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Isolation and identification of insect pathogenic fungus from silkworms with suspected white muscardine disease

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Abstract

The value of silkworms as functional health food materials has increased, as has the interest in its disease control for stable production, and in the economic value of entomopathogenic microorganisms. In this study, we isolated and identified disease-causing fungi from white muscardine silkworms, and confirmed whether this strain could produce white muscardine silkworms. For the analysis of the cause of white muscardine disease in the infected silkworms, the fungi and prokaryotes causing the disease were identified, isolated, and identified using metagenome analysis. Metagenomic analysis detected a large amount of the fungus *Metarhizium rileyi* in silkworms, and a large amount of the bacterium *Enterococcus mundtii*, which was presumed to be the causative agent of the disease. For accurate identification of the fungi, these were purified by culture medium, and sequencing and phylogenetic tree analyses were performed using an internal transcribed spacer. As a result, *M. rileyi*, *Cladosporium cladosporioides*, and *C. tenuissimum* were identified. In general, *M. rileyi* is known to form green conidia, but in this study, white-yellow conidia were formed, indicating that the exact causative agent of the fungal disease cannot be estimated by diagnosing the symptoms. Thus, a diagnostic method is necessary for the continuously collection of required pathogens, and identifying their morphological and genetic characteristics.

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Introduction

Entomopathogenic fungi are commonly found in the agricultural and forestry ecosystem and cause diseases in insect farming farms, resulting in economic losses (Binneck *et al*., 2019). However, some bacteria are used as a source of income for farms through their use as functional materials, and are also used to control the density of pests in agriculture and ecosystems. Such entomopathogenic fungi have various functions and roles in the insect industry, and more than 700 species in 100 genera are known, but correlation between fungi and insects in ecosystems remains unclear (Roberts., 1989).

In East Asian countries such as Korea, China, and Japan, some entomopathogenic fungi have been used as important oriental medicinal drugs for a long time, and extensive research has been conducted to develop new functional materials or bioactive substances. Representative entomopathogenic fungi used for medicinal purposes include *Cordyceps sinensis* and *Bombycis corpus*, and the former is known to have highly effective

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anticancer, immunity-strengthening, and anti-aging effects (Kwon *et al*., 2001). Notably, white muscardine silkworms are highly effective in treating headaches, pulmonary tuberculosis, and diabetes (Kim *et al*., 1997).

C. sinensis is a fungus known to occur in most insects, including silkworms, butterflies, bees, scarabs, cicadas, dragonflies, and stink bugs (Jianzhe *et al*., 1989). It is a mushroom whose spores attach to the epidermis of an insect, invade the insect using a germ tube, kill the insect, and form a fruiting body. Of the seven species of *C. militaris, C. maritialis, C. bassiana*, and *C. beauveri* are currently used for medicinal purposes (Jianzhe *et al*., 1989).

Bombycis corpus is caused by an infection with the bacterium *Beauveria bassiana*, which causes muscardine disease in insects. Similar to *Cordyceps*, it infects a wide range of host insects, including Lepidoptera, Orthoptera, and Coleoptera, and produces cuticle-decomposing enzymes during epidermal infections. It penetrates the epidermis, blocks the insect's immune system, kills the host, and spreads until the host is completely covered with conidia (Bidochka and Khach Atourians, 1987).

Although much research has investigated the infection mechanism and life cycle of *C. sinensis*, not many bacteria have been discovered. Therefore, additional research on new strains and their functionality is necessary (Jianzhe *et al*., 1989). In addition to research on entomopathogenic fungi as physiologically active substances, research is being conducted to use them as environmentally friendly pesticides to control crop pests. Therefore, the discovery and characterization of new resources would have high economic value (Lü *et al*., 2019). Accordingly, interest in discovering and resourcing microorganisms with various functions from soil and pests is increasing worldwide, and there is a need to collect microbial resources and actively study their characteristics.

Therefore, in this study, we collected samples of silkworms with white muscardine disease, isolated and identified the bacteria causing white muscardine disease through metagenomic analysis of the samples, analyzed the relationship with the disease, further and collected resources for functional research.

