

## RESEARCH ARTICLE

# Diversity of Endophytic Fungi Isolated from *Pinus densiflora* and *Juniperus rigida* Distributed in Mt. Baekryeonsan and Mt. Johangsan, Korea

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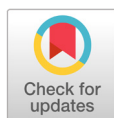
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## ABSTRACT

We investigated the biodiversity of endophytic fungi in *Pinus densiflora* and *Juniperus rigida* from Mt. Baekryeonsan and Mt. Johangsan in Korea. In total, 59 isolates were discovered from 26 host plants and identified using sequences from the rDNA internal transcribed spacer (ITS) region. Twenty taxa of endophytic fungi were identified in this study; 62% of isolates belonged to Leotiomycetes, 34% belonged to Sordariomycetes, and 4% belonged to Agaricomycetes. Results confirmed three previously unreported endophytic fungi in Korea: *Phanerochaete concrescens*, *Creosphaeria sassafras*, and *Scopuloides rimosa*. Community analysis showed that the fungal diversity patterns of the samples from Mt Baekryeonsan and Mt. Johangsan are similar to those from previous studies and varied depending upon host and locality.

**Keywords:** Biodiversity, Endophytic fungi, *Juniperus rigida*, *Lophodermium* spp., *Pinus densiflora*,



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## INTRODUCTION

Endophytic fungi are asymptomatic organisms that live in healthy plant tissues throughout the entire lifecycle or part of the lifecycle [1]. Most are ascomycetes, and some are basidiomycetes [2, 3]. Many recent studies have focused on the biodiversity, secondary metabolites, and resistance to environmental stresses of these fungi [4, 5]. Moreover, various studies of endophytic fungi have demonstrated the potential of available secondary metabolites as bioactive substances [6].

Gymnosperms are distributed worldwide, with approximately 30~60 taxa, including cultivated or introduced species in Korea [7]. Among these taxa, only 3~6 conifer trees are endemic species in Korea [8, 9]. Current worldwide climate change and deforestation have threatened the survival of conifers, including those on the Korean peninsula. However, few studies have evaluated endophytic fungi that have evolved along with these trees in Korea. Therefore, it is necessary to investigate the

biodiversity and ecological function of endophytic fungi in Korea.

In this study, we investigated the biodiversity of endophytic fungi in *Juniperus rigida* and *Pinus densiflora* from Mt. Baekryeonsan and Mt. Johangsan, Korea and compared the distributions of endophytic fungal communities according to host plant and locality.

## MATERIALS AND METHODS

We collected twigs with healthy leaves from six individuals of *J. rigida* and 11 individuals of *P. densiflora* from Mt. Baekryeonsan (N38°27', E126°39') and three individuals of *J. rigida* and six individuals of *P. densiflora* from Mt. Johangsan (N38°27', E126°39'). Collected leaves were subjected to surface sterilization. Surfaces of needle leaves were washed with tap water, and the leaves were treated using 1% NaOCl solution for 3 min and 70% ethanol for 2 min. The leaves were then washed with sterilized water twice, subjected to surface sterilization, and cut into 1 cm pieces. Four pieces were placed on potato dextrose agar (PDA) medium and incubated at 25°C in the dark. New hyphae were finally isolated [10].

Genomic DNA was extracted from the mycelia of isolates of endophytic fungi. DNA extraction was performed using a DNeasy Plant SV mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Extracted DNA was amplified with primers ITS1F and ITS4 for the ITS region [11] and primers Bt2a and Bt2b for the  $\beta$ -tubulin region [12] using 2 × Taq PCR Smart Mix2 (SolGent, Daejeon, Korea).

The polymerase chain reaction products were sequenced using a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) at SolGent, Korea. Analyzed sequences were identified on NCBI using BLAST, and neighbor-joining trees were generated using MEGA7 [13] to confirm the topological position among closest taxa with bootstrap analysis (1,000 replicates). Biodiversity and community structures of endophytic fungi were analyzed according to host plants and locality.

