

## RESEARCH ARTICLE

# First Report on *Lophiostoma macrostomum* and *Pestalotiopsis lespedezae* Isolated from Conifer Leaves of *Pinus densiflora* in Korea

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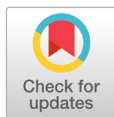
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## ABSTRACT

In this study, we isolated endophytic fungi from the conifer leaves of *Pinus densiflora* inhabiting Mt. Gyeohwasan in Korea. We identified the isolated fungal strains based on their morphological characteristics and phylogenetic analysis using DNA sequences of the internal transcribed spacer, small subunit rDNA, large subunit rDNA, and  $\beta$ -tubulin region. In the process of this study, we confirmed two endophytic fungi that have not been recorded previously in Korea, *Lophiostoma macrostomum* and *Pestalotiopsis lespedezae*. In this report, we described the morphological characteristics of these fungal strains and the results of their molecular analysis.

**Keywords:** Endophytes, *Lophiostoma macrostomum*, *Pestalotiopsis lespedezae*, *Pinus densiflora*



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## INTRODUCTION

Endophytic fungi have a symbiotic relationship with the plants [1]. They live in healthy tissues of the plants and do not cause disease unless there is a stressful condition in the plant [2,3]. However, they can protect the host plants from pathogens or predators through the production and secretion of secondary metabolites such as alkaloids [4,5]. In particular, since these metabolites can exhibit bioactive effects such as anti-proliferative or anti-microbial properties in plant as well as in human body [6,7], it is essential to confirm the diversity of endophytic fungi and secure the strains that can produce metabolites. In this study, we described the morphological characteristics and the results of molecular analysis of two unrecorded endophytic fungi isolated from the conifer leaves of *Pinus densiflora* S. et Z.

## MATERIALS AND METHODS

The sampling was conducted in April 2016, around Gyeohwasan mountain (N35°47'18.6", E126°37'56.1") in Buan-gun, Jeollabuk-do. We sampled healthy conifer leaves without any symptoms. Sampled leaves were placed in polyethylene bags and transported to the laboratory within 24 h. After washing the leaves with distilled water, they were sterilized in 1% NaClO solution for 1 min and 70% EtOH for 2 min, and then divided into appropriate sizes to place into potato dextrose agar (PDA) medium [8]. The culture medium was incubated for 3 d or more in a dark place at 25°C until the mycelium grew out, after which, it was subcultured in the fresh PDA medium using a scalpel to obtain pure culture. Morphological characteristics were observed on a dissecting microscope and an optical microscope after culturing for 7 d in PDA and malt extract agar (MEA) medium, respectively (Table 1). For the identification of fungal strains, genomic DNA was extracted from hyphae using the DNeasy plant mini kit (Qiagen, USA) according to the manufacturer's instructions, followed by amplification of the internal transcribed spacer (ITS) containing 5.8S region by PCR using ITS1F and ITS4 primers [9]. Then, the small subunit (SSU) region of rDNA, the large subunit (LSU) region, and the  $\beta$ -tubulin (TUB) region were amplified using NS1/NS4 [10], LR0R/LR16 [11], and Bt2a/Bt2b primer set [12], respectively. During PCR, the annealing temperature was set to 50, 40, 44, and 55°C for ITS, SSU, LSU and TUB, respectively. After the PCR, agarose gel electrophoresis was performed on 1.5% agarose gel for 20 min to confirm the size of each DNA fragment, followed by DNA sequencing (SolGent, Daejeon, Korea). We used BLAST on the National Biological Information Center (NCBI) to confirm the similarity of the species and presence of the reference DNA sequences (Table 2). The phylogenetic tree was constructed by using neighbor-joining method by concatenating DNA sequences of two or three regions, using a MEGA7 program [13]. The identified fungal strains were deposited to the National Institute of Biological Resources (NIBR, Incheon, Korea). The DNA sequences used for the identification were submitted to the NCBI by obtaining the accession number.

