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First Occurrence of Cobweb Disease on *Hypsizigus marmoreus* Caused by *Cladobotryum varium* in Korea

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Cobweb disease symptoms had been found on *Hypsizigus marmoreus* (Beech mushroom) in Cheongdo-gun, Gyeongbuk province, Korea. The symptoms were white mycelium covered fruit bodies of the mushroom and later, rotten the mushrooms. The fungus isolated from the diseased mushroom caps was identified as *Cladobotryum varium* based on the mycological and molecular characteristics. This is the first occurrence of cobweb disease on *H. marmoreus* caused by *C. varium* in Korea.

Keywords: *Cladobotryum varium*, cobweb disease, *Hypsizigus marmoreus*, mushroom

INTRODUCTION

Hypsizigus marmoreus (Beech mushroom) is a basidiomycete fungus which is a delicious and nutritious food and used for medicinal utilities (Lam and Ng, 2001). It is popular edible mushrooms in Japan and Korea, and successfully cultivated in Korea and Taiwan (Lee *et al.*, 2007). The natural compound of *H. marmoreus* has strong medical activities such as antitumour (Ikekawa *et al.*, 1992; Ikekawa, 1995; Tsuchida *et al.*, 1995), antifungal and antiproliferative activities (Lam and Ng, 2001). Because of these interesting functions, the production of *H. marmoreus* is being increasing worldwide. On mushrooms, the fungus; *Cladobotryum* species caused serious disease in all mushroom-growing countries worldwide (McKay *et al.*, 1998). Several mushroom species had been attacked by *Cladobotryum* spp. such as *C. dendroides*, *C. mycophilum*, *C. varium*, *C. multiseptatum* and *C. verticillatum* (McKay *et al.*, 1999; Adie *et al.*, 2006). Generally, chemical fungicides such as benzimidazole, were used to suppress these fungi in mushroom farms. However, *C. mycophilum* and *C. dendroides* became resistant to benzimidazole fungicides in United Kingdom. As a result of this, *Cladobotryum* species were difficult to manage with these fungicides (Grogan, 2006). Generally, *Cladobotryum* which is causal fungal of cobweb disease produce masses of conidia and they cause brown spotting symptoms on mushroom caps. In 2010, the cobweb-like disease symptoms were observed on *H. marmoreus* in commercial mushroom farms of Cheongdo-gun of Gyeongbuk Province, Korea. The

symptoms found on *H. marmoreus* were white-mycelium covered fruit bodies of mushrooms, and massive spores were found on the fruit bodies. These spores were spread to other spawns by airflow, and the infected fruit bodies eventually became rotten and collapsed. The same symptoms are reported on cobweb disease of *Flammulina velutipes* caused by *C. varium* (Kim *et al.*, 1999). Thus, the cobweb like disease symptoms on *H. marmoreus* was the first occurrence and any diseases on this mushroom have not been reported before in Korea. Therefore, the purpose of this study was to isolate and identify the pathogen caused cobweb disease in *H. marmoreus* using mycological and molecular methods.

MATERIALS AND METHODS

Isolation and identification of fungi

The mycelium were isolated from diseased fruit body of *H. marmoreus* and cultured on potato dextrose agar (PDA) media at 22°C for 3 days. To identify mycological characters, single spore was cultured on PDA at 22°C in the dark for 3–4 days. The shapes and sizes, color, conidia and conidiophores of isolated fungus were observed on 100 conidia by microscope. Then, the fungus was identified following the description of Gams and Hoozemans (1970).

Temperature preferences and pathogenicity test

The mycelia plugs (5 mm in diameter) was punched out from actively growing area of the culture by a cork borer, and placed on the center of PDA media (90 mm in diameter). Then, the plates were incubated in 9 different temperatures; 5, 10, 15, 18, 20, 22, 25, 28 and 32°C. The growth rate of the isolates was determined by measuring the diameter of mycelial colony after 8 days of incubation.