## RESULTS AND DISCUSSION

From 26 individual host plants, 59 morphotypes of endophytic fungi were isolated. The fungi were grouped into 20 taxa using sequence analysis (Table 1, Fig. 1). Most of the isolates showed similarities with the closest taxa above 96%. However, isolates 11F027, 11G007, 11G094, 11G228, 11G293, and 12D012 were not able to be identified at the species level. Fungal strains isolated from two species, *P. densiflora* and *J. rigida*, were divided into three classes: Leotiomycetes (62%), Sordariomycetes (34%), and Agaricomycetes (4%), similar to previous studies [14, 15]. Additionally, three species of endophytic fungi were identified for the first time in Korea. Their culture characteristics are described in Fig. 2, along with the phylogenetic analysis.

### *Phanerochaete concrescens* V. Spirin & S. Volobuev, *Mycological Progress* 14:7 (2015)

Colonies on PDA measured 39~41 mm in diameter after 7 days, with a creamy color in the center,

beige in the margin; colonies spreading to the border with white aerial mycelia, somewhat elevated in the center; reverse in the center, light yellow to pale brown, and ivory in the margin. Colonies on MEA measured 28~32 mm in diameter after 7 days, were restricted, with a creamy surface, somewhat elevated in the center; reverse was pale beige.

**Specimen examined:** Mt. Baekryeonsan, Imsil, Jeollabuk-do, Korea; N38°27'02.97", E126°39'08.01"; July 22, 2011; isolated from leaves of *Pinus densiflora*, strain 11F005, EERSFG0000000345, GenBank no. MK158244.

**Note:** *P. conrescens* was reported from Russia in 2015 by Spirin and Volobuev. This species belonged to Basidiomycota and was isolated from *Betula* sp. [16]. Based on the sequence analysis, the ITS region had 99% similarity with the reference *P. conrescens* KP994380.1. Large subunit (LSU) also showed 99% similarity with the reference *P. conrescens* KP994382.1 (Fig. 3).

***Creosphaeria sassafras* (Schwein.) Y.M. Ju, F. San Martín & J.D. Rogers, Mycotaxon 47: 223 (1993)**

**Table 1.** Molecular identification and the closest taxa of representative isolates using ITS rDNA region from *Pinus densiflora* and *Juniperus rigida* in Mt. Baekryeonsan and Johangsan

