

Fungal Endophytes Isolated from Needle Leaves of Three Coniferous Species on Mt. Seodae of Korea

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ABSTRACT: We investigated endophytic fungi diversity of two Pinaceae species (*Pinus densiflora*, *Pinus rigida*) and one Cupressaceae species (*Juniperus rigida*) on Mt. Seodae, Korea. In total, 34 isolates were obtained from 19 host plants and identified according to internal transcribed spacer (ITS) region. As a result, they were placed in 13 taxa. Among them, 52.9% belonged to Leotiomycetes, 32.3% belonged to Sordariomycetes, 5.8% belonged to Agaricomycetes, and 3.0% for isolates belonged to either Eurotiomycetes, Dothideomycetes or Ustilaginomycetes. In particular, *Lophodermium* growing in pine would be an especially important instance of biodiversity for endophytes, suggesting that further study examining its ecological function in the environment is critical.

KEYWORDS: Diversity, Endophytic fungi, *Juniperus rigida*, *Pinus densiflora*, *Pinus rigida*

Endophytic fungi are harmless symbionts of plants [1]. In general, endophytic fungi have a mutualistic relationship with their host plants to obtain nutrients from plants, while the fungi also provide benefits to the plant, such as protection from the environment, promoting growth [1, 2]. Secondary metabolites from the endophyte have become an important study area and recent studies have been carried out to seek new metabolites [3, 4].

Conifer species are distributed worldwide and it is estimated that about 600 species exist globally [5]. It is reported that there are approximately 54 different species in Korea, including both native and introduced species [6]. Currently, global climate change is affecting forest decline [7], thus studies into the relationships between conifer species and their symbionts, sharing an evolutionary his-

tory, are needed to biodiversity. The purpose of this study was to isolate and identify endophytic fungi from needle leaves to compare their diversities among different coniferous species in Korea.

Sampling and Isolation Leaf samples were collected in Mt. Seodae (36° 13 N, 127° 56 E), which is located in Chungnam Province. We collected healthy needle leaves, selecting leaves randomly, from 5 individual pines (*Pinus densiflora*), 4 individual juniper trees (*Juniperus rigida*), and 10 individual pitch pines (*Pinus rigida*). These were washed with tap water and then surface-sterilized by 1% NaOCl solution for 3 min, 70% EtOH solution in 2 min and finally rinsed with sterile water. After that, the leaves were cut into 5 mm pieces using sterile scissors and then placed on potato dextrose agar medium. In total, 4 pieces were placed on the medium and grown on 25°C in the darkness. Pure isolates were obtained by continuous sub-culture.

DNA extraction, amplification, and analysis The DNA of each endophyte strain was extracted using the Exgene Plant SV mini kit (GeneAll, Seoul, Korea) according to the manufacturer's manual. The extracted DNA was amplified using ITS1F and ITS4 primers [8]. Polymerase chain reaction (PCR) conditions were as follows: 5 min at 94°C for pre-denaturation, 30 sec at 94°C for denaturation, 30 sec at 50°C for annealing, and 1 min at 72°C for elongation. These steps from denaturation to elongation