Pathogenicity tests for verifying Koch's postulates were performed on about 15 day-old mushrooms. Inoculum was prepared from 7 to 10 day-old cultures on PDA media and adjusted to 3×10^5 spores ml⁻¹. The spore

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suspension (50 ml) was sprayed on fruit bodies of *H. marmoreus*. The inoculated mushrooms were covered with plastic bags to keep the 100% humidity for 24 hours, and then, incubated at 20°C. The disease development was observed visually after 3 days.

DNA extraction and PCR amplification

Total genomic DNA was extracted from each fungal isolate as described by Liu (Liu *et al.*, 2000). Using a sterile toothpick, a small lump of mycelia was scraped from a 3 day-old fungal culture and transferred to a 1.5 ml tube containing 400 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). After disruption of mycelia by sterilized plastic pestle, the tube was left at room temperature for 10 min. The 3M sodium acetate (150 µl), pH 5.2 was added to the tube and vortexed it briefly. After centrifugation at $10,000 \times g$ for 2 min, the supernatant was transferred to new 1.5 ml tube and added equal volume of isopropyl alcohol. The tube was mixed by briefly inversion and centrifuged ($10,000 \times g$, 2 min) and the supernatant was discarded. The resultant DNA pellet was washed with 300 µl of 70% ethanol and centrifuged ($10,000 \times g$, 3 min). The DNA pellet was air dried and dissolved in 50 µl of sterilized distilled water. The total genomic DNA was frozen at -20°C and used as template DNA for PCR.

The total genomic DNA was used to amplify the internal transcribed spacer (ITS) region and partial 28S rDNA of ribosomal DNAs. The ITS region and 28S rDNA region were amplified with the primer pairs of ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3')/ ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990) and NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3')/ NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (O'Donnell, 1993), respectively. PCR amplification was performed in 20 µl of the reaction mixture containing 20 ng of fungal genomic DNA, 5 unit of Taq polymerase (Solgent, Daejeon, Korea), 2 µl 10X reaction buffer (100 mM Tris-HCl, 400 mM KCl, 15 mM MgCl₂, pH 9.0), 10 mM dNTPs mixture and 5 pmol of each primer using Applied Biosystems 2720 thermal cycler programmed for 94°C for 3 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C

for 1 min; and 72°C for 7 min for the final extension. Amplified DNA fragments were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) and followed by directly sequencing (Solgent, Daejeon, Korea) with the same primers.

Sequence determination and phylogenetic analysis

The obtained sequences were aligned using DNASTAR computer package (DNASTAR Inc.) and phylogenetic trees were constructed using the neighbor-joining method in the CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic trees for ITS region and partial of 28S rDNA were obtained from the data using the program TreeView (Win32, ver. 1.6.1). Bootstrap analysis with 100 replications was performed to determine support for various clades.

RESULTS AND DISCUSSION

A cobweb-like disease of *H. marmoreus* was observed on mushroom cultivation in Chungdo-gun of Gyeongbuk province, Korea. This disease symptom was found on beech mushrooms cultivated in a greenhouse under high humidity conditions. Early symptom was white mycelium covered on the fruit bodies of young mushroom (Fig. 1A). Late symptom progressed as the infected fruit bodies became rot and masses of dry spores covered entirely on rotten mushrooms (Fig. 1B). Then, the mycelium growth spread to the caps and other fruit bodies, and finally, the diseased fruit bodies rotted rapidly. The described symptoms similar those caused by *Cladobotryum* species on other mushrooms.

The fungal isolates formed white mycelium on PDA media at 22°C, and changed the color into cream color after 10 days (Fig. 2A and 2B). The conidiophores were simple branches; conidia were 2-celled, 8.6–15.8 µm long, 6.4–8.6 µm thick, ovoid shape and with big truncate basal hilum (Fig. 2C and 2D). The shape and color of fungal colony, and the shape of conidia and conidiophores were similar to those of description of *Cladobotryum varium* (Gams and Hoozemans, 1970). Therefore, the isolated fungus was identified as *C. varium* by its morphological characters.