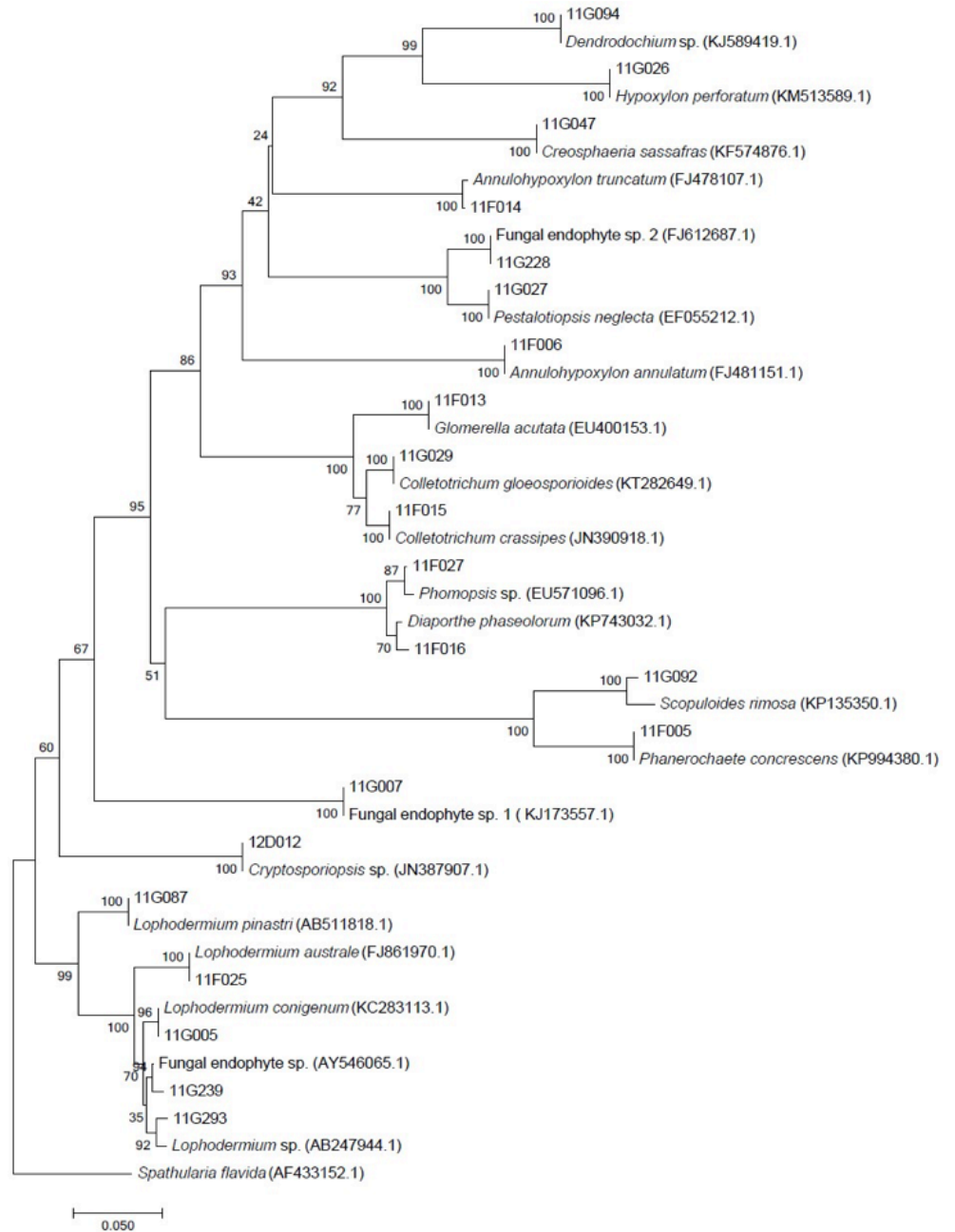
Isolates no.	Accession no.	The closest GenBank taxa	Relative abundance (%) <sup>a</sup>			
			Mt. Baekryeonsan		Mt. Johangsan	
			<i>P. densiflora</i>	<i>J. rigida</i>	<i>P. densiflora</i>	<i>J. rigida</i>
11F005	KP994380.1	<i>Phanerochaete conrescens</i>	3			
11F006	FJ481151.1	<i>Annulohyphoxylon annulatum</i>				17
11F013	EU400153.1	<i>Colletotrichum acutatum</i>		9		
11F014	JQ303335.1	<i>Annulohyphoxylon truncatum</i>	6	27		
11F015	JN390918.1	<i>Colletotrichum crassipes</i>	3			
11F016	KP743032.1	<i>Diaporthe phaeolorum</i>	3			
11F025	FJ861970.1	<i>Lophodermium australe</i>			9	
11F027	EU571096.1	<i>Phomopsis</i> sp.		9		
11G005	KC283113.1	<i>Lophodermium conigenum</i>	68		73	
11G007	KJ173557.1	Fungal endophyte sp. 1				17
11G026	KM513589.1	<i>Hypoxylon perforatum</i>		9		
11G027	EF055212.1	<i>Pestalotiopsis neglecta</i>		9		17
11G029	KT282649.1	<i>Colletotrichum gloeosporioides</i>	3	18		
11G047	KF574876.1	<i>Creosphaeria sassafras</i>				17
11G087	AB511818.1	<i>Lophodermium pinastri</i>			9	
11G092	KP135350.1	<i>Scopuloides rimosa</i>	3			
11G094	KJ589419.1	<i>Dendrodochium</i> sp.	10			
11G228	FJ612687.1	Fungal endophyte sp. 2				33
11G293	AB247944.1	<i>Lophodermium</i> sp.			9	
12D012	JN387907.1	<i>Cryptosporiopsis</i> sp.		18		
Total isolates			31	11	11	6
Shannon diversity index (H')			1.22	1.85	0.89	1.56
Species richness			8	7	4	5