**Table 1.** Morphological characteristics of fungal strains isolated from conifer leaves of *Pinus densiflora*

Strain	<i>Lophiostoma macrostomum</i> 16B060	<i>Pestalotiopsis lespedezae</i> 16B115	<i>Pestalotiopsis lespedezae</i> [20]
Colony	PDA, 25°C, 7 days	PDA, 25°C, 7 days	PDA, 25°C, 7 days
Color	Bright gray; reverse white	Bright white	Pink to white; reverse pink to orange
Size	14-16 mm in diam.	38-40 mm in diam.	Growing 10-14 mm per day
Shape	Raised, slight aerial mycelium in center, margin crushed oval	Raised, cottony aerial mycelium, margin entire	Flat to raised, cottony, margin fimbriate
Conidia	Conidiophore globose to subglobose, develop into chlamydospore, hyaline, colorless, globose to cylindrical, 0-1 septate, (12.65-) 13.90 (-15.02) × (9.14-) 12.16 (-14.24) $\mu$ m in diam.	Pale brown, translucent, fusiform, 3-4 septate, (11.22-) 13.34 (-17.63) × (5.31-) 6.11 (-7.24) $\mu$ m in diam.	Pale brown, fusiform, curved, 3-4 septate, (20-25) × (6.3-10) $\mu$ m in diam.

**Table 2.** DNA sequences used in phylogenetic analysis, including sequences used as the outgroups

Species	OTU name	Genbank No.*			
		SSU	ITS	LSU	TUB
<i>Lophiostoma compressum</i>	KT534	JN941376.1	JN942962.1	JN941379.1	
<i>Lophiostoma macrostomoides</i>	CBS 123097	FJ795482.1	MH863108.1	FJ795439.1	
<i>Lophiostoma macrostomum</i>	KT508	AB618691.1	JN942961.1	AB619010.1	
<i>Lophiostoma macrostomum</i>	KT635	AB521731.1	MG572385.1	AB433273.1	
<i>Lophiostoma macrostomum</i>	KT709	AB521732.1	MH389072.1	AB433273.1	
<i>Lophiostoma macrostomum</i>	16B060	MT323102.1	MT323120.1	MT323121.1	
<i>Lophiostoma rosae</i>	TASM 6105	MG829125.1	MG828908.1	MG829018.1	
<i>Lophiostoma rugulosum</i>	CBS 123093	FJ795483.1	MH863273.1	FJ795440.1	
<i>Lophiostoma semiliberum</i>	KT622	AB618694.1	JN942966.1	AB619012.1	
<i>Lophiostoma semiliberum</i>	KT828	AB618696.1	JN942970.1	AB619014.1	
<i>Lophiostoma vagabundum</i>	CBS 628.86	FJ795485.1	AF383954.1	FJ795442.1	
<i>Lophiostoma vaginatipora</i>	d43	KJ591575.1	KJ591577.1	KJ591576.1	
<i>Pestalotiopsis arceuthobii</i>	CBS 434.65		KM199341.1		KM199427.1
<i>Pestalotiopsis diploclisia</i>	CBS 115587		KM199320.1		KM199419.1
<i>Pestalotiopsis hawaiiensis</i>	CBS 114491		KM199339.1		KM199428.1
<i>Pestalotiopsis lespedezae</i>	SY16E		EF055205.1		EF055242.1
<i>Pestalotiopsis lespedezae</i>	YY12A		EF055206.1		EF055243.1
<i>Pestalotiopsis lespedezae</i>	PN3DH		EF055203.1		EF055240.1
<i>Pestalotiopsis lespedezae</i>	16B115		MT323122.1		MT613344.1
<i>Pestalotiopsis malayana</i>	CBS 102220		KM199306.1		KM199411.1
<i>Pestalotiopsis oryzae</i>	CBS 353.69		KM199299.1		KM199398.1
<i>Pestalotiopsis papuana</i>	CBS 887.96		KM199318.1		KM199415.1
<i>Pestalotiopsis portugalica</i>	CBS 393.48		KM199335.1		KM199422.1
<i>Pestalotiopsis spathulata</i>	CBS 356.86		KM199338.1		KM199423.1
<i>Pestalotiopsis telopeae</i>	CBS 114137		KM199301.1		KM199469.1
<i>Preussia lignicola</i> (outgroup)	CBS 363.69	DQ384087.1	GQ203783.1	DQ384098.1	
<i>Seiridium podocarpi</i> (outgroup)	CBS 137995		KJ869150.1		LT853248.1

\*SSU: Small subunit rDNA; ITS: Internal transcribed spacer; LSU: Large subunit rDNA; TUB:  $\beta$ -tubulin DNA.

