

Molecular Identification of Endophytic Fungi Isolated from Needle Leaves of Conifers in Bohyeon Mountain, Korea

Jae-Joon Yoo and Ahn-Heum Eom*

Department of Biology Education, Korea National University of Education, Cheongwon 363-791, Korea

(Received November 13, 2012. Revised December 3, 2012. Accepted December 7, 2012)

Fungal endophytes are microfungi that live in plants without causing apparent symptoms of infection. This study was conducted to identify endophytic fungi isolated from leaves of coniferous trees in Bohyeon Mountain of Korea. We collected leaves of two species of coniferous trees, *Pinus densiflora* and *Pinus koraiensis*, from 11 sites in the study area. A total 58 isolates were obtained and identified using molecular and morphological characteristics. Four species of endophytic fungi were isolated from *P. densiflora*: *Lophodermium conigenum*, *Leotiomyces* sp., *Septoria pini-thunbergii*, and *Polyporales* sp., while two fungal species were isolated from *P. koraiensis*: *Eurotiomyces* sp. and *Rhytismataceae* sp. The most frequently isolated species were *L. conigenum* and *S. pini-thunbergii*.

KEYWORDS : Fungal endophytes, *Lophodermium conigenum*, *Pinus densiflora*, *Pinus koraiensis*, *Septoria pini-thunbergii*

Introduction

Fungal endophytes are microfungi that colonize and live within healthy plant tissues without inducing symptoms of disease [1]. These organisms are thought to be ubiquitous among terrestrial plants, having been recovered from all major plant lineages in a wide range of terrestrial communities. Additionally, numerous investigations over the past three decades have shown that endophytes are abundant in asymptomatic leaves of many conifers [2-6]. Endophytes may offer significant benefits to their host plants by producing a plethora of substances that provide protection and survival value, such as enhancement of stress-, insect-, and disease-resistance, productivity improvement, and herbicide activity [7]. Accordingly, endophytes likely play an important role in ecological communities [8]. However, few studies of the diversity, host plant affinities, and environmental and medicinal effects of fungal endophytes in Korea have been conducted to date [9, 10]. Therefore, the present study was conducted to isolate and identify endophytic fungi from the leaves of coniferous trees on Bohyeon Mountain of Korea.

Materials and Methods

Sampling. The study area was Bohyeon Mountain, Kyeonbuk Province, Republic of Korea. A total of 11 plants of two coniferous species (five *Pinus densiflora*

and six *Pinus koraiensis*) were randomly selected at various altitudes on the mountain. The healthy leaves of the host plants were collected, packed into polyethylene bags and transported to the laboratory.

Fungal endophyte isolations. The surface of each coniferous leaf was washed with running tap water and treated with 1% NaOCl solution for 3 min and 70% ethanol for 2 min, after which it was washed with sterilized distilled water. Surface-sterilized samples were then cut into 1-cm sections and four segments per plate were cultured in water agar, potato dextrose agar (PDA), yeast mold agar, and malt extract agar. Plate media were sealed and incubated at 25°C for up to 8 wk, during which time they were checked often for hyphal growth. The mycelia were transferred to PDA for isolation and axenic culture.

DNA extraction and PCR. All isolates were grouped into morphotypes based on colony shape, height, and color of the aerial hyphae, as well as the base color, growth rate, margin characteristics, surface texture, and depth of growth into the medium [11, 12]. One or two isolates of each morphotype were then selected for molecular identification. DNA was extracted using a DNeasy Plant Mini kit according to the manufacturer's protocols (Qiagen Co., Valencia, CA, USA). PCR was then conducted to amplify the internal transcribed spacer (ITS) region of

*Corresponding author <E-mail : eomah@knue.ac.kr>

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Table 1. Molecular identification of endophytic fungi isolated from needles of *Pinus densiflora* and *Pinus koraiensis* in Korea