ITS, internal transcribed spacer.

<sup>a</sup>Relative abundance indicates the percentages of the number of isolates in the study sites of the total numbers of isolates.

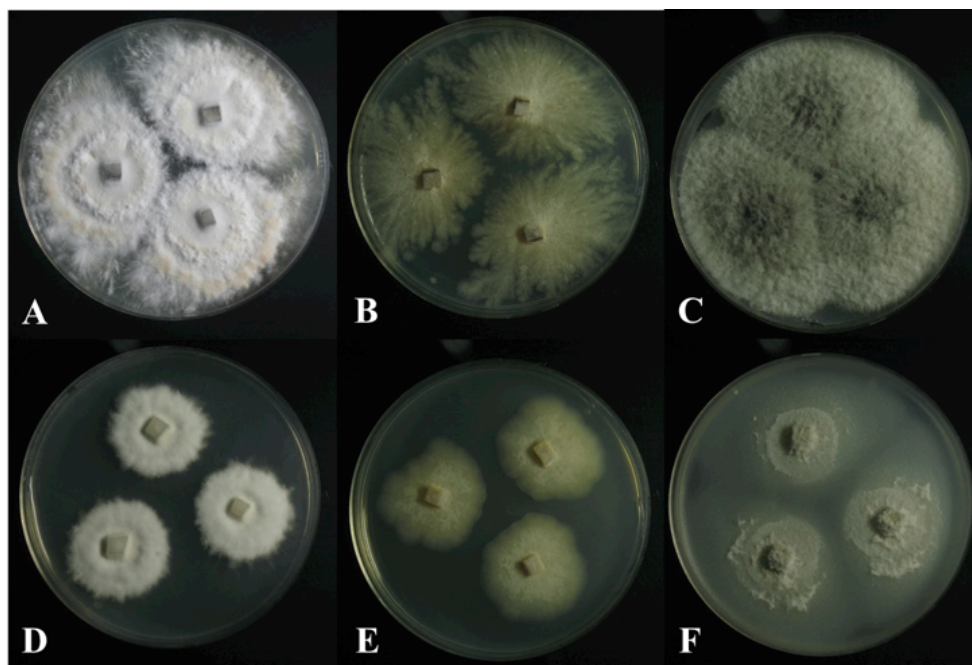
Colonies on PDA measured 37~40 mm in diameter after 7 days, showed white to pale beige surfaces; flat colonies showed spreading to the border with irregular and radial shapes; reverse was dark yellow. Colonies on malt extract agar (MEA) measured 28~33 mm in diameter after 7 days, showed whitish surfaces; reverse was ivory. Colonies were restricted with irregular margins.

