

Distribution of Rhizosphere and Endosphere Fungi on the First-Class Endangered Plant *Cypripedium japonicum*

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Abstract Endangered native plant habitats and populations are rapidly disappearing because of climate and environmental changes. As a representative, the abundance of the first-class endangered wild plant, *Cypripedium japonicum*, has been rapidly decreasing in Korea. The purpose of this study was to evaluate the distribution of rhizosphere and endophytic fungi on *C. japonicum* in its native habitat. A total of 440 rhizosphere and 79 endosphere fungi isolates were isolated and identified on the basis of their molecular characteristics. Sixty-five genera and 119 fungi species were identified in this study. The genus *Trichoderma* showed the highest abundance among both rhizosphere and endosphere fungi. *Mortierella*, *Hypocrea*, and *Penicillium* spp. were also relatively dominant species on *C. japonicum*. The community structures of rhizosphere and endosphere fungi were similar, but endosphere fungi showed greater diversity.

Keywords Endangered plant, Endosphere, Korean ladyslipper, Rhizosphere

Korean ladyslipper (*Cypripedium japonicum* Thunb.) is an angiosperm that belongs to the genus *Cypripedium*, family Orchidaceae. *Cypripedium* is composed of more than 40 varieties, including Korean ladyslipper, big-flower ladyslipper (*C. macranthos*), spotted ladyslipper (*C. guttatum*), and ladyslipper orchid (*C. calceolus*) [1]. The Korean ladyslipper is native to Korea, China, and Japan. However, because of climate change and accelerated global warming, the number of plants in the Korea peninsula has dramatically decreased. The Korean ladyslipper has been classified as a first-class endangered wild plant by the Ministry of Environment of

Korea. It has been estimated that only 800 individual plants are alive in three natural habitats nationwide [1].

Orchid plant seeds typically do not have an endosperm and are composed of unfractionated and unorganized cells. Therefore, the symbiotic relationship with orchid mycorrhizal fungi (OMF) is essential for orchid seed germination and development [2-4]. OMF colonize plant roots and affect various aspects of plant physiology, such as nutritional status, development, and tolerance against abiotic or biotic stresses [2]. OMFs do not enter or damage the root tissues, but rather colonize on the root surface as ectomycorrhizal fungi and supply and exchange nutrients with the orchid plant via its mycelium [5, 6]. *Rhizoctonia* spp. are well-known OMF and the most frequently isolated species from the orchid [7]. The fungal genera *Ceratobasidium*, *Sebacina*, and *Tulasnella* are also frequently isolated and reported from the orchid root as epiphytic fungi [3]. Endomycorrhizal fungi are also considered significant components for orchid seed germination and development [8, 9]. A previous study on Korean ladyslipper OMF detected the presence of putative mycorrhizal fungi such as *Leptodonitidium orchidicola*, *Humicola fuscoatra* var. *fuscoatra*, *Umbelopsis dimorpha*, and *Phialocephala fortinii* [10]. However, some previous studies reported that orchid-associated fungi species did not show fungal diversity or community in endangered plants. Therefore, the aim of this study was to identify rhizosphere and endosphere fungi populations on Korean ladyslipper. This information improves the understanding of the relationship

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Table 1. Sampling date and number of samples and isolated fungi

Sampling date	No. of roots	No. of rhizospheres ^a	Log CFU/g of rhizosphere	No. of total isolates
March 24, 2016	3	4	3.38×10^2	90
May 20, 2016	5	4	3.21×10^2	215
July 27, 2016	4	7	1.61×10^2	120
September 20, 2016	2	12	0.5×10^2	205

CFU, colony-forming units.

^aAt least 1 g of rhizosphere soil was taken for each sample.

between fungi and Korean ladyslipper, a first-class endangered wild plant.