Isolate No.	ITS-5.8S		LSU rDNA		Identification
	Nearest match	Max identity (%)	Nearest match	Max identity (%)	
12A001	<i>Lophodermium</i> sp. (AB247944)	96	<i>L. conigenum</i> (HM140537)	99	<i>Lophodermium conigenum</i>
12A006	<i>Lophodermium</i> sp. (AB247944)	96	<i>L. conigenum</i> (HM140537)	99	<i>L. conigenum</i>
12A007	<i>Lophodermium</i> sp. (AB247944)	96	–	–	<i>L. conigenum</i>
12A009	<i>Lophodermium</i> sp. (AB247944)	96	–	–	<i>L. conigenum</i>
12A010	Fungal endophyte EMS38 (AY546074)	100	Leotiomycetes sp. (JQ759938)	99	Leotiomycetes sp.
12A012	<i>Lophodermium</i> sp. (AB247944)	96	<i>Lophodermium</i> sp. BLE32 (FN868860)	99	<i>L. conigenum</i>
12A013	<i>Lophodermium</i> sp. (AB247944)	96	<i>L. conigenum</i> (HM140537)	99	<i>L. conigenum</i>
12A016	<i>S. pini-thunbergii</i> (DQ019397)	100	–	–	<i>Septoria pini-thunbergii</i>
12A023	<i>S. pini-thunbergii</i> (DQ019397)	100	–	–	<i>S. pini-thunbergii</i>
12A024	<i>Lophodermium</i> sp. (AB247944)	96	<i>L. conigenum</i> (HM140537)	99	<i>L. conigenum</i>
12A026	Fungal endophyte 5683 (DQ979771)	92	Rhytismataceae sp. (HM595596)	97	Rhytismataceae sp.
12A028	<i>Lophodermium</i> sp. (AB247944)	96	<i>L. conigenum</i> (HM140537)	99	<i>L. conigenum</i>
12A029	<i>S. pini-thunbergii</i> (DQ019397)	100	–	–	<i>S. pini-thunbergii</i>
12A036	<i>S. pini-thunbergii</i> (DQ019397)	100	Dothideomycetes sp. (JQ760115)	98	<i>S. pini-thunbergii</i>
12A054	<i>S. pini-thunbergii</i> (DQ019397)	100	–	–	<i>S. pini-thunbergii</i>
12A057	Eurotiomycetes sp. (JQ759942)	99	<i>Phaeomoniella effuse</i> (GQ154618)	99	Eurotiomycetes sp.
12A060	<i>S. pini-thunbergii</i> (DQ019397)	100	–	–	<i>S. pini-thunbergii</i>
12A062	Polyporales sp. (EF687930)	99	–	–	Polyporales sp.

ITS, internal transcribed spacer; LSU, large subunit.

the extracted DNA, including the 5.8s rDNA, using the primers ITS1F and ITS4 [13] under the following conditions: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and final extension at 72°C for 5 min. In addition, 28s rDNA was amplified using the primers LR0R and LR16 by PCR under the following conditions: 94°C for 3 min followed by 36 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min, and final extension at 72°C for 10 min.

Sequencing and phylogenetic analysis. PCR products were sequenced by Solgent (Daejeon, Korea). The sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST searches. Phylogenetic analyses were conducted using the neighbor joining (NJ) methods in MEGA5 [14], and a NJ tree was constructed using the Tamura-Nei distance [15]. All characters were equally weighted and unordered. Gaps and missing data were treated as complete deletions. Support for specific nodes on the NJ tree was estimated by bootstrapping 1,000 replications.

Results and Discussion

A total of 58 fungal endophytes were isolated from 132 leaf fragments from 11 plants of the two species of conifers evaluated in this study. All the isolates were grouped into 18 morphotypes based on morphological characteristics such as colony shape, height, and color of aerial hyphae, base color, growth rate, margin characteristics

and surface texture. Molecular identification using ITS-5.8S and large subunit (LSU) rDNA sequences from the isolates revealed seven taxa of endophytic fungi: Eurotiomycetes sp., Leotiomycetes sp., *Lophodermium conigenum*, Polyporales sp., Rhytismataceae, and *Septoria pini-thunbergii* (Table 1). All of the isolated species belonged to Ascomycetes, except for isolate 12A062, which was a basidiomycetous fungi, *Polyporales* sp. Only two taxa, *L. conigenum* and *S. pini-thunbergii*, were identified at the species level with more than 99% sequence identifies based on both the ITS and LSU rDNA. However, four taxa showed sequences related at above the family level of fungi: Eurotiomycetes, Leotiomycetes, Polyporales and Rhytismataceae.