**Specimen examined:** Mt. Johangsan, Muju, Jellabuk-do, Korea; N38°27'52.00", E126°39'13.00",

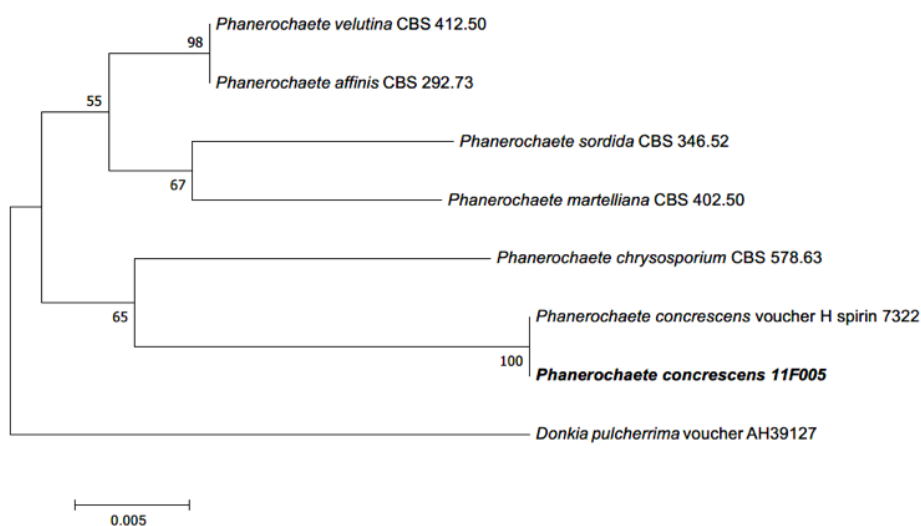


**Fig. 1.** Neighbor-joining phylogenetic tree of endophytic fungi from *Pinus densiflora* and *Juniperus rigida* in Mt. Baekryeonsan and Johangsan. Internal transcribed spacer regions were used these sequence analysis to confirm the topological appropriation of isolates (1,000 replicates). *Spathularia flavida* was used as an out-group.

July 15, 2011; isolated from leaves of *Juniperus rigida*, strain 11G047, EERSFG000000342, GenBank no. MK158314.