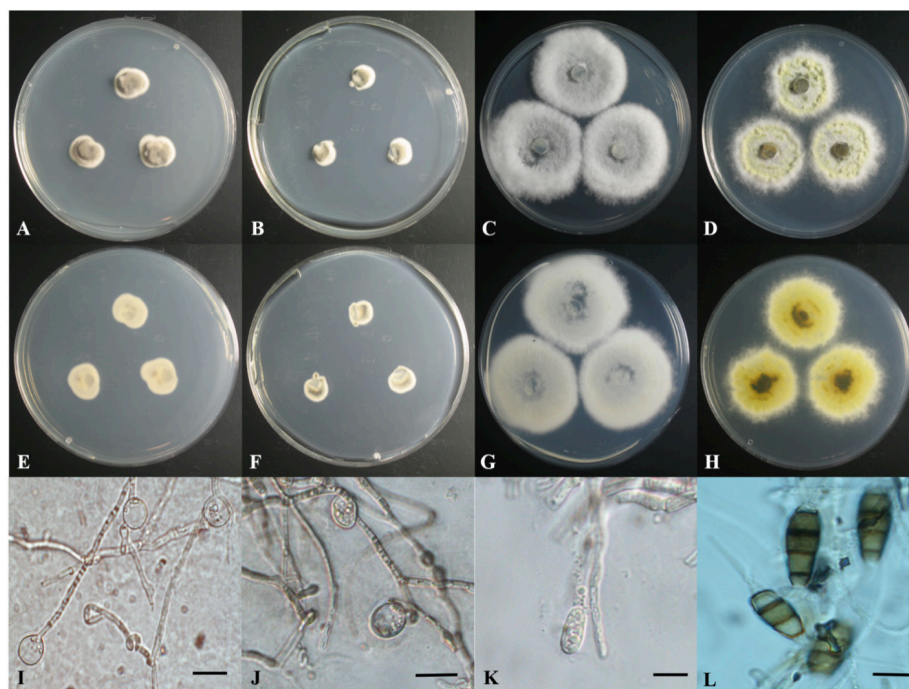
## RESULTS AND DISCUSSION

### *Lophiostoma macrostomum* (Tode) Ces. & De Not., Commentario della Società Crittogamologica Italiana 1:219 (1863) MB#149287

The colonies cultured for 7 d in the PDA medium had the diameters of about 14 to 16 mm. The top side of colony was observed to be bright gray, and the bottom side was observed to be white in color. The colonies were slightly elevated the medium, and their margin was close to a crushed oval. Aerial mycelium was slightly covered at the center of the colony (Fig. 1A and 1E). Diameters of the colonies cultured for 7 d in the MEA medium were about 11 to 12 mm, indicating slower growth than in the PDA. The color of the colonies was observed to be white on both the obverse and the reverse. The colonies were slightly raised upward the medium, and their margin was irregular (Fig. 1B and 1F). Conidiophores were circular, formed at the end of the hyphal growth (Fig. 1I), which swelled up to develop into a chlamydospore (Fig. 1J).