Only three sites of Korean ladyslipper natural habitats have been reported in the Korea peninsula. Therefore, we do not provide detailed information of the location of the natural habitat. In 2016, rhizosphere fungi and the root of *C. japonicum* were sampled at a restricted site in Mt. Deogyusan National Park (Table 1). Each plant was labeled with an individual discrimination tag, which allowed for the collection of samples from the same plant during the sampling period. However, the number of samples varied because of the state of the plants (Table 1). Rhizosphere soil was diluted in 9 mL sterilized distilled water up to 10^{-4} and smeared on 1/5 potato dextrose agar (1/5 PDA; potato dextrose 5 g, agar at 15 g per 1 L), as well as rifampicin (50 mg/mL) to prevent bacterial growth. The 1/5 PDA plants were incubated at 27°C for 3 days, and then colony-forming units (CFU) were determined for each sample and a single colony of fungus was transferred onto new 1/5 PDA media to purify the culture. Root specimens (0.5 cm in length) were sterilized with 70% ethanol for 30 sec followed by 1%

NaClO for 30 sec, and then washed 5 times with sterilized distilled water. The sterilized specimens were transferred onto a 1/5 PDA plate and incubated at 27°C for 3 days. A single hypha tip was isolated with a sterilized needle under a dissecting microscope and transferred onto new 1/5 PDA plates to purify the culture. Rhizosphere and root samples were collected 4 times. The population in rhizosphere fungi was 3.38×10^2 CFU/g of soil in the first sample collected, 3.21×10^2 CFU/g in the second sample, 1.61×10^2 CFU/g in the third sample, and 0.5×10^2 CFU/g in the fourth sample (Table 1). Compared to other plants, *C. japonicum* showed relatively low rhizosphere fungal density. This may be because of the well-developed hairy root system and soil composition type. Similar to other orchid species, *C. japonicum* was found in complete sandy soil conditions. These characteristics may cause the plant to have limited interactions with soil particles or microbes. Fungi with different morphological characteristics were isolated from among rhizosphere and endosphere fungi of *C. japonicum* (Fig. 1). Ninety, 215, 120, and 205 isolates were obtained during the first through fourth collections, respectively (Table 1). A total of 630 isolates