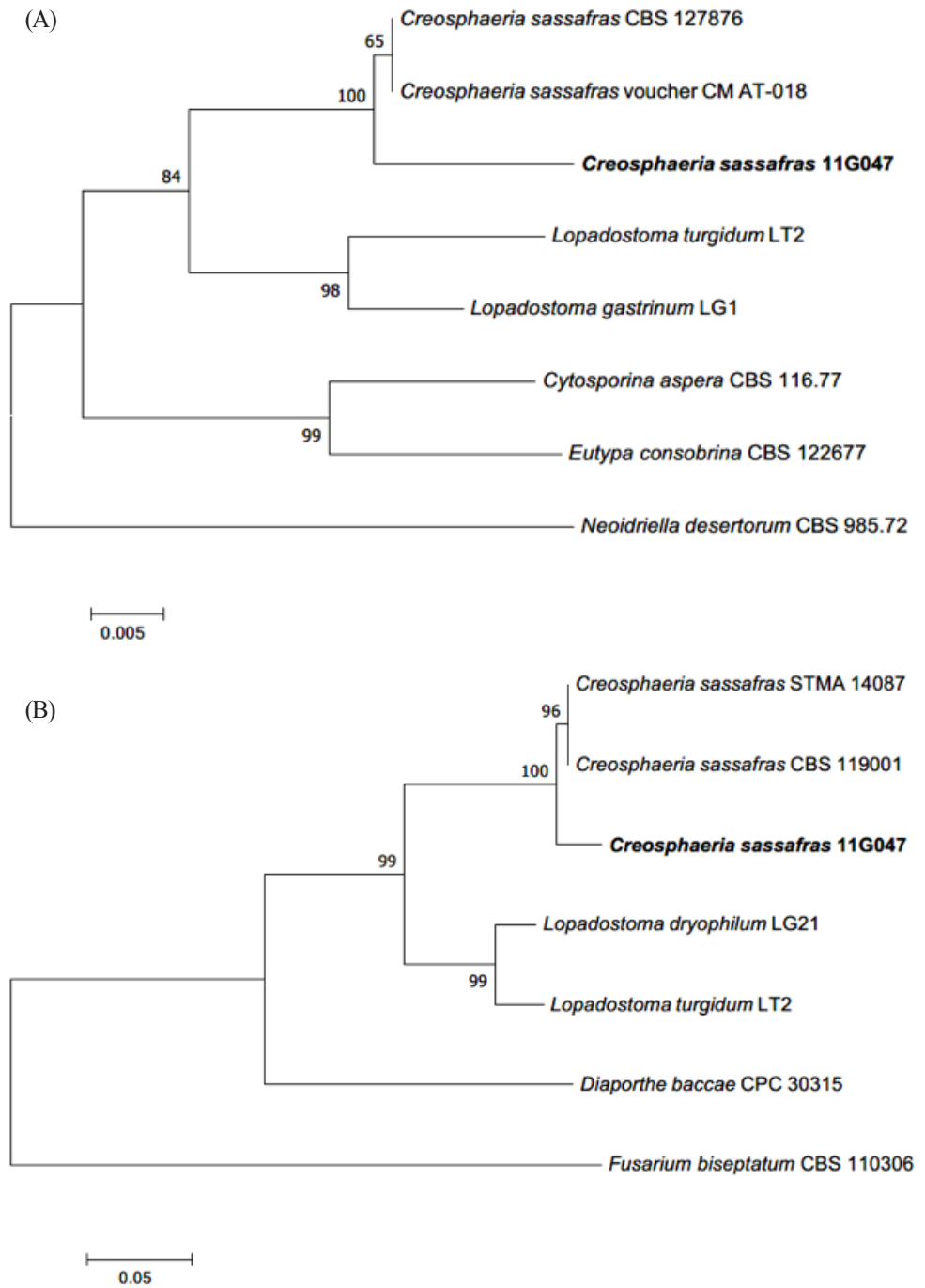


**Fig. 2.** Colonies of strain 11F005 (*Phanerochaete concrescens*) grown for 7 days on PDA (A) and MEA (D). Colonies of strain 11G047 (*Creosphaeria sassafras*) grown for 7 days on PDA (B) and MEA (E). Colonies of strain 11G092 (*Scopuloides rimosa*) grown for 7 days on PDA (C) and MEA (F). PDA, potato dextrose agar; MEA, malt extract agar.



**Fig. 3.** Neighbor-joining phylogenetic tree based on a large subunit rDNA sequences. *Donkia pulcherrima* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in bold.

**Note:** *C. sassafras* was reported by Ju et al. in 1993 and belonged to Xylariaceae in Ascomycota [17]. In China, *C. sassafras* was isolated from liverwort (*Scapania verrucosa*) as an endophytic fungus [18]. This species can secrete antimicrobial matter as sassafrins [17]. Based on genetic analysis, the ITS



**Fig. 4.** Neighbor-joining phylogenetic tree based on a large subunit rDNA (A) and  $\beta$ -tubulin gene (B) sequences. *Neoidriella desertorum* and *Fusarium bisepatum* were used as an outgroup in each tree. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in bold.



region had 99% similarity with the reference *C. sassafras* KF574876.1.  $\beta$ -tubulin (TUB) also showed 97% similarity with the reference *C. sassafras* KU684126.1 (Fig. 4).

