


RESEARCH NOTE

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First Report of Gray Mold Disease on Endangered Species *Cypripedium japonicum*

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ABSTRACT

Cypripedium japonicum is known to be the indigenous plant to Korea, Japan, and China. However, *C. japonicum* represents the most critically endangered plant species in South Korea. The plant is esthetically pleasing due to its flower, which is larger than any other orchidaceous species. Disease symptoms relating to gray mold were observed on *C. japonicum* in May 2019. The suspected pathogen was successfully isolated from the symptomatic leaf tissue and conducted a pure culture of the fungi. The conidia formed consisted of a colorless or light brown single cell, which was either egg or oval-shaped with a size of 7.1 to 13.4 × 5.2 to 8.6 μm. Molecular phylogenetic relationship analysis was also confirmed that the pathogen concerned belonging to the family of *Botrytis cinerea*. Therefore, the findings confirmed that the pathogen isolated from *C. japonicum* was consistent with the unique properties of *B. cinerea*.

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The constant deterioration of the earth's ecological system and indiscriminate destruction of nature caused by civilization have given rise to a reduction in biological diversity and a rise in the rate of extinction to a critical level. Many experts suggest that ~20% of 250,000 species of the vascular plants thriving on the earth will become extinct within the next 50 years [1–3]. Plants belonging to the orchid family are especially sensitive to such external threats, resulting in the number of their genera and species that may continuously drop. A total of 50 taxonomic groups including *Cypripedium japonicum*, *Sedirea japonica*, *Dendrobium moniliforme*, and *Cymbidium orchids* have been designated as rare plant species in South Korea and the figure corresponds to an 8 percent of 571 rare plant species worldwide.

Cypripedium japonicum is a critically endangered species designated by Korea Forest Service. The plant is esthetically pleasing due to its flower, which is larger than other orchidaceous species. For this reason, coupled with a rising interest in growing wild vegetation in households, the general public is quick to take possession of the plant as soon as its natural habitat becomes known, further worsening its endangered status [4]. Despite numerous studies being conducted on *C. japonicum*, which include

the study of growing conditions of its natural habitat [5], the study of its seed germination [6], orchid mycorrhizal fungi's properties [7] and its genetic diversity [8], studies in relation to its pathology is still non-existent, yet. Therefore, this study seeks to provide a fundamental set of data for long-term preservation and restoration of *C. japonicum* by investigating its habitats located in South Korea to elucidate the diseases prevalent in the plant.

In 2019, the outbreak of gray mold disease occurrences was primarily observed at the natural habitats of *C. japonicum* (sites not disclosed) (Figure 1(A,B)). The disease was manifested by softening of the leaves by water soaking symptoms during the initial stage, which was followed by browning caused by the decomposition of the leaf tissue in the later stage of the disease progress. The leaf tissues affected by the pathogen were characterized by the formation of gray-colored conidia and in cases where the severity of the disease was critical, the entire plant eventually withered away to decompose.

To isolate the pathogens concerned, the boundary between the section of leaving that infected, and the section of health leaf was cut into 5 mm × 5 mm dimensions. The specimen was disinfected using 70% ethanol for 1 min and 1% NaOCl solution for 30 s, respectively. Then the samples were washed