Materials and Methods

Silkworm sample preparation

In this study, we used the silkworm BaekokJam (Jam 123X124), a dormant breed. The silkworms were reared under the standard conditions (temperature, 24–27 °C, humidity, 70-90%) provided by the Ministry of Agriculture and Biology of the Rural Development Administration (Park *et al*., 2022). Among the reared silkworms, silkworms with white muscardine disease were selected at the fifth instar stage. Each individual was placed in a pad dish and cultured at 27 °C for 20 days; white silkworms were stored in an ultra-low temperature freezer at −80 °C.

DNA extraction and metagenome sequencing

Five normal and white muscardine silkworms were ground and mixed into one sample. Genomic DNA was extracted using the MagMax Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. For metagenome sequencing, the V3-V4 region of the 16S ribosomal RNA gene was amplified for analysis of prokaryotic community, and the 3F-4R region of the internal transcribed spacer (ITS), amplified using the Nextera XT index kit (Illumina, San Diego, CA, USA), was used to analyze eukaryotic bacteria. Sequence analysis was performed by Macrogen Co. Ltd. (Daejeon, Korea).

Isolation and identification of pathogens

Five milliliters of phosphate-buffered saline were added to 50 mg of the pulverized silkworm sample and homogenized. The homogenized suspension was then serially diluted up to 1010-fold, and 100 μl of each diluted sample was plated on Sabouraud dextrose agar plus yeast extract. For observing morphologically distinct mycelia, plates were incubated at 27 °C for 10 days. To compare the growth of the fungi, we purchased the *B. bassiana* KACC43988 strain from the Korean Agricultural Culture Collection (KACC, Wan-Ju, Korea). For isolating pure bacteria, the cells were subcultured three or more times; next, genomic DNA was extracted from the cultured strains by using a MagMax DNA purification kit (Applied Biosystems), and sequence analysis was performed after amplification through PCR by using ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') primers (White et al., 1990). PCR was performed with a denaturing temperature of 94°C for 1min, annealing at 50°C for 30 sec and extension at 72°C for 30 sec for 30 cycles using Pfx DNA Polymerase (Invitrogen, Hercules, CA, USA) for gene amplification. Thermal cycler (Takara, Japan). The base sequences generated from the ITS4 and ITS5 primers were integrated, and sequence alignment was performed using the GenBank database of the National Center for Biotechnology Information (http://www.

ncbi.nlm.nih.gov/). Subsequently, distances and clusters were analyzed using ClustalW and MEGA 11 program packages (Taumra *et al*., 2021), and a phylogenetic tree was created using the neighbor-joining (NJ) method (Saitou *et al*., 1987). The evolutionary history of the NJ method is based on the Tamura-Nei Model (Tamura and Nei, 1993).

Results and Discussion

Morphological differences among silkworm samples

The collected samples are shown in Fig. 1. Silkworms infected with infectious disease, a typical form of muscardine disease, shrink and harden (Lu *et al*., 2019), and white conidia grow. To classify green muscardine disease and white muscardine diseases, we cultured the conidia at 27 °C for an additional 10 d until no further growth of conidia was observed. However, no color change was observed even after 10 days. According to Lee *et al*. (2012), in the case of green muscardine disease, the initially white conidia changed to green over time. However, in this study, all collected samples remained white; therefore, we judged them to be white muscardine silkworms.

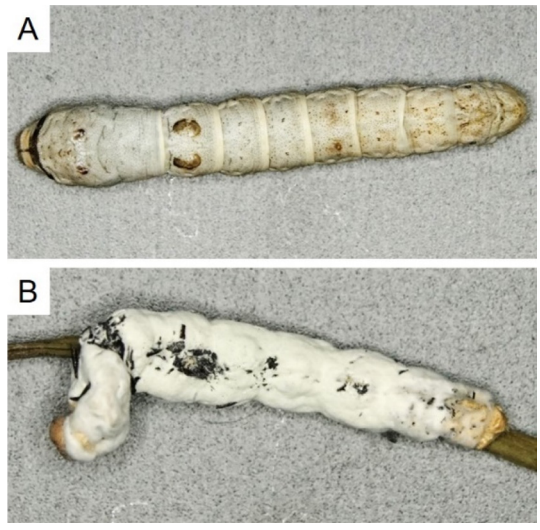

Fig. 1. Normal 5th instar silkworm (A) and fungal-infected silkworm (B). While normal silkworms have a smooth and elastic epidermis, fungus-infected silkworms have a hard body and white mold grows on the epidermis.