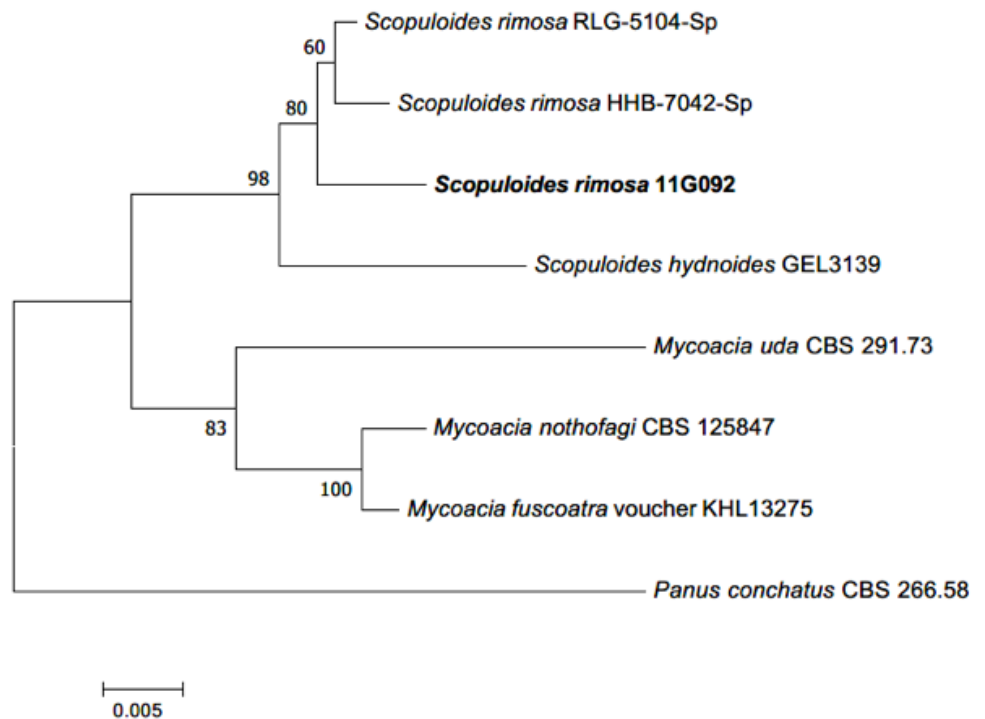
***Scopuloides rimosa* (Cooke) Jülich, Persoonia 11 (4): 422 (1982)**

Colonies on PDA measured 43~45 mm in diameter after 7 days, with olivaceous to light gray color in the center; colonies showed spreading to the border with woolly aerial mycelia; reverse in the center was olivaceous and pale beige in the margin. Colonies on MEA measured 35~37 mm in diameter after 7 days; colonies were restricted, with slightly dense irregular mycelium in the center; the margin was complete, with a grayish green surface; the reverse was yellowish green.

**Specimen examined:** Mt. Baekryeonsan, Imsil, Jeollabuk-do, Korea; N38°27'02.97", E126°39'08.01", July 22, 2011; isolated from leaves of *Pinus densiflora*, strain 11G092, EERSFG0000000344, GenBank no. MK158344.

**Note:** *S. rimosa* was reported by Jülich in 1982 and belongs to Basidiomycota. Clampless hyphae is a specific characteristic of the genus *Scopuloides* [19]. Based on sequence analysis, the ITS region had 96% similarity with *S. rimosa* KP135350.1. LSU also showed 99% similarity with *S. rimosa* KP135352.1 (Fig. 5).

In Mt. Baekryeonsan, seven taxa of endophytic fungi were isolated from *J. rigida*, eight taxa were isolated from *P. densiflora*, whereas only one taxa, *Annulohypoxyylon turcatum*, was isolated from