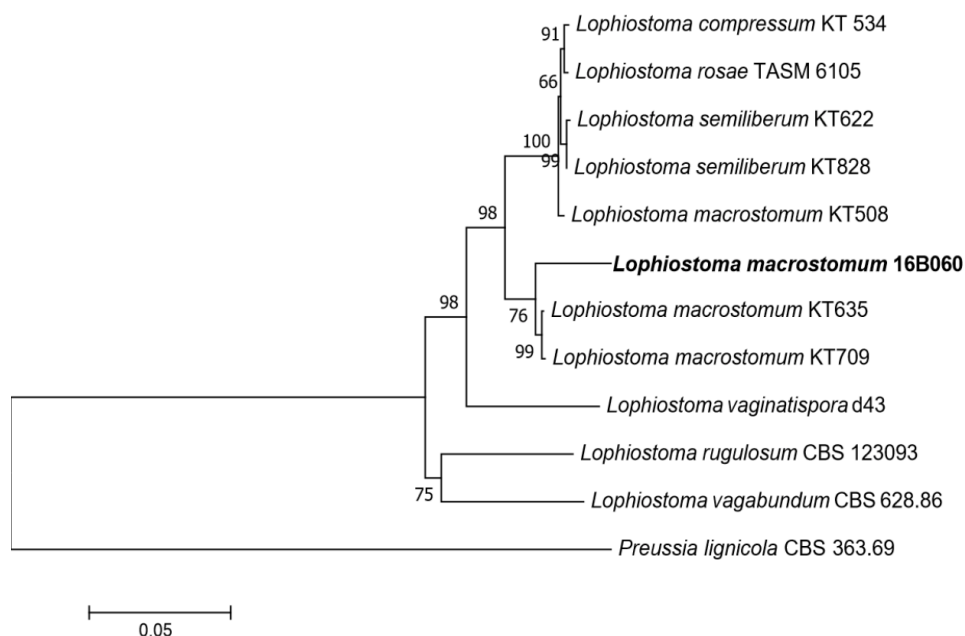
Chlamydo-spores were observed to be globose or cylindrical, hyaline, and colorless, with 0-1 septate, (12.65-) 13.90 (-15.02) × (9.14-) 12.16 (-14.24) μm in diameter (n=10).

**Specimen examined.** Mt. Gyehtwasan, Buan-gun, Jeollabuk-do, Korea, N35°47'19.4", E126°37'54.0", April 8, 2016, *Lophiostoma macrostomum*, isolated from conifer leaf of *Pinus densiflora*, strain 16B060, NIBRFG0000503375, GenBank Nos. MT323102 (SSU), MT323120 (ITS), and MT323121 (LSU).



**Fig. 1.** Colonies of strain 16B060 (*Lophiostoma macrostomum*) grown for 7 days on potato dextrose agar (PDA) (A, E) and malt extract agar (MEA) (B, F); Conidiophore and chlamydo-spore (I, J). Colonies of strain 16B115 (*Pestalotiopsis lespedezae*) grown for 7 days on PDA (C, G) and MEA (D, H); conidiophore and conidia (K, L). (scale bars: I, J=20 μm; K, L=10 μm)

**Notes.** *L. macrostomum* was first reported as a *Sphaeria macrostoma* by Tode in 1791 and became a type species of the genus *Lophiostoma* in 1863 when the genus *Lophiostoma* was suggested by Cesati & De Notaris [14]. Only the teleomorph forming the asci was recorded in the original literature and other references [14-16]. However, we confirmed anamorph and chlamydo-spores in this study. There is a record about this species isolated from the branches of mulberry (*Morus bombycis* Koidz.) as an endophytic fungus in Japan [17]. The BLAST result showed that the SSU rDNA sequence of 16B060 was closely related to that of *L. macrostomum* AB521731.1 with 98.92% similarity, the ITS sequence was closely related to that of *L. macrostomum* MH389072.1 with 99.40% similarity, and the LSU sequence was closely related to that of *L. macrostomum* EU552140.1 with 99.52% similarity. The concatenated DNA sequences of 16B060 also formed a monophyletic group with those of *L. macrostomum*, strains KT635 and KT709 (Fig. 2).



