Characterization of Species of Cladobotryum which Cause Cobweb Disease in Edible Mushrooms Grown in Korea

Chang-Gi Back¹, Chang-Yun Lee², Geon-Sik Seo³ and Hee-Young Jung⁴*

¹School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Korea
²Greenpeace Mushroom Co., Cheongdo 714-853, Korea
³Department of Industrial Crops and Mushrooms, Korea National College of Agriculture and Fisheries, Hwaseong 445-760, Korea

Four Cladobotryum isolates were collected from four different commercially grown mushroom types infected with cobweb disease in Cheongdo-gun and Chilgok-gun of Gyeongbuk Province, Korea in 2010. The isolates were identified as C. mycophilum from Agaricus bisporus and Pleurotus eryngii, C. varium from Flammulina velutipes and Hypsizygus marmoreus. The cultural characteristics of the four isolates were investigated using potato dextrose agar (PDA) media under nine different temperatures ranging from 5–32°C. Rapid growth of the isolates to colony diameters of 47–82 mm was observed at conditions of 18–22°C. No growth was observed at 32°C. C. mycophilum produced a yellowish red pigment while C. varium produced a cream colored pigment after cultivation for 25 days on PDA. Phylogenetic analysis of the internal transcribed spacer region revealed that the two isolates of C. mycophilum were highly pathogenic toward three mushroom types, but not toward H. marmoreus. The two isolates of C. varium were less pathogenic than those of C. mycophilum, but were pathogenic toward all mushroom types evaluated.

KEYWORDS: Cobweb disease, Cross pathogenicity, ITS region, Phylogenetic analysis, 28S rDNA

Introduction

Cobweb disease is caused by several species of Cladobotryum and is characterized by the growth of coarse mycelium over the affected mushrooms [1]. The disease is found in all mushroom-growing countries worldwide and causes economic loss in areas it impacts [2-4]. There have been numerous reports of cobweb disease affecting Agaricus bisporus, which is known to be infected by species of Cladobotryum including C. dendroides, C. mycophilum, C. varium, C. multiseptatum, and C. verticillatum [2, 5]. C. mycophilum and C. varium are known to be the dominant pathogens for A. bisporus. In Korea, numerous types of mushrooms including A. bisporus, Pleurotus eryngii, Flammulina velutipes and Hypsizygus marmoreus are commercially cultivated for domestic consumption.

In recent years, two species of cobweb fungi, C. mycophilum on A. bisporus, and C. varium on P. eryngii and F. velutipes, have been reported in Korea [6, 7]. Cladobotryum was identified by the morphological and cultural characteristics of its sporocarp and spores, as well as its internal transcribed spacer (ITS) region and partial 28S rDNA genetic characteristics. However, these two species produce nearly identical symptoms during mushroom cultivation. In order to manage cobweb disease effectively, correct identification of pathogens is important as cobweb disease can be spread through spore distribution. However, the possible infection by Cladobotryum species of different types of mushrooms has yet to be investigated. Therefore, in this study we investigated Cladobotryum isolates from four mushroom types based on their morphological and genetic characteristics, and their cross pathogenic ability.

Materials and Methods

Fungal isolation and identification. Cladobotryum isolates were collected from the fruiting bodies of four different cobweb disease infected mushrooms: A. bisporus, P. eryngii, F. velutipes and H. marmoreus, which were obtained from Cheongdo-gun and Chilgok-gun in Gyeongbuk Province, Korea in 2010. All cultures used in the experiments were derived from a single spore and were grown on potato dextrose agar (PDA) at 20°C in the dark for 3–4 days. The shape, size and color of 100 conidia and conidiophores of the isolates were microscopically observed. The isolates were then identified based on the morphological
characteristics of the conidia and conidiophores according to the descriptions from Gams and Hoozemans [8].

**Growth conditions.** The isolates were cultured on PDA media at 20°C for four days under dark conditions. From these cultures, small mycelia plugs (5 mm in diameter) were punched out from the actively growing area using a cork borer and placed at the center of a culture plate (90 mm in diameter) containing PDA media. The influence of temperature on their growth was investigated by incubating the plates at 5, 10, 15, 18, 20, 22, 25, 28, and 32°C, and measuring the resulting colony diameters after four days. Data are presented as the means of three replicates. To investigate the resulting pigmentation on the PDA, the cultures were further kept at 22°C for 5–25 days.