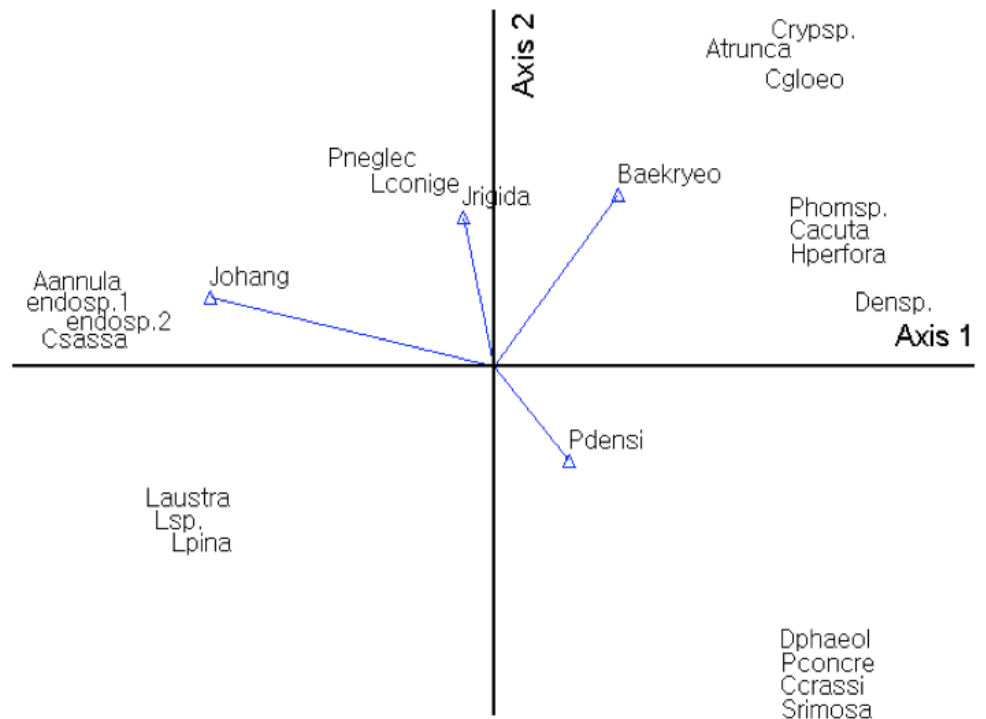
**Fig. 5.** Neighbor-joining phylogenetic tree based on a large subunit rDNA sequences. *Panus conchatus* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in bold.

both. In Mt. Johangsan, five fungal taxa were isolated from *J. rigida*, and four taxa were isolated from *P. densiflora*, but no common endophytic fungi were found.

*Lophodermium conigenum* was the most dominant endophytic fungi in *P. densiflora* in both mountains. *A. truncatum* was the dominant species isolated from *J. rigida* from Mt. Baekryeonsan, whereas at Mt. Johangsan, fungal endophyte sp. 2 was the dominant species.

Using Bray-Curtis ordination, endophytic fungi were analyzed according to host plants and locality. The results showed that the endophytic fungal communities varied depending on host plants and locality. *P. neglecta* and *A. annulatum* were affected by host plant (*J. rigida*) and locality (Mt. Johangsan). *Diaporthe phaeolorum* and *Colletotrichum gloeosporioides* were affected by host plant (*P. densiflora*) and locality (Mt. Baekryeonsan; Fig. 6).

*Lophodermium* spp. were reported as pathogens of needle cast in pine trees, and approximately 103 species have been found worldwide [20]. These pathogens are known to have a unique ecological habitat according to the host plant. *L. pinastri* was found in senescent leaves, *L. conigenum* was found in conifer twigs or leaves, and *L. seditiosum* was found in leaves of conifer seedlings. Not all *Lophodermium* species showed pathogenic properties; however, some have been shown to act as endophytic fungi in conifers. In Korea, *Lophodermium* spp. were found to be associated with needle-cast disease [21], and six species of *Lophodermium* (*L. austral*, *L. durilabrum*, *L. nitens*, *L. pinastri*, *L. pini-excelsae*, and *L. pini-pumilae*) and their relationships with host plants were reported. In



**Fig. 6.** Use of Bray-Curtis ordination to validate the effective factors that contribute the endophytic fungal community. Radiating lines are host plants and locality. Axis 1 and 2 have 90.16 % explanatory power.



previous biodiversity studies of endophytic fungi in conifer trees from Korea, *L. conigenum*, *L. pinastri*, and *Lophodermium* sp. were found in *P. densiflora*, *P. koraiensis*, *P. rigida*, and *P. thunbergii* [3, 10, 12, 13]. Thus, *Lophodermium* spp. are expected to be a crucial component of fungal endophytes in host plants, and additional comparative studies of the functional roles of endophytes and conifer host plants are needed.

## ACKNOWLEDGEMENTS

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