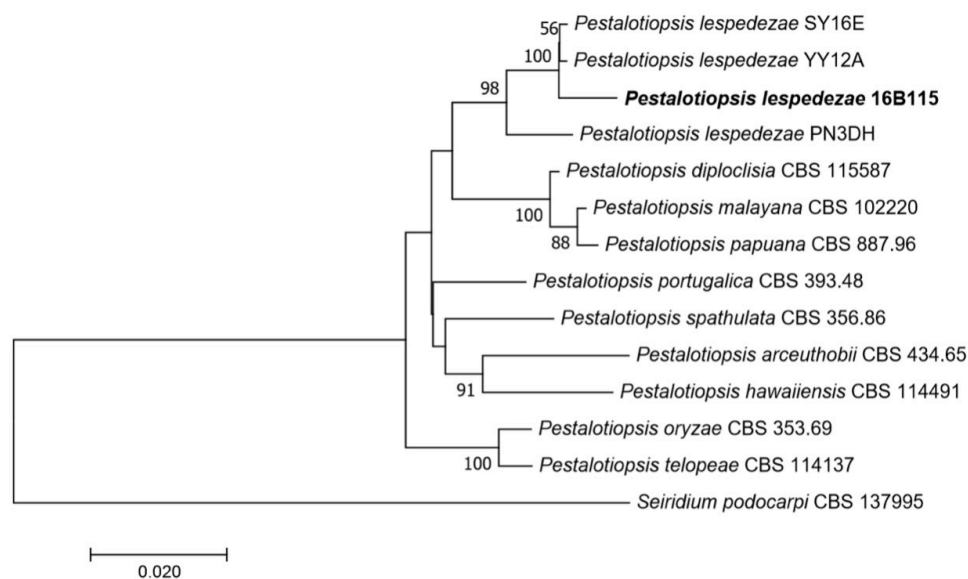
**Fig. 2.** Neighbor-joining phylogenetic tree based on a concatenated alignment of small subunit (SSU), internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequences. *Preussia lignicola* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in bold.

### ***Pestalotiopsis lespedezae* (Syd.) Bilgrami, Proceedings of the National Academy of Sciences of India 33:441 (1963) MB#336017**

The diameters of the colonies cultured for 7 d in PDA medium were about 38 to 40 mm, and the color was observed to be bright white on both the obverse and the reverse. The colonies were slightly raised upward the medium. The aerial mycelium was highly covered; therefore, the overall shape of the margin was close to the entire (Fig. 1C and 1G). The diameters of the colonies cultured for 7 d in the MEA medium were about 33 to 35 mm. The obverse of the colony was observed to be ivory at the center, and white at the margin. The reverse of the colony was observed to be dark yellowish-brown at the center, and yellow at the margin. The colonies were slightly raised upward the medium, and aerial mycelium spread irregularly toward the margin (Fig. 1D and 1H). At the distal end of the hyphal growth, the formation of cylindrical conidiophore was observed. Conidiophores were hyaline, with 1-2 septate (Fig. 1K), and developed into the fusiform conidia. Conidia were translucent, pale brown, with 3-4 septate, and the color of the middle cells appeared darker than the base (Fig. 1L),  $(11.22-13.34(-17.63)) \times (5.31-6.11(-7.24)) \mu\text{m}$  in diameter (n=20).

**Specimen examined.** Mt. Gyehwasan, Buan-gun, Jeollabuk-do, Korea, N35°47'19.4", E126°37'54.0", April 8, 2016, *Pestalotiopsis lespedezae*, isolated from conifer leaf of *Pinus densiflora*, strain 16B115, NIBRFG0000503376, GenBank No. MT323122 (ITS), MT613344(TUB)

**Notes.** *P. lespedezae* was first reported as *Pestalotia lespedezae* in 1900 and then recombined into genus *Pestalotiopsis* by Bilgrami in 1963 [18]. There is a report on *P. lespedezae* isolated as an endophytic fungus from the conifer leaves and bark of *Pinus armandii* in China [19]. The length of the conidia was slightly longer than that isolated in the present study; however, the color of the middle cells was darker than the base, and the fusiform conidia that divided by the septa were consistent with that isolated in this study [19]. It has been reported that *P. lespedezae* inhibits the growth of pathogenic fungi belonging to genus *Alternaria*, when cultured together in one medium [20]. The BLAST result showed that the ITS sequence was closely related to that of *P. lespedezae* EF055203.1 with 99.01% similarity and the  $\beta$ -tubulin DNA sequence was closely related to that of *P. lespedezae* EF055243 with 99.33% similarity. The concatenated DNA sequences of 16B115 also formed a monophyletic group with the concatenated sequences of *P. lespedezae*, strains SY16E and YY12A (Fig. 3).



**Fig. 3.** Neighbor-joining phylogenetic tree based on a concatenated alignment of internal transcribed spacer (ITS) and beta-tubulin (TUB) sequences. *Seiridium podocarpus* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in bold.

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