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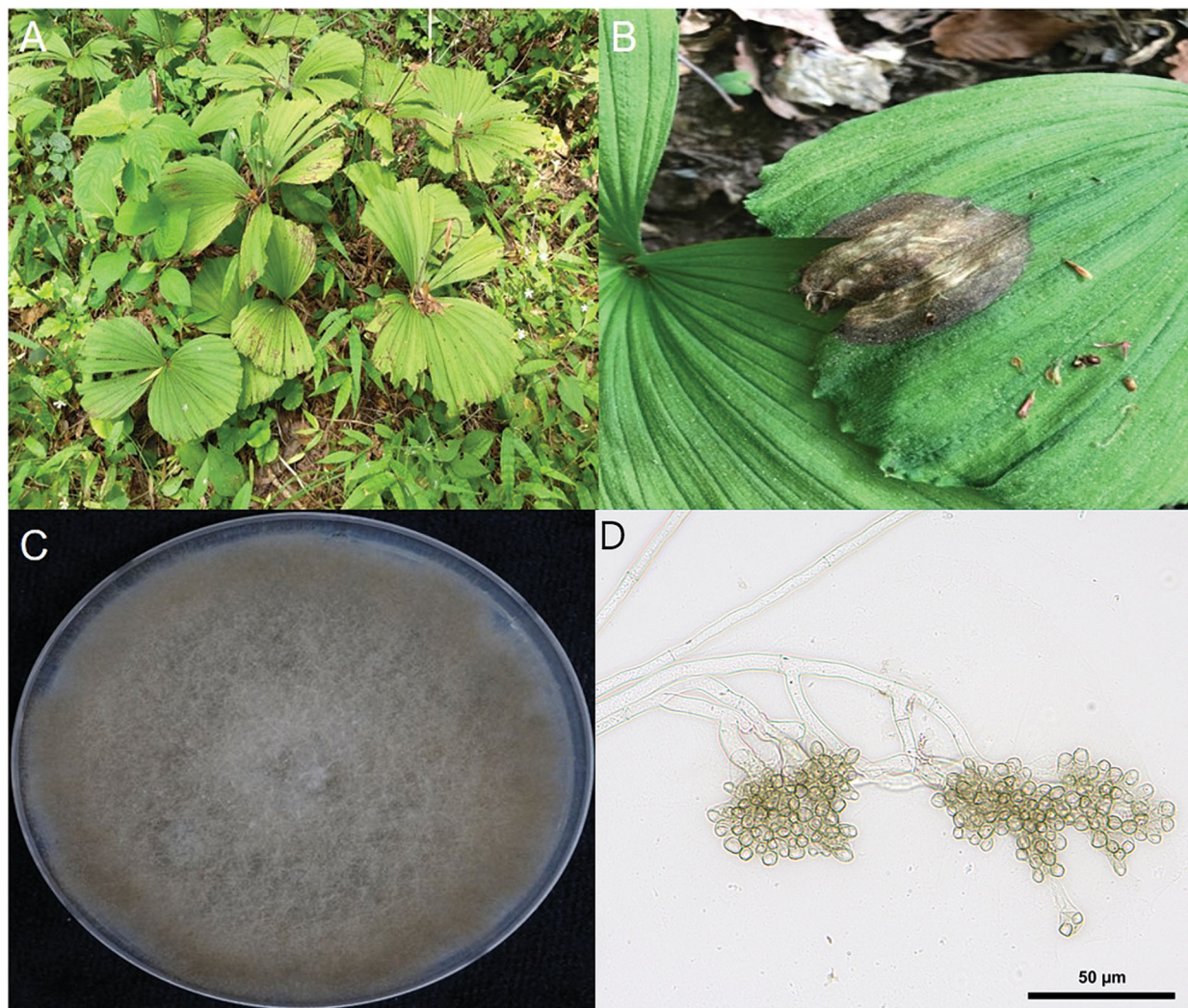


Figure 1. Symptoms of gray mold disease on *C. japonicum*. (A, B) Gray mold symptom of *C. japonicum* at a natural habitat; (C) Colony morphology of *B. cinerea* on PDA media; (D) Conidia of *B. cinerea*, isolated from *C. japonicum*. Red bar presents 50 μm .

with sterile water on three occasions and dried on the sterile filter paper for 10 min. Upon completion of the drying procedure, the specimens were implanted on water agar (WA: agar 20 g per L) and incubated at 25 °C for 72 h. To investigate the properties of the pathogen such as conidia, shapes, sizes, the fore-ends of hyphae, to the fungi were grown for 72 h on a potato dextrose agar medium (PDA: potato dextrose broth 24 g, agar 20 g/L) and incubated at 25 °C without exposure to light for 10 days. The outcome of this process has also been utilized for the identification of the pathogen by molecular characteristics. Colonies of the isolated pathogen that were incubated on the PDA medium exhibited grayish-brown color and formed gray conidia on the surface of the mycelia (Figure 1(C)). Total of three isolates of pathogens (Funbio 11, 12, and 13) that appeared to have identical morphological characteristics have been secured *via* the process of isolation. The conidia formed consisted of colorless or light brown single cells, which were either egg or oval-shaped with a size of 7.1 to 13.4 \times 5.2 to 8.6 μm

Table 1. Comparison of morphological characteristics of gray mold fungus isolated from *Cyripedium japonicum*.