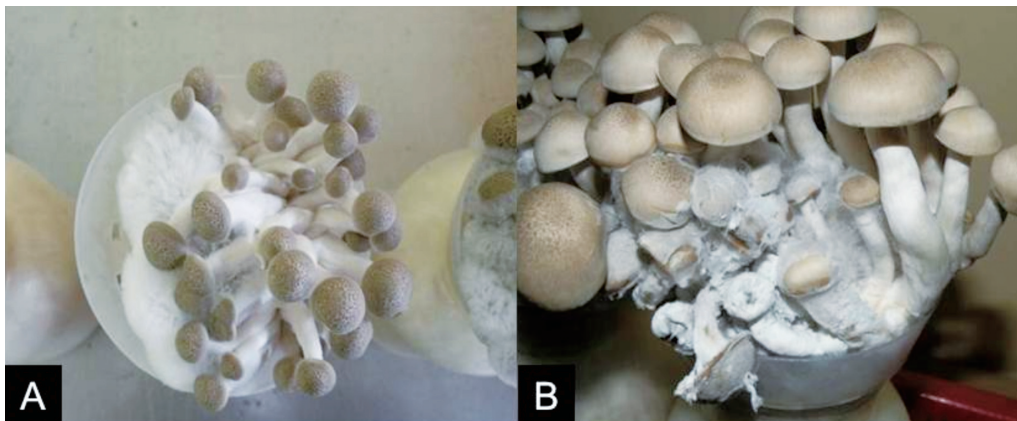


Fig. 1. Cobweb disease symptoms on *H. marmoreus*; Fruit bodies and caps covered with white mycelium (A); Young stage of *H. marmoreus* infected by cobweb mycelium (B).

