate. The basidiospores are pigmented and ovoid to globose. Hymenial setae are 17~35×6~8 μm. Nineteen isolates of *Phellinus* species, including *Phellinus linteus*, were used for sequencing of the internal transcribed spacer (ITS) region of the nuclear rDNA. Based on these sequence data, specific primers were designed for identification of *Phellinus linteus* isolates in Korea. The specific primers were within the ITS1 and ITS2 regions and were nested within the universal primers flanking the spacer regions. A total of four primers (the universal primers ITS-1F and ITS-4, and the specific primers PL-F and PL-R) were used for detection of *Phellinus linteus* collected in Korea. The length of the four amplification products of *Phellinus linteus* DNA were 800 bp (ITS-1F/ITS-4), two bands of about 720 bp (ITS-1F/PL-R and PL-F/ITS-4), and 610 bp (PL-F/PL-R). Among 23 isolates of *Phellinus* species collected in Korea, Thirteen isolates were identified as *Phellinus linteus* based on the presence of the four bands. The other species produced only the single ITS-1F/ITS-4 product.

KEYWORDS: *Phellinus linteus*, Basidiocarp, Setae, Specific primer

The genus *Phellinus* belongs to the Hymenochaetceae in the basidomycetes, and was erected by Quelet (1886). These fungi are widely distributed in subtropical and tropical regions and include both annual and perennial forms (Donk, 1973; Patouillard, 1900). Donk (1960) selected *Polyporus rubripnus* Quel (= *Polyporus torulosus* Pers.) as the generic nomenclature type. *Phellinus* species are known to cause white pocket rot and severe plant diseases such as root rot, canker or heartrot in living trees, as well as destroying slash and other woody residues. Substrata include stumps and logs of various hardwoods, including *Quercus*. Despite such severe diseases, the taxonomic concept of *Phellinus* has not been established due to disagreements among taxonomists. Even though several taxonomists proposed various criteria to establish the generic concept, such criteria are not accepted by all taxonomists (Fisher, 1987; Mark and Cook, 1993; Parmasto, 1985).

In particular, *P. linteus* attracts great attention due to its medicinal value in Asia. This fungus was originally described as *Polyporus linteus* by Berkeley and Curtis (1860). Later, Teng (1964) renamed this species *Phellinus linteus*. This fungus is distributed throughout southeastern USA, Mexico, India, China, and Japan on various hosts, occurring on dead trees with limited occurrence on living trees (Ahmad, 1972). 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. In Korea, *P. linteus* occurs mainly on *Morus*. Currently, this fungus is highly prized due to its medicinal value; the inhibition rate of the hot water extract of basidiomes against cancerous growths (sarcoma 180) in white mice is 96.7% (Ikekawa, 1968). Several studies were reported that a complex group of polysaccharides isolated from the basidiomes stimulates the immune system (Chung and Kim, 1994; Oh and Han, 1993).

Despite such great medicinal value, the species concept of this fungus is not well studied. Criteria to identify *P. linteus* can include macroscopic features, anatomy of basidiomes, habitat, and cultural characteristics. However, such criteria are not sufficient in distinguishing *P. linteus* from other *Phellinus* species such as *P. igninarius* and *P. pini*. In order to resolve this problem, molecular marker of *P. linteus* and other *Phellinus* species should be investigated.

The nuclear ribosomal DNA repeat unit (rDNA) has

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been used to analyse major evolutionary events, and the internal transcribed spaces (ITS) regions of the rDNA, being less conserved, have been used successfully for investigating phylogenetic relationship of fungi (Bruns *et al.*, 1991).

In this study, phylogenetic relationships of nineteen isolates of *Phellinus* species were investigated by sequence analysis of rDNA ITS regions of and the use of specific primers constructed for *P. linteus*. Twenty three isolates of *Phellinus* species collected in Korea were identified using the specific primer pairs. The goals of our studies were to introduce a more efficient identification system of *P. linteus*.

Materials and Methods

Isolates sources and culture condition

Twenty three isolates of *Phellinus* species collected from Korea and eighteen isolates from other regions were used for identification by specific primers (Table 1). All cultures are grown on malt-agar (2% agar, 2% malt extract, 0.2% yeast extract) at 28°C.

DNA extraction

For the preparation of total genomic DNA from *Phellinus* spicies, isolates were transferred to flasks containing malt-yeast broth (2% malt extract, 0.2% yeast extract). After 10 days culturing, mycelium was harvested by filtration and dehydration with Whatman paper and then stored at -20°C. DNA isolation was performed using the modifications of Yoon *et al.* (1991). DNA pellets were air-dried and resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Concentrations of DNA in samples were estimated by comparing the intensity of DNA bands in 1% agarose gels with a series of DNA dilutions and viewing under UV light after staining with ethidium bromide.