Analysis of microbial diversity via high-throughput sequencing

The bacterial compositions of samples obtained from normal silkworms and silkworms affected by muscardine disease were investigated using high-throughput sequencing of 16S rRNA

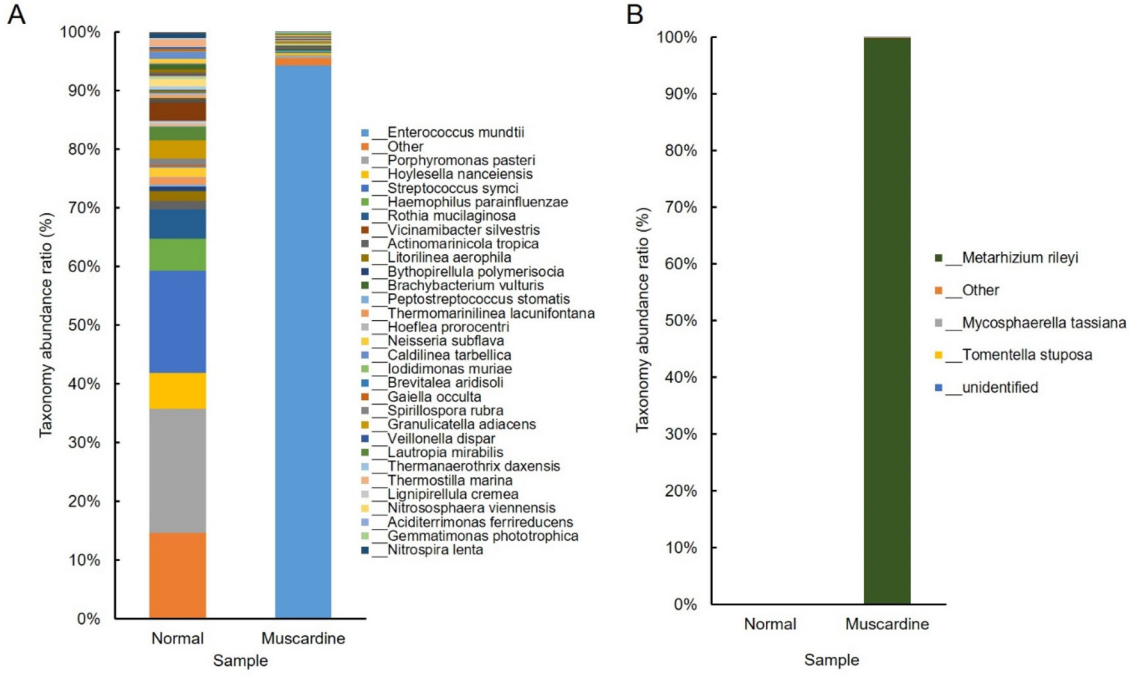

Fig. 2. Analysis of microbial diversity through metagenomic analysis. Prokaryotic species detected through 16S rRNA amplicon sequencing (A); eukaryotic species detected through ITS amplicon sequencing (B).

and ITS amplicons (Fig. 2). Using 16S rRNA amplicon analysis, 681 types of bacteria were detected in normal silkworms, and 131 types of bacteria were detected in silkworms infected with muscardine disease. As shown in Fig. 2A, bacterial diversity was high in normal silkworms; *Porphyromonas pasteri* was the most abundant bacterium (approximately 17%), and in silkworms with muscardine disease, bacterial diversity decreased by more than fivefold. *Enterococcus mundtii*, which causes diseases in insects, accounts for more than 76% of the microbial community (Cappellozza *et al*., 2011). By contrast, in the ITS amplicon analysis shown in Fig. 2B, no fungal ITS were detected in normal silkworms; however, approximately five types of fungi were detected in silkworms with muscardine disease, and the most prevalent fungus was *Metarhizium rileyi* (accounting for 99%). According to Binneck *et al*. (2019), *M. rileyi* is the causative agent of green muscardine disease and is known to form green conidia; however, in this study, it formed white conidia. It has been reported that the diversity of microbial communities in disease-infected insects were destroyed and that disease-causing bacteria account for a high proportion (Sun *et al*., 2022). Because *E. mundtii* is found in the intestines of normal silkworms, we propose that the abnormal growth in the samples collected in this study occurred due to disruption of the balance of the microbial community during infection with *M. rileyi*.

