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Accurate Delimitation of *Phanerochaete chrysosporium* and *Phanerochaete sordida* by Specific PCR Primers and Cultural Approach

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Abstract White rot fungi, *Phanerochaete chrysosporium* and *Phanerochaete sordida,* have been mostly studied in a variety of industrial processes like biopulping and pulp bleaching as well as in bioremediation. Whereas *P. sordida* is widely distributed in the North Temperate Zone, *P. chrysosporium* is reported in the restricted area and hundreds of reports have been described from a few strains of *P. chrysosporium*, which are deposited at various fungal collections in the world. The isolates of two species are not easily discriminated because of their morphological and molecular similarity. Through the ITS sequence analyses, a region containing substantial genetic variation between the two species was identified. PCR amplification using two specific primers was successfully used to differentiate *P. chrysosporium* from *P. sordida.* These results were supported by cultural studies. The growth rates at 37°C on PDA, MEA, and Cza and the microscopic features of conidia on PDA and YMA were also very useful to differentiate those two species.

Keywords: *Phanerochaete chrysosporium*, *Phanerochaete sordida*, specific primer, cultural approach

White rot fungi degrade lignin more extensively and rapidly than any other group of organisms [15, 25, 34]. Many of them have been mostly applied in a variety of industrial processes like biopulping and pulp bleaching, as well as in bioremediation because of their enzymatic abilities. *Phanerochaete chrysosporium* Burds. has been studied extensively for remediation of soils contaminated with hazardous organic compounds [9, 26–29] and degradation of a variety of xenobiotic chemicals *via* a free radical mechanism mediated by extracellular peroxidases [1, 4]. *Phanerochaete sordida* (Karst.) Erikss. & Ryv. was also extensively studied for organopollutant degradation [10, 17, 26]. Hatakka [18] proposed three taxonomically heterogeneous groups of fungi based on the patterns of ligninolytic enzymes. *P. chrysosporium* is a typical representative of the LiP-MnP group [18, 37]. Although lignin peroxidase activity was not detected in *P. sordida* cultures, LiP-like genes were amplified using reverse transcription-coupled PCR from *P. sordida* [35]. The pattern of ligninolytic enzymes might not be a useful tool for discriminating the *Phanerochaete* in the species level.

P. chrysosporium belongs to the form complex of *P. sordida* from which it may be distinguished by the longer, thin-walled, and more or less enclosed cystidia [12]. *P. sordida* is widely distributed in the North Temperate Zone and is probably one of the most common corticioid fungi, typically inhabiting fallen branches of hardwood trees [3, 12, 32]. However, *P. chrysosporium* is restricted in the southern region of North America [6] and rarely reported in South Africa [41]. For that reason, hundreds of reports have been described from a few strains of *P. chrysosporium*, which are deposited at various fungal collections in the world. Although the two species have different geographical habitats, the differentiation of the two species on culture is not easy. Major problems also arise when misidentified strains were deposited in culture collections or original strains were contaminated during subsequent transfers. Therefore, accurate identification of the two *Phanerochaete* species has been required at many laboratories and fungal collections. PCR with species-specific primers is used very effectively for differentiation of closely related fungal species, as well as for identification of fungal pathogens both in laboratory cultures and in plant tissues [16, 39, 40]. In the study reported here, cultural studies and PCR

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Table 1. Fungal species used in this study and their locality and sources.

Fungal species	Type of material	Locality	Sources[a]
Phanerochaete chrysosporium	Culture	Sweden	KCTC 6293 (=ATCC 32629, CBS 246.84, CCRC 36201, CECT 2777, DSM 1547)
P. chrysosporium	Culture	Maine, USA	KCTC 6728 (=ATCC 34541, IFO 31249)
P. sordida	Culture	France	KCTC 6713 (=CBS 346.52)
P. sordida	Culture	Chungchung Nam-do, Korea	KCTC 26213
P. sordida	Specimen	Kwanak, Korea	SFC 900616-5
P. sordida	Culture	Belgium	KCTC 6757 (=CBS 797.85)
P. martelliana	Culture	France	CBS 401.50
P. velutina	Specimen	Kyongki-do, Korea	SFC 970816-20
P. calotricha	Specimen	Kunwu, Korea	SFC 970918-2
P. giganteum	Specimen	Kyungki-do, Korea	SFC 971025-2-2
Pulchericium caeruleum	Culture	Japan	IFO 4974
Phlebia rufa	Specimen	Seoul, Korea	SFC 980527-4
Irpex lacteus	Specimen	Kangwon-do, Korea	SFC 951007-39
Bjerkandera adusta	Culture	-	KCTC 6717 (=IFO 4983)