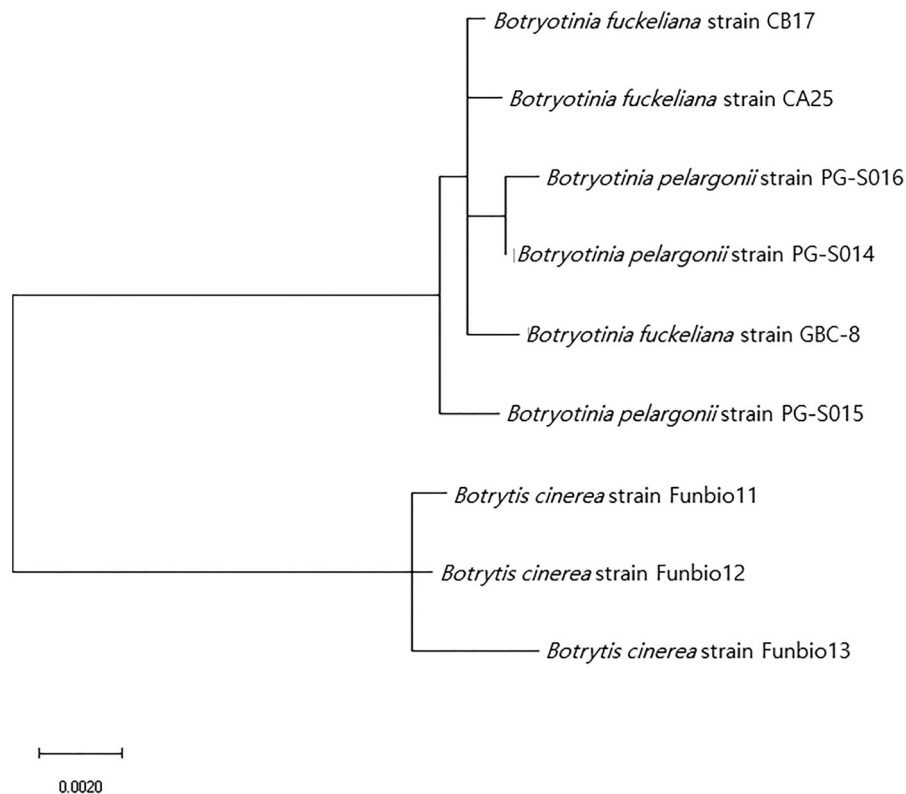
Characteristics	Isolate in this study	<i>B. cinerea</i> [9]
Colony		
Color	Grayish brown	Grayish brown
Conidia		
Shape	Ellipsoidal or ovoid	Ellipsoidal or ovoid
Size (μm)	7.1 ~ 13.4 \times 5.2 ~ 13.4	8 ~ 16 \times 5 ~ 10
Color	Colorless or pale brown	Colorless or pale brown
Conidiophore		
Size (μm)	16 ~ 31 \times 1.8 ~ 2	16 ~ 31 \times 2

($n = 50$) (Figure 1(D); Table 1). The conidiophores were ramified to resemble the appearance of tree branches and on their tips where the conidia were developed. Taken together, the morphological characteristic of the fungi confirmed that the pathogen isolated from *C. japonicum* was consistent with the unique properties of *Botrytis cinerea*. Even though, we secured the pathogen, it was impossible to perform artificial inoculation because *C. japonicum* was an endangered species.

DNA extraction was performed based on a technique involving the use of cetyltrimethylammonium

Table 2. List and information of primers for fungal identification.

Target gene	Primer name	Primer sequence 5'-3'	Reference
G3PDH	G3PDHfor	ATTGACATCGTCGCTGTCAACGA	Staats et al. [11]
	G3PDHrev	ACCCCACTCGTTGTCGTACCA	
HSP60	HSP60for	CAACAATTGAGATTGCCACAAG	Staats et al. [11]
	HSP60rev	GATGGATCCAGTGGTACCGAGCAT	
ITS	ITS1	TCCGTAGGTGAACCTGCGG	Staats et al. [11]
	ITS4	TCCTCCGCTTATTGATATGC	

**Figure 2.** Phylogenetic tree obtained through the maximum-likelihood and maximum parsimony analyses using MEGA 10 program based on the (A) ITS, (B) *HSP60*, and (C) *G3PDH* sequence of the isolates of *B. cinerea* and that of other *B. cinerea* isolates were retrieved from GenBank.

bromide (CTAB) [10] and the genomic DNA was used to perform the sequencing processes. Identification task specifically looked at three nuclear DNA genes (*G3PDH*, *HSP 60*, and ITS) with the specific primer sets (Table 2) corresponding to each of the genes was used to amplify through polymerase chain reaction (PCR) [12]. The amplicons of the PCR were subjected to electrophoresis using 1% agarose gel. The sequencing analyses were carried out at Macrogen (Seoul, Korea). The sequences were assigned an accession number MN267696 (ITS), MT233447 (*HSP 60*), and MN267728 (*G3PDH*) by the National Center for Biotechnology Information (NCBI). The phylogenetic analyses were conducted using GenBank BLAST provided by the NCBI, which involved maximum likelihood and maximum parsimony method in MEGA 10 program. As a result of conducting sequencing analyses based on the three genes ITS, *HSP 60*, and *G3PDH* the isolated pathogens were showed 100% similarity with previously registered *B.*

cinerea stains in GenBank. The result of a phylogenetic relationship analysis also confirmed that the pathogen concerned belonged to the same family as that of *B. cinerea* (Figure 2).

With a constant decrease in the number of plant species caused by the latest climate change, a large proportion of genus and species of the orchid family, in particular, are becoming increasingly susceptible to such a phenomenon. Furthermore, the incidence of disease outbreak is also accretion each year due to climate change, which poses a significant threat to the many plant species including the orchids. In accordance with the criteria suggested by the International Union for Conservation of Nature and Natural Resources (IUCN), *C. japonicum* is the major to species that has been assigned the code EN (endangered) at the global level and the code CR (critically endangered) at the domestic level in South Korea [13–15]. This study has confirmed that *B. cinerea* caused gray mold disease on *C. japonicum*. The findings are anticipated to play a

significant role in devising long-term strategies to preserve and restore *C. japonicum* and at the same time, the ongoing pathological investigation is necessary to promote the preservation of the plant in the Korean peninsula.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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