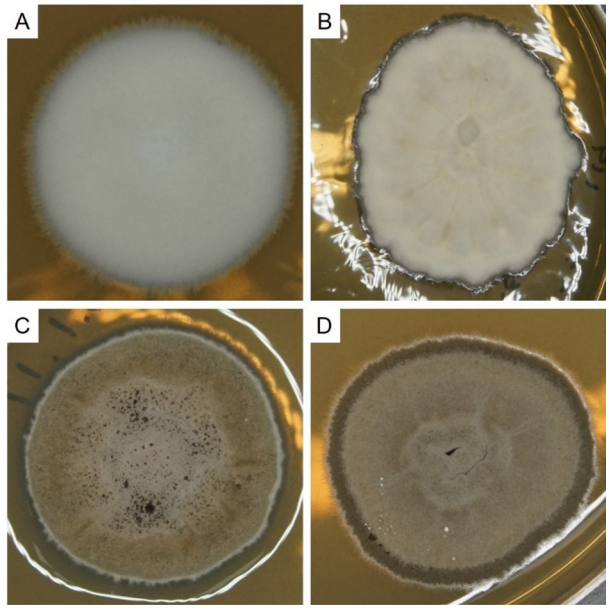

Fig. 3. Comparison of the morphology of various fungi. The fungus was isolated from silkworms infected with white mold by using Sabouraud dextrose agar plus yeast extract medium and sub-cultured four times. Cell growth of *B. bassiana* purchased from KACC (A), yellow-white mold; fungi 1 (B), gray mold; fungi 2 (C) and green mold; fungi 3 (D) isolated from the same silkworm sample. Compared with *B. bassiana*, the growth rate of white fungus isolated from silkworms presumed to be infected with white muscardine disease is slower, and the outer shell of the mycelium is uneven. In addition, mycelia with different colors and two fungi showing growth patterns were isolated.

Isolation and identification of fungi from muscardine silkworms

To determine whether the white mold present in the silkworms with muscardine disease was *M. rileyi*, we isolated and cultured the fungi present in the silkworm samples (Fig. 3). Three types of fungi with different characteristics were isolated from silkworms infected with muscardine (fungi 1, 2, and 3). To compare morphological differences between the isolated fungi and the causative agent of white muscardine, a standard strain of *B. bassiana* (KACC 43988) was cultured and compared. In the case of *B. bassiana*, (Fig. 3A), white conidia grew rapidly on the Sabouraud dextrose agar plus yeast extract medium and had a cloud-like fluffy texture; the color of isolated fungi 1 was yellow-white in color. (Fig. 3B); these results are similar to those of Lu *et al*. (2019). The hyphae were tightly packed and grew slowly. In addition, unlike *B. bassiana*, fungi 2 and 3 formed gray (Fig. 3C) or blue-green (Fig. 3D) conidia. These results support those in the literature of analyses of microbial diversity through high-throughput sequencing and indicate that the samples collected in this study were not the white muscardine disease caused by *B. bassiana*.

Molecular phylogeny of isolated fungal strains

For phylogenetic analysis of the isolated strains, the sequences of fungi 1, 2, and 3 were analyzed in both directions using ITS 4 and 5 primers (Fig. 4A), and approximately 600 overlapping nucleotides were compared with the genes and sequences of other fungi. After alignment, a phylogenetic tree was constructed using the NJ method. Referring to Fig. 4B, we confirmed that fungi 2 and 3 were closely related, but fungi 1 showed no similarity. Analysis of the genetic position of fungi (Fig. 4C) demonstrated that fungi 1 was closely related to *M. rileyi* (MH860365.1); and the sequence identity was 100%. By contrast, fungi 2 was closely related to *Cladosporium cladosporioides* (MK120287.1); and the sequence identity was greater than 99%. Fungi 3 was closely related to *Cladosporium tenuissimum* (OQ076461.1) (Fig. 4D);