[a]CCRC=Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan; CECT=Spanish Type Culture Collection, Spain; CBS=Centraalbureau voor Schimmelcultures; DSM=Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFO=Institute for Fermentation; KCTC=Korean Collection for Type Cultures; SFC=Seoul National University Fungus Collections.

amplification using specific primers in the ITS region were performed to confirm the delimitation of two species, *P. chrysosporium* and *P. sordida*.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

Fungal strains used in this study are listed in Table 1. Strains other than *P. chrysosporium* and *P. sordida* were chosen in accordance to previous phylogenetic studies [3, 19, 31]. Specimens were obtained from the Seoul National University Fungus Collection (SFC). To obtain growth rate and microscopic characters, cultures were first inoculated onto freshly prepared Malt Extract Agar (MEA) in Petri dishes. When growth was observed in each colony, 5-mm-diameter plugs were taken from the actively growing edge and inoculated onto various media including MEA, Potato Dextrose Agar (PDA), Yeast Extract-Malt Extract Agar (YMA), and Czapek Agar (Cza). They were grown at 10, 24, and 37°C in the dark. Measurements (three replicates) were taken once in every two days after inoculation.

DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted from mycelia grown on MEA plates or completely dried specimens. Agar plate-grown mycelia or dried specimen pieces were placed into an Eppendorf tube and 700 ml of lysis buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 3% SDS] was added. The mixture was vortexed for 30 s and incubated at 70°C for 1 h. DNA was purified through phenol-chloroform extraction. RNA was removed by RNase A treatment at 37°C for 10 min before chloroform extraction. The purified DNA was precipitated with 0.5 vol of isopropanol and then centrifuged at 12,000 rpm for 10 min at room temperature. The supernatant was removed. The pellet was washed with 70% ethanol, dried in air, and then resuspended in 50 ml of TE. Extracted DNA was electrophoresed through a 0.7% agarose gel in TAE buffer, stained with ethidium bromide, and visualized with UV light. DNA was stored at -20°C until required.

The ITS region was amplified using the fungal universal primers ITS5 and ITS4 [38]. PCR amplification was carried out in an *i*-cycler (Bio-Rad, U.S.A.) for 30 cycles of 94°C for 30 s denaturing, 52°C for 30 s annealing, and 72°C for 40 s extension. Initial denaturing at 94°C was extended to 4 min and the final extension was at 72°C for 7 min. Amplification reactions contained 10 pM primers, 250 mM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 2 U of *Taq*-DNA polymerase (Bioneer, Korea), and 1 ml of template DNA in a final volume of 50 ml. The PCR products were electrophoresed and visualized with UV light after staining with ethidium bromide. The amplified PCR products were purified using Wizard PCR prep (Promega, U.S.A.). The nucleotide sequences of 2 strains of *P. chrysosporium* and 4 strains of *P. sordida* were determined using BigDye terminator cycle sequencing kits (Applied Biosystems, U.S.A.) following the manufacturer's instructions. The gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, U.S.A.). The sequences were proofread, edited, and merged into comparable sequences using the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/) [21]. The sequences were then submitted to the GenBank database and assigned accession numbers AF475146-AF475147 (*P. chrysosporium*) and AF475148-AF475151 (*P. sordida*).

Specific PCR Amplification for *P. chrysosporium* and *P. sordida*

Through the sequence analysis of *P. chrysosporium*, *P. sordida*, and related fungi, specific primers for *P. chrysosporium* and *P. sordida* were designed from the ITS region and synthesized by Bioneer, Korea. The primer was named Pchr-F for *P. chrysosporium* with the sequence 5'-GAGCATCCTCTGATGCTTT-3' (forward), and Psor-F for *P. sordida* with the sequence 5'-CAGAAGGGCGAGTGTAA-3' (forward). For specific PCR amplification, two forward specific primers were used with primer ITS4 (reverse). To find the optimal annealing temperature for each specific primer, the temperature gradient option implemented in *i*-cycler (BIO-RAD, U.S.A.) was used. PCR amplification conditions were the same as described above except for the annealing temperature at 50°C to 60°C.

RESULTS

Cultural Studies on Various Culture Media

Because the colony growth morphology was similar for all the strains for each media type, we could not find a difference in mat morphology between the two species (Fig. 1). Mats of all strains on MEA and Cza were very thin, appressed, radiating, forming a granular surface after the margin reached the edge of the plate, and on PDA and YMA, white floccose. Mycelia of all cultures were hyaline, well branched, and lacking clamp-connections except for one strain of *P. sordida* (KCTC6757). Three types of conidia, aleuriospore, arthrospore, and chlamydospore, were observed in cultures of *P. chrysosporium* (KCTC6293 and KCTC6728) as described by Burdsall and Eslyn [6], and their conidial productions were abundant on PDA and YMA, but poor on MEA and Cza (data not shown). However, only one type of sporangium-like structure (chlamydospores) was detected in cultures of *P. sordida* (KCTC 6713 and KCTC 26213).