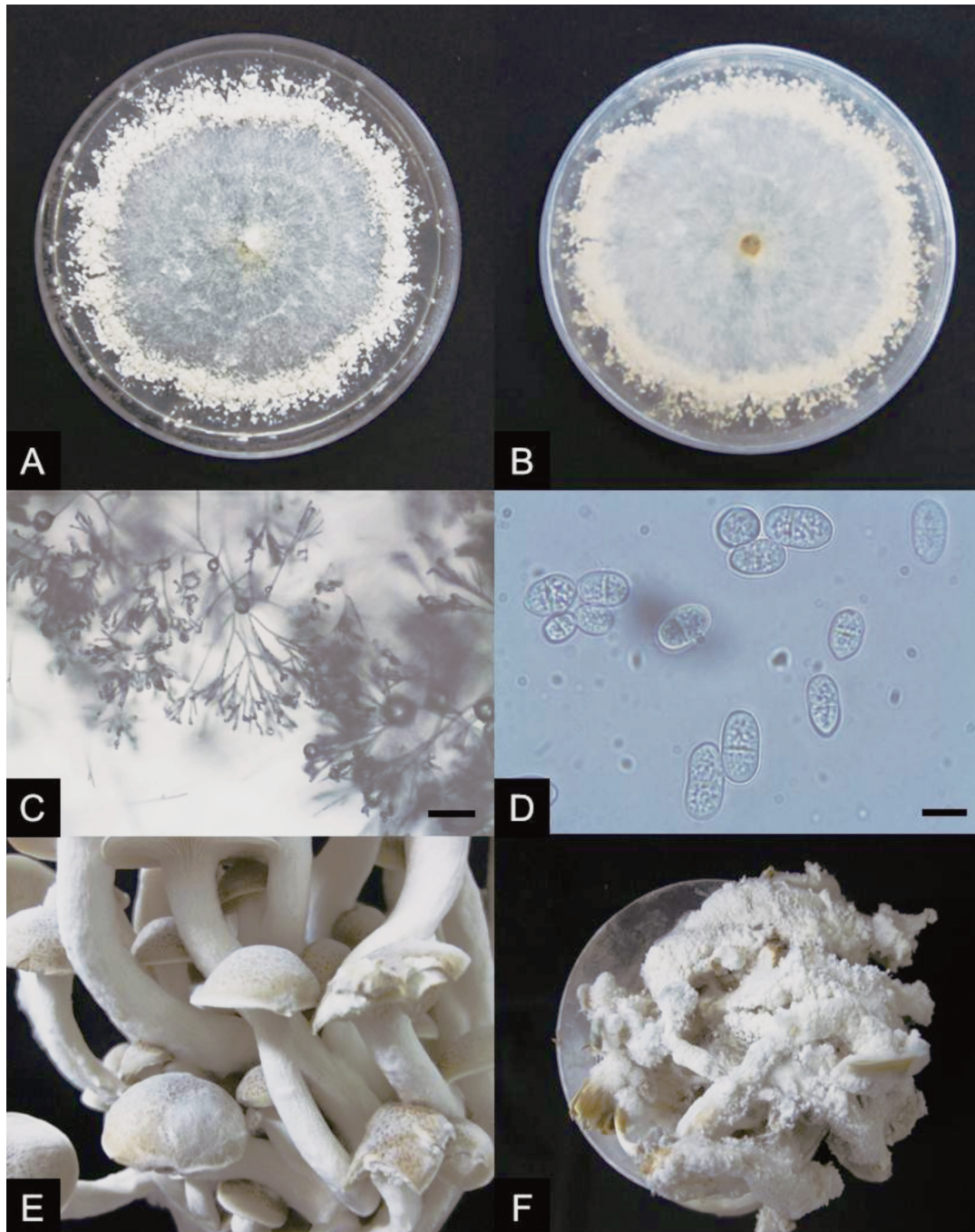


Fig. 2. Morphological characteristics of *Cladobotryum varium*; Front view of grey white-mycelia and dry masses of conidia in the margin of the colony (A), and back view (B) on PDA at 22°C for 14 days. Simple branched conidiophores (C, scale bar=100 µm), and 2-celled, ovoid shaped conidia (D, scale bar=10 µm) of *C. varium*. White mycelium development of *C. varium* on fruit bodies at 8 days (E) (E), and rotten fruit bodies after 19 days (F) after inoculation.

The influence of temperatures on the fungal growth was determined by colony diameter on PDA after 8 days of incubation. The rapid fungal growth was observed on 18~22°C with the colony diameter of 47~54 mm and any growth was found over 32°C with 0 mm. The fungus grew slowly a range of 10~15°C with 14~36 mm (data not shown). In mushroom farms, cobweb disease outbreak was observed on temperatures ranged 15~18°C. It can be assumed that other factors such as relative humidity in mushroom farms might favor the growth of fungus even under 18°C.

The Koch's postulate was completed by inoculation of isolated fungi on healthy mushrooms. The same cobweb-like disease symptoms were developed on the inoculated mushroom within 8 days after inoculation (Fig. 2E). The rotted fruit bodies and massive spores were observed within 11 days later (Fig. 2F). The disease developments on the inoculated mushrooms resembled with those on natural infection. Moreover, the symptoms were the same as reported on cobweb disease of *Flammulina velutipes* caused by *C. varium* (Kim *et al.*, 1999).

Molecular characterization of the isolated fungus was determined based on ITS region and partial of 28S rDNA. Direct sequencing of the PCR products amplified ITS region and partial of the 28S rDNA and resulted in 642 bp and 606 bp respectively. All sequences of the identified isolates for each gene were 100% identical with each other. The ITS sequence was closely related to those of Japanese isolate, *H. aurantius* (anamorph; *C. varium*, 99.5%; AB298700) and the partial of the 28S rDNA sequence was 100% identical with *H. aurantius* (anamorph; *C. varium*, AF160230). The obtained sequences of the fungus were deposited in the DDBJ/GenBank database under the accession number of AB591044 for the ITS region and AB591045 for partial of 28S rDNA. By phylogenetic analyses of ITS region and partial of 28S rDNA, the Korean isolate corresponded to *Cladobotryum varium* (Fig. 3A and 3B). According to results of morphological and molecular evidences, the fungal isolates from *H. marmoreus* were identified as *C. varium*.