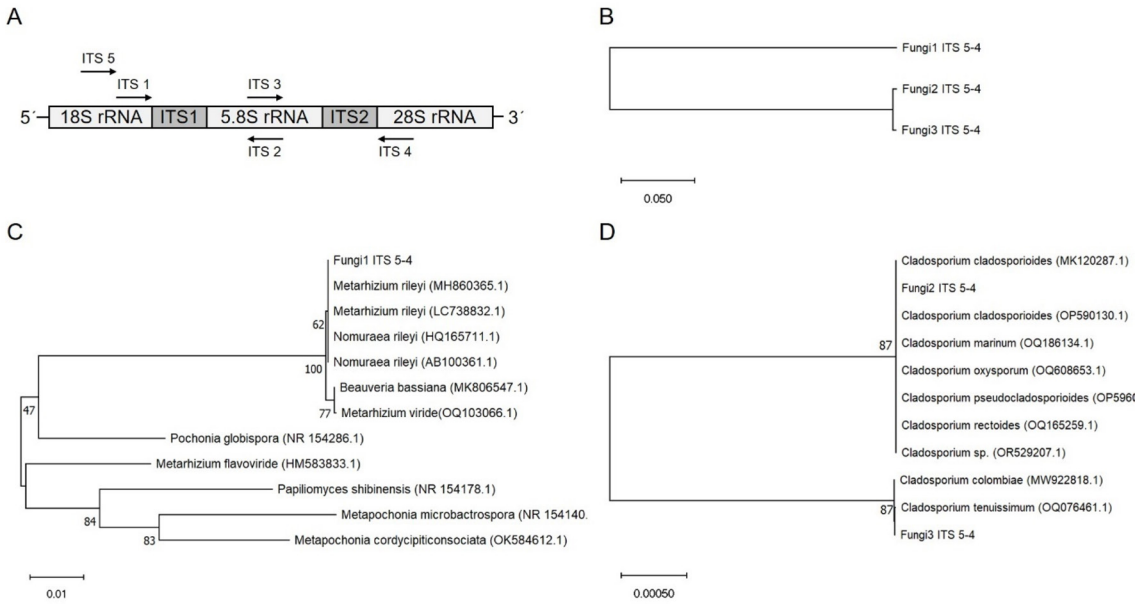

Fig. 4. Identification of three fungi isolated from white muscardine disease silkworms. Schematic diagram of rRNA gene and primers (A). Phylogenetic trees based on ITS 1 to 2. Relationship among three isolated types of fungi (B) and taxonomic relationships between yellow-white fungi 1 (C), gray mold 2, and green mold 3 (D). After nucleic acid isolation from each fungus, PCR and sequencing were performed to determine the ITS sequence. We identified the species of fungi by referring to the National Center for Biotechnology Information blast using the analyzed nucleotide sequence. These trees were constructed by using the neighbor-joining method.

and fungi 1 appeared to be closely related to *Nomuraea rileyi* (HQ165711.1) and *B. bassiana* (MK806547.1) (Fig. 4C). This result because they are called by the same scientific names *as M. rileyi* and *N. rileyi* (Binneck *et al*., 2019). Moreover, at one time, the genus *Beauveria* was known to contain only the common species *B. bassiana*, the less common *B. brongniartii*, and the rare *B. album* (deHoog., 1972) because it was maintained as a *bassiana* species, although it could be classified as a different species during the subsequent addition of species such as *caledonica, amorpha, vermiconia,* and *velata* (Samson and Evans 1982; Rehner *et al*. 2006). Thus, fungi 1 represents *M. rileyi*, fungi 2 represents *C. cladosporioides*, and fungi 3 represents *C. tenuissimum*. The isolated *Cladosporium sp.* sometimes classified as *Davidiella* or *Mycosphaerella which was* plants pathogenic fungus with unknown entomopathogenicity and was likely present in the intestinal tract through the mulberry leaves (Bensch *et al*., 2012).

On the basis of these results, the cause of the disease in the collected white muscardine silkworms was considered to be *M. rileyi* (Fig. 2A), and unlike what is commonly known, it did not form green hyphae but yellow-white hyphae (Fig. 3B). Further, the isolate was confirmed as *M. rileyi* (Fig. 4C). Although the collected bacteria were not *B. bassiana* as initially expected, *M. rileyi* a well-known insect pathogen, was newly isolated from silkworms infected with white muscardine. In addition, although the pathogenicity of silkworms is not known, it is significant that *C. tenuissimum* and *C. cladosporioides* were isolated from silkworms, thereby securing material for future research on insect diseases. In the future, it is believed that research on the insect pathogenicity of the two additionally isolated *Cladosporium* sp. bacteria will be necessary.

Acknowledgments

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