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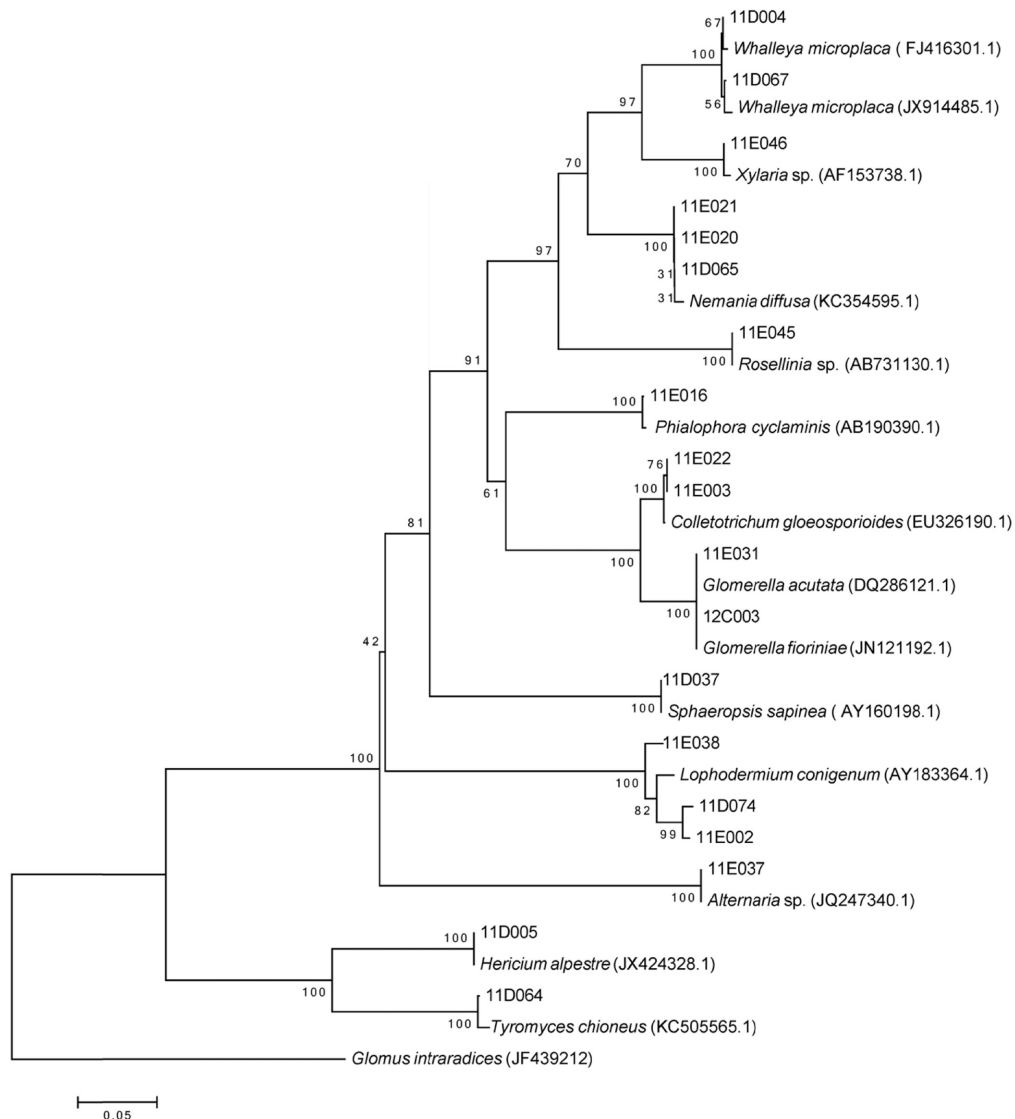


Fig. 1. Phylogenetic tree of endophytic fungi from *Juniperus rigida*, *Pinus densiflora* and *Pinus rigida* in Mt. Seodae. Internal transcribed spacer and 5.8S rDNA region were used to confirm the topological appropriation of the isolates. *Glomus intraradices* was used as an outgroup.

were repeated for 30 cycles, after which the samples were held 5 min at 72°C for stabilization. PCR products were then stored at 4°C. PCR products were examined by electrophoresis with 1.5% agarose gel using RedSafe gel stain (iNtRON Biotechnology, Seongnam, Korea), the amplified bands' images were captured by Digital Gel Documentation (Korea Bio-Tech, Korea), and sequencing was conducted in SolGent (Daejeon, Korea).

The sequences were analyzed using BLAST (Basic Local Alignment Search Tool) on the NCBI. To identify them, we selected the taxon with the greatest identity. We then aligned these sequences to construct a phylogenetic tree by neighbor-joining method to confirm their topological

position on the tree (Fig. 1). Bootstrap analysis was performed with 1,000 replicates using MEGA5 [9] and outgroup was set as *Glomus intraradices* to complete the tree.

Altogether, 34 fungal isolates were obtained from 19 host plants. The isolates were able to divide into 13 strains (Table 1). They exhibited over 99% similarity with reference sequences on GenBank of NCBI, so it was inferred that they belong to the same taxon [10]. However, some stains, such as 11E037, 11E045, and 11E046, could not be identified at the species level. They were divided into 6 classes: Leotiomycetes (52.9%), Sordariomycetes (32.3%), Agaricomycetes (5.8%), Dothideomycetes (3.0%), Ustilaginomycetes (3.0%) and Eurotiomycetes (3.0%).