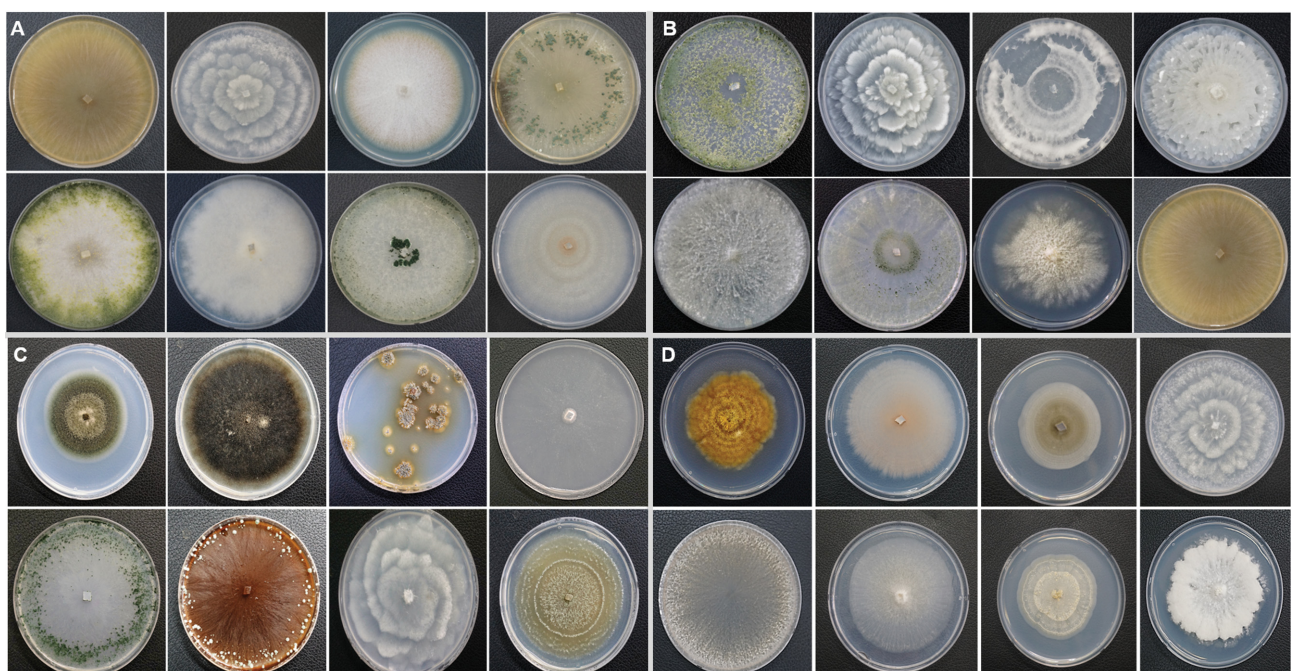


Fig. 1. Morphological diversity of isolated fungi from *Cypripedium japonicum* rhizosphere or endosphere. Sampling in March (A), May (B), July (C), and September 2016 (D).

of rhizosphere or endosphere fungi were maintained as pure cultures on 24-well plates for fungal identification.

To identify the fungi, genomic DNA of the fungi was extracted using the CTAB protocol [11]. Primers internal transcribed spacer 1 (ITS1) and ITS4 [12] were used to amplify the partial ITS region. PCR was carried out in a total volume of 20 μ L, which contained 50 ng of template gDNA, 5 U of *Taq* DNA polymerase, 10 pmol of each primer, 200 μ M of dNTP mixture, 2 μ L of 10 \times reaction buffer, and ddH₂O up to the final volume. PCR was performed as follows: pre-denaturation at 94°C 5 min; 30 cycles of denaturing at 94°C for 30 sec; annealing at 55°C for 45 sec and extension at 72°C for 40 sec; and final extension at 72°C for 10 min. To obtain sequences of the amplified ITS region, an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) was used. Sequence information was compared with that in GenBank through a BLAST search (<https://www.ncbi.nlm.nih.gov>) and phylogenetic analyses were conducted by a neighbor-joining algorithm using R (<http://www.r-project.org>). Among the 630 isolates, 519 were identified, while 111 remained as unknown. Among the identified isolates, 440 fungi were isolated from rhizosphere fungi and 79 isolates were derived from endosphere fungi. A total of 65 genera and 119 species were identified from *C. japonicum* rhizosphere and endosphere fungi (Supplementary Table 1). Among the 119 species, the following 32 fungi species were commonly present in rhizosphere and endosphere fungi: *Chaetomium* sp., *Cladosporium* sp., FJ450035.1, KF212314.1, *Gibberella moniliformis*, *Hypocrea lixii*, *Hypocrea pachybasioides*, *Hypocrea* sp., *Microdiplodia* sp., *Mortierella minutissima*, *Mortierella* sp., *Mortierellaceae* sp., *Nectriaceae* sp.,

Paecilomyces sp., *Penicillium polonicum*, *Phoma medicaginis*, *Staphylotrichum coccosporum*, *Talaromyces cellulolyticus*, *Trichoderma atroviride*, *Trichoderma effusum*, *Trichoderma harzianum*, *Trichoderma koningiopsis*, *Trichoderma polysporum*, *Trichoderma* sp., *Trichoderma virens*, *Tritirachium* sp., KJ555186.1, DQ421181.1, *Verticillium* sp., *Xylaria primorskensis*, and *Xylariales* sp. Among commonly presented fungi, *Trichoderma* spp. were the most abundant species (182 isolates). Interestingly, Ordóñez *et al.* [13] suggested that *Trichoderma*, *Phoma* sp., and *Epidendrum* sp. were not dominant in orchard plants. These differences may be because of host plant species, environmental conditions, and geographical distance. *Xylariales* spp. are lignin and cellulose decomposers [13, 14]. *Xylariales* spp. may positively affect *C. japonicum* growth and survive under nutrient-insufficient conditions. As fungi unique to the endosphere, only 8 species were identified: *Alternaria alternata*, *Coprinopsis strossmayeri*, *Eutypella scoparia*, *Neonectria* sp., *Neurospora tetrasperma*, *Penicillium vasconiae*, *Scytalidium lignicola*, and *Tritirachium* sp. Zelmer [15] reported *Alternaria* sp. as an endophyte fungi of genus *Cypripedium* in Canadian territory. Other endophytes, such as *Chaetomium* sp., *Cylindrocarpon* sp., *Epicoccum purpureum*, *Phialocephala* sp., *Phoma* sp., *Acremonium killense*, *Humicola* sp., and *Fusarium* sp. were also isolated and reported in the genus *Cypripedium* [10, 15]. The remaining 78 species of fungi were present only in the rhizosphere fungi of *C. japonicum* (Supplementary Table 1). However, the distribution of fungi was similar between rhizosphere and endosphere fungi. The genus *Trichoderma* was the most frequently isolated from both rhizosphere and endosphere fungi. Genera *Mortierella* and *Hypocrea* showed high density among both rhizosphere and endosphere fungi (Fig. 2). The phylogenetic trees of