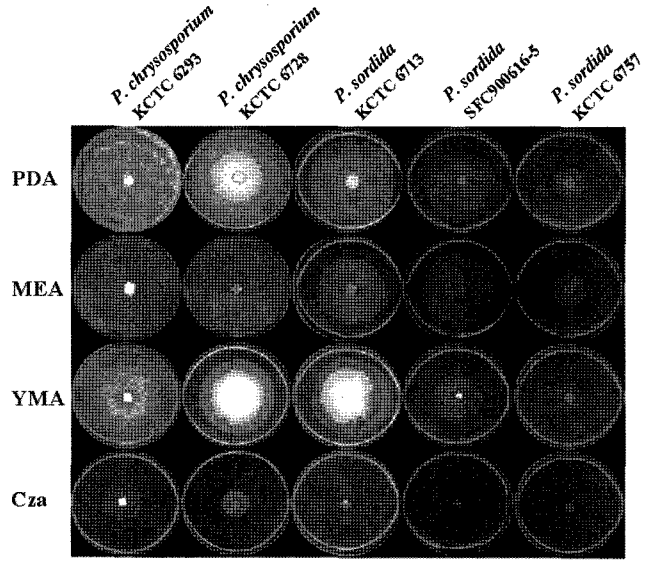

Fig. 1. Growth morphologies on various media. All cultures were grown at 24°C for two days.

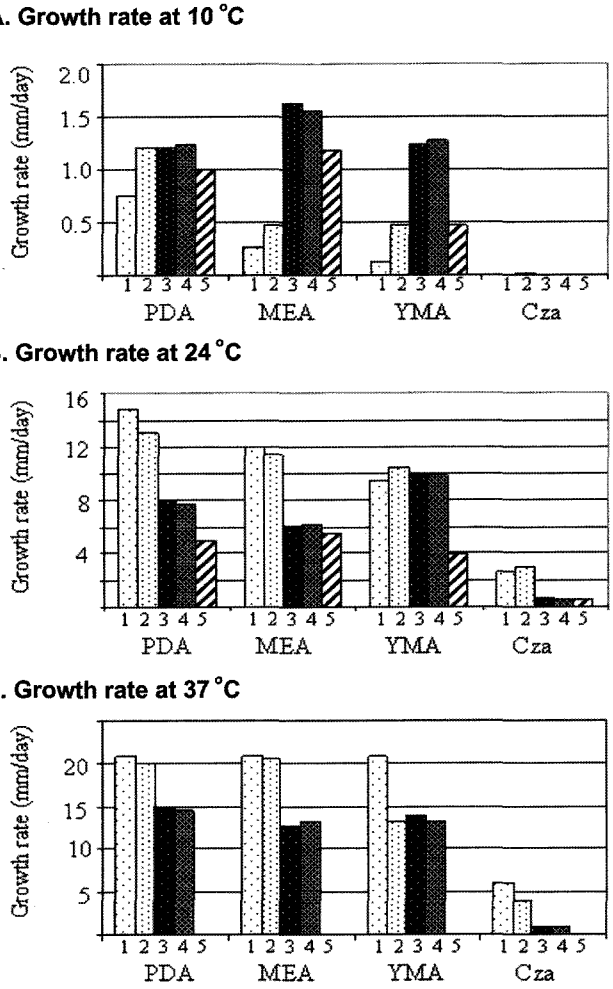

Fig. 2. Growth rate at 10°C (**A**), 24°C (**B**), and 37°C (**C**) on various media.
1, *P. chrysosporium* (KCTC 6293); 2, *P. chrysosporium* (KCTC 6728); 3, *P. sordida* (KCTC 6713); 4, *P. sordida* (SFC 900616-5); 5, *P. sordida* (KCTC 6757).

The strains of *P. chrysosporium* and *P. sordida* exhibited slightly different growth rates on various media at three different temperature conditions. Most strains grew well on PDA, MEA, and YMA except on Cza, which is an important medium in identification of imperfect fungi. Growth rates were tested on the four media types at 10°C, 24°C, and 37°C. The growth rate of *P. chrysosporium* was different than that of *P. sordida* when grown at 10°C on MEA and YMA. The growth rates of *P. chrysosporium* and *P. sordida* were also different from each other when grown at 24°C and 37°C on PDA , MEA, and Cza (Fig. 2).