PCR amplification of rDNA by specific primers

Specific primers for detection of Korean *P. linteus* were designed based on unique regions in ITS1 and ITS2 as compared to other *Phellinus* species (Table 1). Most of these ITS sequences were provided by D. M. Rizzo and Thomas Harrington. For diagnosis, PCR products of the ITS regions were amplified with four primers (the universal primers ITS-1F and ITS-4, and the specific primers PL-F and PL-R). The *P. linteus* specific primers PL-F and PL-R were nested within the ITS-1F and ITS-4 primers, within the ITS1 and ITS2 regions, respectively. The primer sequences are given in Table 2. Amplification reaction mixtures (100 μl) contained 10 mM Tris-Cl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (Sigma), 0.1% Triton X-100, 100 mM each of dATP, dCTP, dGTP and dTTP (Promega), 50 pmol of each primer, 50 ng of genomic DNA, and 2.5 units of Taq DNA polymerase (Promega). Control reactions omitted genomic DNA in order

Table 1. The List of Phellinus species in this study

Code no.	Isolates	Scientific name	Origin
1	Ph-1	*Phellinus* sp.	Hongcheon
2	Ph-5	*Phellinus* sp.	Samcheok
3	Ph-8	*Phellinus* sp.	Wonju
4	Ph-11	*Phellinus* sp.	Hongcheon
5	Ph-13	*Phellinus* sp.	Wonju
6	Ph-23	*Phellinus* sp.	Jungsun
7	Ph-28	*Phellinus* sp.	Chuncheon
8	Ph-29	*Phellinus* sp.	Yangyang
9	Ph-30	*Phellinus* sp.	Hongcheon
10	Ph-32	*Phellinus* sp.	Hongcheon
11	Ph-33	*Phellinus* sp.	Pyungchang
12	Ph-34	*Phellinus* sp.	Gosung
13	Ph-35	*Phellinus* sp.	Mt. Taebaek
14	Ph-39	*Phellinus* sp.	Mt. Chiak
15	Ph-66	*Phellinus* sp.	Mt. Odae
16	Ph-93	*Phellinus* sp.	Hongcheon
17	Ph-97	*Phellinus* sp.	Andong
18	Ph-98	*Phellinus* sp.	China
19	Ph-99	*Phellinus* sp.	Junju
20	Ph-103	*Phellinus* sp.	Wonju
21	Ph-109	*Phellinus* sp.	Hongcheon
22	Ph-132	*Phellinus* sp.	Hongcheon
23	Ph-138	*Phellinus* sp.	Andong
24	Ph-41	*Phellinus linteus*	USA
25	Ph-42	*Phellinus linteus*	USA
26	Ph-44	*Phellinus linteus*	USA
27	Ph-46	*Phellinus igniarius*	USA
28	Ph-48	*Phellinus nigricans*	USA
29	Ph-49	*Phellinus pini*	USA
30	Ph-50	*Phellinus verri*	USA
31	Ph-51	*Phellinus* sp.	USA
32	Ph-53	*Phellinus puctatus*	USA
33	Ph-54	*Phellinus robustus*	USA
34	Ph-55	*Phellinus everhartii*	USA
35	Ph-56	*Phellinus chrysoloma*	USA
36	Ph-58	*Phellinus igniarius*	USA
37	Ph-59	*Phellinus robineae*	USA
38	Ph-60	*Phellinus gilvus*	USA
39	Ph-64	*Phellinus laevigatus*	USA
40	Ph-75	*Phellinus tremulae*	USA
41	Ph-87	*Phellinus pini*	USA

Table 2. Primer sequence used for this study

Primer	Sequence(5'-3')
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'
ITS4	5'-TCCTCCGCTTATTGATATGC-3'
PL-F	5'-ACCTGCTGCTGGTGCGAAAATCG-3'
PL-R	5'-CGGACGGCTAGAAGCAAGCTCG-3'

to distinguish amplified DNA fragments from artifactual results. Amplification reactions were preceeded by 4 min of preincubation at 94°C to enhance denaturation of genomic DNA. The amplification protocol consisted of 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C. Amplified DNA fragments were resolved by electrophoresis in 2% agarose gels. A 100 bp DNA molecular weight ladder (Promega) was also run on the 1 gel to serve as a size marker. Photographs were taken of each gel over a UV transilluminator, using a Polaroid camera.

Results and Discussion

In Korea, *Phellinus linteus* (Berk. & Curt.) Teng occurred mainly on *Morus* species and various species of *Quercus*. This fungus is perennial and causes a white pocket rot in living trees. The basidiome is sessile, dimidiate to elongate, hard and woody. The context is thin to thick and the upper surface becomes dark brown or blackened and slightly rimose in age. The basidiospores are yellowish brown and ovoid to globose, 4.5~5.5×5~7 μm. Hymenial setae are 17~35×6~8 μm.

P. linteus has been well known as a plant pathogen, but it is also known for its medicinal uses in the Orient. Recently in Korea, the interest of *P. linteus* has increased due its inhibition of sacroma 180. However, more than 250 species belong to *Phellinus*, and there is no suitable diagnositic test for *P. linteus* in Korea. The basidiospores are very important in distinguishing *P. linteus* from closely related *Phellinus* species, but it is difficult to botain basidiospores.