The cobweb disease caused by *C. varium* on *H. marmoreus* had been already recorded in Japan but mor-

phological characters of the fungus were not mentioned. In Korea, the cobweb diseases had been reported on *Pleurotus eryngii* and *Flammulina velutipes* (Kim *et al.*, 1998; Kim *et al.*, 1999). In cobweb disease development, temperature preference of *C. varium* growth on *P. eryngii* and *H. marmoreus* varied from 18–22°C. In addition, conidiospore size of *C. varium* on *H. marmoreus* was smaller than those on *Flammulina velutipes*. In Korea, two species of *C. varium* and *C. mycophilum* infection had been found in mushroom farms (Kim *et al.*, 1999; Back *et al.*, 2010). These two species have potential to infect other mushroom species. Since the commercial impatiens are popular edible mushrooms in Japan, Korea and other Asian countries, cobweb disease has the potential to cause significant economic losses in mushroom farms, like the recent outbreaks occurred in Europe, the USA, Australia and Spain (Gaze and Fletcher, 2008; Gea *et al.*, 2011). This agent is regarded as one of the severe pathogens in mushroom farms. To our knowledge, this is the first report of cobweb disease caused by *C. varium* on *H. marmoreus* in Korea.

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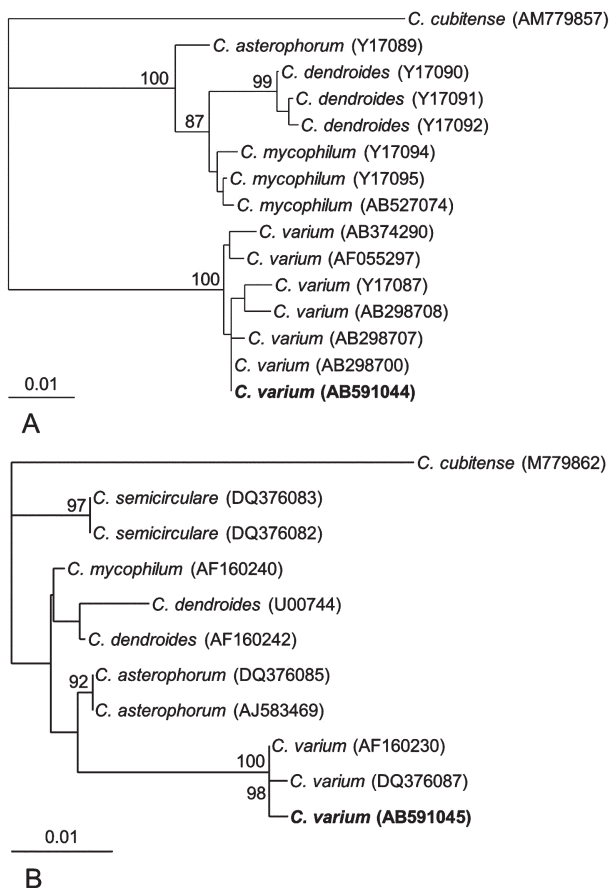


Fig. 3. Phylogenetic distance trees constructed by the neighbor-joining method, comparing the ITS region (A) and partial of 28S rDNA (B) sequences of *Cladobotryum varium* with those of other *Cladobotryum* species from GenBank. *Cladobotryum cubitense* was used as the outgroup. Accession numbers are in parentheses. Numbers on branches are confidence values obtained for 100 replicates (only values above 80% are shown). The bar represents a phylogenetic distance of 1%.

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