Table 1. The closest taxa of representative fungal strains isolated from needle leaves of conifers

Fungal Strains (accession No.)	The Closest GenBank taxa (accession No.)	Similarity (%)
11E003 (LC062614)	<i>Colletotrichum gloeosporioides</i> (EU326190.1)	99
11E016 (LC062621)	<i>Phialophora cyclaminis</i> (AB190390.1)	99
11E020 (LC062622)	<i>Nemania diffusa</i> (KC354595.1)	99
11E031 (LC062617)	<i>Glomerella acutata</i> (DQ286121.1)	99
11E037 (LC062619)	<i>Alternaria</i> sp. (JQ247340.1)	99
11E038 (LC062610)	<i>Lophodermium conigenum</i> (AB247944.1)	99
11E045 (LC062613)	<i>Rosellinia</i> sp. (AB731130.1)	99
11E046 (LC062611)	<i>Xylaria</i> sp. (AF153738.1)	100
11D005 (LC062612)	<i>Hericium alpestre</i> (JX424328.1)	99
11D037 (LC062618)	<i>Sphaeropsis sapinea</i> (AY160198.1)	99
11D064 (LC062615)	<i>Tyromyces chioneus</i> (KC505565.1)	99
11D067 (LC062616)	<i>Whalleya microplaca</i> (JX914485.1)	99
12C003 (LC062620)	<i>Glomerella fioriniae</i> (JN121192.1)	99

Table 2. Diversity of endophytic fungi isolated from leaves of three conifer species

Fungal strains	Relative abundance (%)*		
	<i>Juniperus rigida</i>	<i>Pinus densiflora</i>	<i>Pinus rigida</i>
11E003			10
11E016	16		
11E020		20	10
11E031	16		
11E037	16		
11E038		20	70
11E045			5
11E046	16		
11D005			5
11D037	16		
11D064		20	
11D067		40	
12C003	16		
Total number of isolates	6	5	23
Species richness	6	4	5
Shannon diversity index (H')	1.79	1.33	0.78

*Relative abundance indicates the percentages of the number of isolates in the study sites of the total numbers of isolates.

The largest portion fungal isolates identified were found to be *P. rigida*, but species diversity in *J. rigida* (Shannon diversity index; $H' = 1.79$) is higher than that in *P. rigida* ($H' = 0.78$) (Table 2). These are very different families, even though both of them are included in conifer. Studies on Pinaceae species in the world showed much more abundance than that for Cupressaceae species. Few previous studies have provided results about juniper tree's endophytes. In comparison with one other study, which

isolated 4 fungal endophytes from Juniper trees in Mt. Taehwa, this study was able to isolate 6 fungal endophytes, none of which have been previously identified [11]. In the cases of Japanese red pine and pitch pine, some endophytes, such as *Nemania diffusa* and *Lophodermium conigenum*, are commonly found in hosts that are the same at the genus level. Endophytes that were isolated from juniper tree and pine trees in this study, however, had not previously been found on the same species of host.

Basically, endophyte diversity is affected by the host plant diversity, therefore even when growing in similar microclimate conditions, abundant diversity of host plants are the most important resource for facilitation growth of multiple biological niches in the nature.

In other studies of endophytes in the *Pinus* spp., the same fungal species have not been found on the sample plant, but species of the same genus have been as in the case of *Lophodermium* [11-14]. *Lophodermium* sp. has been found in all of the pine tree endophyte studies previously performed in Korea, but the occurrence of other fungal species has been inconsistent. Host specificity for endophytic species has mainly consisted of herbaceous plants, and thus it is thought that there is a unique association between herbaceous plants and endophytes. Meanwhile, specificity of endophytes for woody plants has been seldom reported because many fungal endophytes experience horizontal transfer and this yields a highly random distribution of endophytes. This study has set a precedent for the existence of a host-specific taxon, *Lophodermium*, and our results also suggest that certain taxa are affected by environmental factors other than host specificity. Many endophytes will be distributed to new biological niches via the various interactions among them. Therefore, continuous isolation and identification of endophytes is needed to monitor the level of biological diversity and to understand the functional diversity of *Lophodermium* spp. growth on pine trees in nature.

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