The growth rate, mat morphology, and microscopic features of *P. sordida* (KCTC 6757) differed from the other *P. sordida* strains. It grew slower than other *P. sordida* strains at 10°C and 24°C, and did not grow at all at 37°C on all media (Fig. 2). Its mat was downy to low cottony producing a radiating growth form. Many hyphae with clamp connections were also observed from this strain.

PCR, Sequence Analysis, and Specific PCR Amplification

A single PCR product was obtained from each of the *P. chrysosporium* and *P. sordida* strains following PCR amplification of the ITS region. The amplicons of *P. chrysosporium* and *P. sordida* were 669 bp and 665 bp, respectively. There was no intraspecific nucleotide variation in *P. chrysosporium* and *P. sordida*. Sequence similarity was 96.10–96.11% between *P. chrysosporium* and *P. sordida*. However the sequence of *P. sordida* (KCTC 6757) differed from the others strains of *P. sordida* and *P. chrysosporium* (below 93% similarity). To identify it, we achieved a BLAST search in GenBank. The closest matching species was *Phanerochaete laevis* (AY219347) with 98% sequence similarity. Its sequence analysis also allowed us to examine the morphology of *P. laevis*. Cultures of *P. laevis* are characterized by tufted mats, multiple clamps on the marginal hyphae, and capilliform hyphae [5]. Therefore, this strain might be also regarded as a misidentified species and may need to be corrected.

Even though the ITS sequences between *P. chrysosporium* and *P. sordida* were very similar, a region containing substantial genetic variations was identified, starting at position 117 of *P. chrysosporium* (KCTC 6293). Because these variable sites were gathered in the ITS 1 region, specific primers were designed only in the forward direction in the ITS 1 region. The specificity of the primers, Pchr-F and Psor-F, was assessed on 13 strains of 10 species (Fig. 3). The extracted DNAs were amplified from all the strains when fungal universal primers (ITS5 and ITS4) were used. However, the PCR fragments were only amplified from *P. chrysosporium* and *P. sordida* when their specific primers were used. The size of their amplicons was 500 bp. The results of specific PCR amplification are shown in Fig. 3. Occasionally, non-targeting upper fragments were observed at the annealing temperature of lower than 55°C. The optimal annealing temperature for Pchr-F and Psor-F fell in the range from 55°C to 59°C, where the amplifications gave strong signals and no other detectable bands.

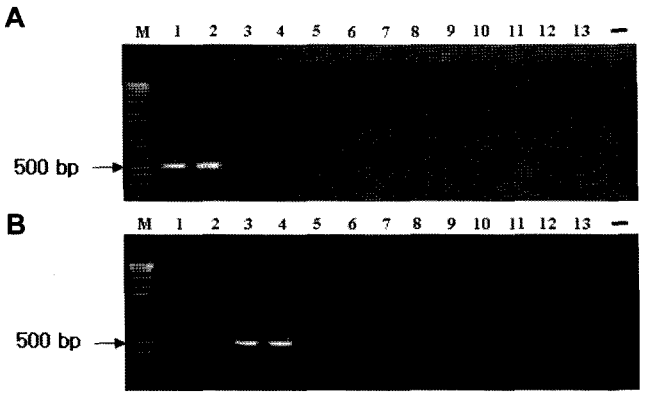

Fig. 3. Species-specific PCR products for the detection of *P. chrysosporium* using Pchr-F primer (**A**), and *P. sordida* using Psor-F (**B**).

Lanes M, DNA molecular weight marker (1 kb plus); 1, *P. chrysosporium* (KCTC 6293); 2, *P. chrysosporium* (KCTC 6728); 3, *P. sordida* (KCTC 6713); 4, *P. sordida* (SFC 900616-5); 5, *P. sordida* (KCTC 6757); 6, *P. martelliana* (CBS 797.85); 7, *P. velutina* (SFC 970816-20); 8, *P. calotricha* (SFC 970918-2); 9, *P. giganteum* (SFC971025-2-2); 10, *Pulcherricium caeruleum* (IFO 49749); 11, *Phlebia rufa* (SFC 980527-4); 12, *Irpex lacteus* (SFC 971007-39); 13, *Bjerkandera adusta* (KCTC 6717); –, negative control without template DNA.