**DNA extraction and PCR amplification.** Total genomic DNA was extracted from each fungal isolate using lysis buffer according to the procedure described by Liu et al. [9]. Total genomic DNA was used to amplify the ITS region and partial 28S ribosomal DNA (rDNA). The ITS rDNA regions and the partial 28S rDNA were amplified using the primer pairs ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3')/ITS4 (5'-TCC TCC GCT TAT TTC GAG GAA GTA A-3') [10] and NL1 (5'-GCA TAT CAA TAA GAG GAA GTA A-3')/ITS4 (5'-TCC TCC GCT TAT TTC GAG GAA GTA A-3').

PCR amplification was conducted in a 20 µL reaction mixture containing 2 µL of fungal DNA (20 ng), 0.2 µL Taq polymerase (5 units/µL), 2 µL 10 reaction buffer (100 mM Tris-HCl, 400 mM KCl, 15 mM MgCl2, pH 9.0), 0.4 µL dNTPs mixture (10 mM), and 2 µL of each primer (5 pmol/µL), 2 µL of each primer (5 pmol/µL) using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions included preheat at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min and final extension at 72°C for 7 min. The amplified DNA fragments were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) and then subjected to direct sequencing (Solgent, Daejeon, Korea) using the same primers.

**Sequence and phylogenetic analysis.** The obtained sequences were aligned using the DNASTAR computer package (DNASTAR Inc., Madison, WI, USA) and phylogenetic trees were constructed using the neighbor-joining method in ClustalW [12]. The phylogenetic trees based on the ITS region and partial sequence of the 28S rDNA were generated using TreeView (Win32 ver. 1.6.1). Bootstrap analysis with 100 replications was performed in order to determine the support the data will provide for various clades.

**Cross pathogenicity test.** The infection ability of the isolates was studied by inoculating each isolate onto four mushroom types. Inoculums were prepared from 10–15-day-old isolation cultures on PDA media and adjusted to 5 × 107 conidia/mL. Inoculation was conducted by spraying the fruiting bodies of each mushroom thoroughly with the spore suspension (50 mL). After being kept in plastic bags in order to maintain 100% humidity for 24 hr, the inoculated mushrooms were incubated at 20°C. The development of disease symptoms was observed visually one day after inoculation and disease severity was rated based on the following score index: +, 1–30% disease severity; +++, 31–50%; ++++, > 51%; and nd, no disease development.

**Results and Discussion**

**Morphological characteristics of the isolates and their identification.** The shape and size of the conidia of the four isolates were observed for 100 conidia (Table 1). The shapes of the four isolates were almost obovoid and consisted of 2–4 cells. The conidia of the four isolates were divided into two groups based on size, one ranging from 11–26 × 7–12 µm and the other from 8–14 × 6–11 µm. These morphological characteristics corresponded to the characteristics of *C. mycophilum* isolated from *A. bisporus* and *C. varium* isolated from *F. velutipes* [4, 7]. The characteristics of the four isolates also agreed with the description of *C. mycophilum* and *C. varium* offered by Gams and Hoozemans [8]. Based on the observed morphological characteristics of the conidia, two isolates from *A. bisporus* and *P. eryngii* were identified as *C. mycophilum* while two isolates from *F. velutipes* and *H. marmoreus* were identified as *C. varium*.

The color of the mycelia from the two *Cladobotryum* species differed over time. The mycelia of all isolates were initially white or grayish as grown on PDA media at 22°C. The mycelia of *C. mycophilum* became yellowish after 5 days of growth and gradually turned reddish over

| Table 1. Morphological characteristics of *Cladobotryum mycophilum* and *C. varium* isolated from four mushrooms on potato dextrose agar media |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Characteristics | *C. mycophilum* | *C. mycophilum* | *C. varium* | *C. varium* |
|                 | *(Agaricus bisporus)* | *(Pleurotus eryngii)* | *(Flammulina velutipes)* | *(Hypsicygus marmoreus)* |
| Conidia shape   | 2–4 cell, obovoid | 2–4 cell, obovoid | 2–3 cell, obovoid | 2–3 cell, obovoid |
| Conidia size (µm) | 11.1–26.6 × 7.7–12.2 | 11.7–23.7 × 10.5–12.7 | 10.1–17.3 × 6.6–10.1 | 8.0–14.8 × 6.1–11.5 |
| Mycelial color  | Yellow, reddish | Yellow, reddish | White, cream | White, cream |