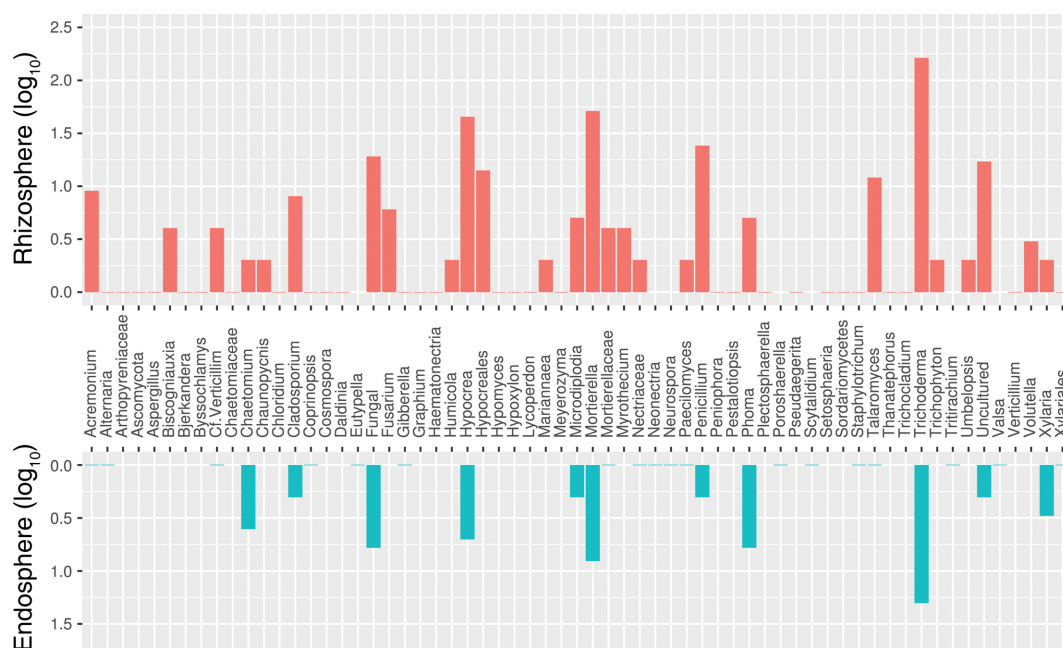


Fig. 2. Distribution of rhizosphere and endosphere fungi on *Cypripedium japonicum*.

the rhizosphere and endosphere fungi showed that the community of endosphere fungi was more diverse than that of rhizosphere fungi (Supplementary Fig. 1). Rhizosphere fungi was involved in macromolecule decomposition to supply nutrients to the plant. Among the various rhizosphere fungi, some fungi species may enter the root tissue and contribute to plant protection.

In this study, we isolated and identified both rhizosphere and endosphere fungi for 1 year from *C. japonicum*, a first-class endangered wild plant. The obtained fungi may be applied to protect the natural habitat of *C. japonicum* and contribute to the propagation of the plants.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including one table and one figure can be found with this article online at <http://www.mycobiology.or.kr/src/sm/mb-45-97-s001.pdf>.

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