DISCUSSION

Phanerochaete P. Karsten is a genus of saprophytic homobasidiomycetes on woody debris and logs. Because of its ability to degrade lignin selectively and because lignin peroxidases were first isolated from this species, *P. chrysosporium* has become a model for describing lignin biodegradation and bioremediation of heterogeneous xenobiotics [4, 37]. It has also become the model to reveal the genetic repertoire of white rot fungi using whole genome sequences [33]. Because of their superiority in the ability to mineralize the wood preservative PCP [36], several strains of *P. sordida* were also used in soil remediation. Restricted local habitat made some identical strains of *P. chrysosporium*, deposited at worldwide culture collections, center to numerous studies dealing with organopollutant degradation and biomechanical pulping. On the other hand, *P. sordida* was found worldwide as a saprophyte on hardwood and occasionally on softwood [5]. As these two species have a high morphological similarity of fruiting body, many misidentified isolates could be mistakenly deposited at various culture collections. Subsequent contamination in one of the cultures can be problems if there is no confirmation.

Our unambiguous differentiation of *P. chrysosporium* from *P. sordida* demonstrated the effectiveness of specific PCR amplification, although limited strains were tested. Simultaneous detection and identification of important pathogens may be possible by the concomitant use of several species- or genus-specific primer pairs in the same PCR assay [8, 22, 24]. Species, specific primers have been frequently designed from ITS sequences [2, 7, 16]. ITS regions show high levels of interspecific polymorphisms, and multiple copies are present in fungal cells. This permits the amplification of such regions from minute amounts of DNA [14]. The ITS-specific primers designed in this study amplified only *P. chrysosporium* and *P.*

sordida strains when their specific primers were applied. No cross-reactions were observed when using this PCR assay with phylogenetically related *Phanerochaete* species and other fungi.

We compared our sequence data with previously aligned sequences from strains of *P. chrysosporium* and *P. sordida* [23]. Surprisingly, sequences from *P. sordida* (HHB-7423-sp and HHB-8922-sp) were the same as sequences from *P. chrysosporium* (KCTC 6293=ATCC 32629, CBS 246.84, CCRC 36201, CECT 2777, DSM 1547) and (KCTC 6728=ATCC 34541, IFO 31249). The sequence of *P. chrysosporium* HHB-6261 (ATCC 34540) was identical to the sequences of *P. sordida* (KCTC6713, KCTC26213, SFC900616-5). Therefore, the cultures of *P. sordida* (HHB-7423-sp and HHB-8922-sp) and *P. chrysosporium* (HHB-6261=ATCC 34540) should be re-identified. If they were misidentified, numerous studies that have reported on *P. sordida* (HHB-7423-sp and HHB-8922-sp) and *P. chrysosporium* HHB-6261 (ATCC 34540) should be reevaluated.

Although DNA methods are becoming common practice in many laboratories, there are still facilities without such molecular capabilities. Researchers in such labs may be able to use cultural approaches to differentiate between species. Although selective media for isolating *P. chrysosporium* from soil has been developed [11, 20], those soils did not contain fungal populations that were phylogenetically related to species of *P. chrysosporium*. Many species of *Phanerochaete* exhibited growth over the range of 14 to 28°C, and as such would be classified as mesophiles [29]. We grew *P. chrysosporium* and *P. sordida* at 10°C and 37°C as well as at 24°C to differentiate them. Most of the strains tested here grew slowly at 10°C on all four culture media (Fig. 2A). However, they grew fast at 24°C and 37°C. The highest growth rate of *P. chrysosporium* was observed at 37°C. This fungus has been isolated from wood chip piles [13, 30] and its optimal temperature has been known at *ca.* 40°C [6]. Considering its geographic habitat and its optimal growth temperature, *P. chrysosporium* can be considered to be thermophilic. Therefore, growth at between 37°C and 40°C can differentiate *P. chrysosporium* from *P. sordida* on PDA, MEA, and CZA media (Fig. 2).

The conidial shapes are also very useful when differentiating between the two species. Three types of conidia have been reported in *P. chrysosporium* [6]. The first type is terminal persistent conidia (aleuriospores), which are ovate to elliptical, 5–12×3–9 μm. The second type is arthric, retrogressive conidia (arthrospores), but its size and shape are various. The third type of conidia is chlamydospores, which are pyriform or spherical in shape, and have thick walls. These conidia were easily observed in *P. chrysosporium* on PDA and YMA. However, only one type of conidia, sporangium-like structures (chlamydospores), was observed in *P. sordida* (KCTC 6713 and KCTC 26213).

In conclusion, both cultural and molecular approaches can be used to differentiate the two fungal isolates *P. chrysosporium* and *P. sordida*. It also can be applicable to determine the distribution and existence of the two *Phanerochaete* species in natural environments.

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