This present study was performed to provide new diagnostic criteria for *P. linteus*. The specific primers were nested within the ITS1 and ITS2 regions of rDNA and used in conjunction with universal primers flanking the spacer regions. In most eukaryotes, including all true fungi, ribosomal DNA (rDNA) exists as a tandemly repeated array of three largest rRNA genes separated by transcribed and nontranscribed spacers, which are highly variable. Such variable regions in rDNA have been sucessfully used as a tool of fungal taxonomic research (Fisher, 1995; Vilgalys and Hester, 1990).

A total of four primers (the universal primers ITS-1F and ITS-4, and the specific primers PL-F and PL-R) were used for detection of *Phellinus* linteus collected in Korea. The length of the four amplification products of *P. linteus* DNA were 800 bp (ITS-1F/ITS-4), two bands of about 720 bp (ITS-1F/PL-R and PL-F/ITS-4), and 610 bp (PL-F/PL-R). Among 23 isolates of *Phellinus* species collected in Korea, 13 isolates were identified as *P. linteus* based on the presence of the four bands. The other species produced only the single ITS-1F/ITS-4 product (Fig. 1) The isolates diagnosed as *P. linteus* had similar cultural characteristics such as pigmentation and mycelial morphology. However, some of the isolates not identified as *P. linteus* also had similar morphological characteristics.

The detection of *P. linteus* using PCR-amplification is more rapid and less labor-intensive than other methods such as PCR-RFLP and mating tests, and the test proved to be highly specific. However, the specific primers were designed according to the ITS sequences of Korean *P. linteus*, and the primers may not detect *P. linteus* from other regions.

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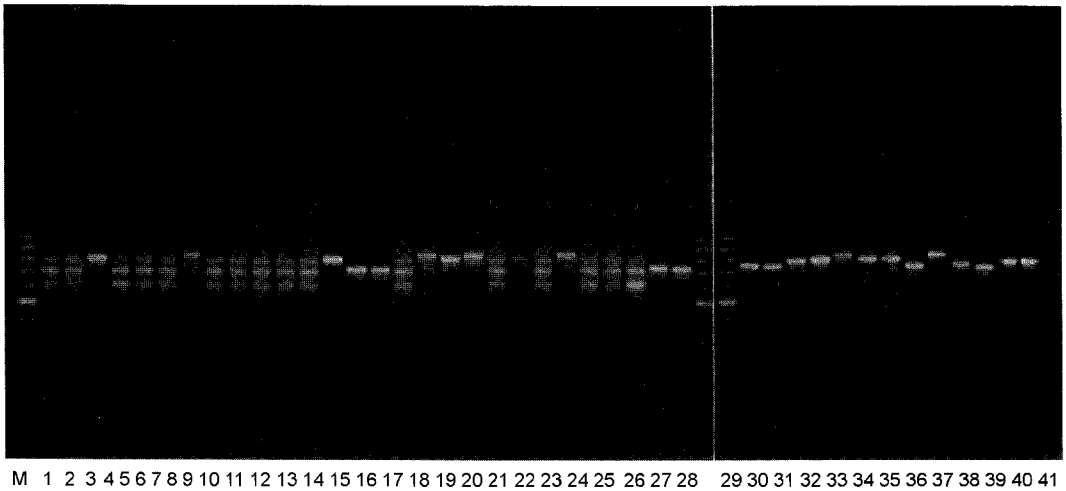

Fig. 1. PCR product of *Phellinus* spp. by specific primrts for detection of *Phellinus linteus* Lane M: 100 bp DNA ladder; Lane 1-41: Sample No. of Table 1.

ences and Engineering Foundation (KOSEF 9704034). David M. Rizzo kindly provide ITS sequences of some of the *Phellinus* species.

적 요

rDNA 내의 ITS region의 염기서열 분석 결과를 토대로 19종의 *Phellinus* 속균중 *Phellinus linteus*를 특이적으로 동정할 수 있는 primer를 제작하였다. 이 특이 primer는 ITS1과 ITS2 내에 위치하며 이들 spacer region에 인접해 있는 universal primer 내에 위치해 있다. 총 4개의 primer (universal primer인 ITS-1F와 ITS-4 그리고 특이 primer인 PL-F와 PL-R)가 한국에서 채집된 *Phellinus* 속균중 *Phellinus linteus*를 동정하는데 사용되었다. *Phellinus linteus*의 증폭된 DNA 크기는 800 bp(ITS-1F/ITS-4)와 720 bp(ITS-1F/PL-R과 PL-F/ITS-4)에 해당하는 2개의 band, 그리고 610 bp(PL-F/PL-R)인것으로 나타났다. 한국에서 채집된 23종의 *Phellinus* 속균중 13종이 *Phellinus linteus*인 것으로 확인되었다.

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