Based on analysis of the ITS sequences, ten sequences belonged to Leotiomycetes, most of which were closely related to the *Lophodermium* sp. (Fig. 1). Moreover, isolate 12A026 was grouped with *Coccomyces mucronatus* and isolate 12A010 was grouped with an undetermined taxon, fungal endophyte EMS38. Six sequences belonged to Dothideomycetes, and these were closely related to *Septoria pini-thunbergii* with 99% sequence similarity. The sequence of 12A057 was grouped with Eurotiomycetes, while isolate 12A062 was grouped with Polyporales and Agaricomycetes. These results provide evidence that some leaf endophytes are Basidiomycota, even though most endophytes of conifer leaves are filamentous Ascomycota [16, 17].

LSU rDNA sequences analysis and ITS rDNA were amplified and sequenced for ambiguous sequences (Fig.

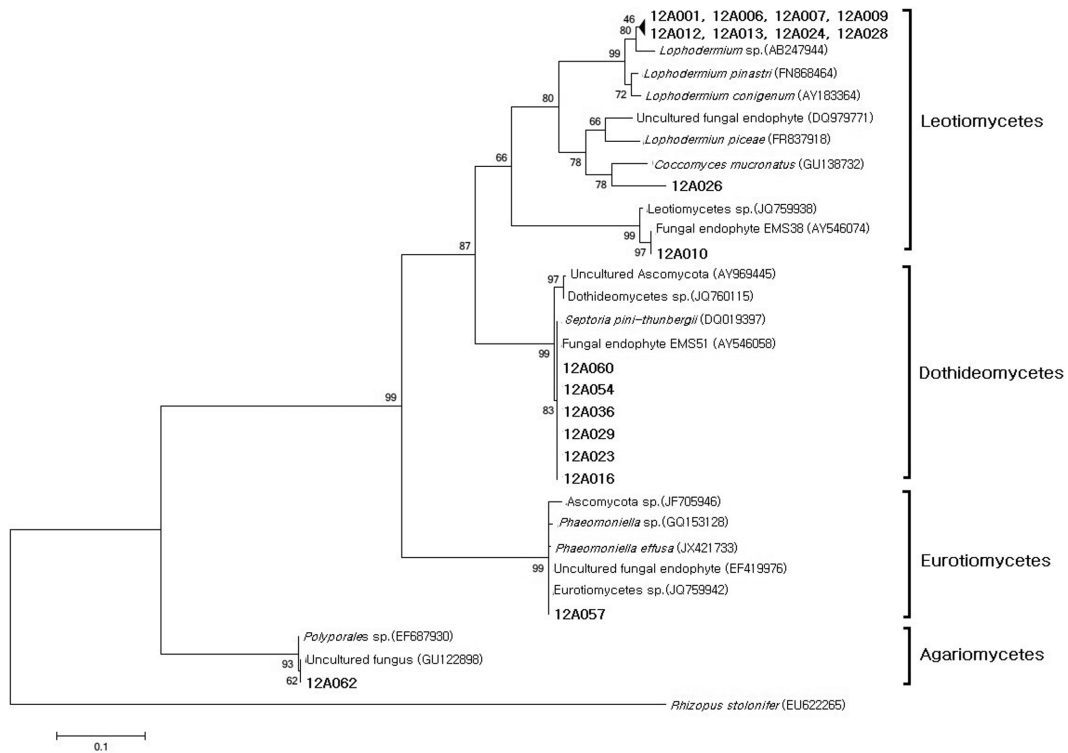


Fig. 1. Phylogenetic analysis based on the internal transcribed spacer-5.8S sequences showing the relationships of endophytic fungi isolated from conifer leaves with reference taxa. The tree shown was derived using the neighbor joining method.

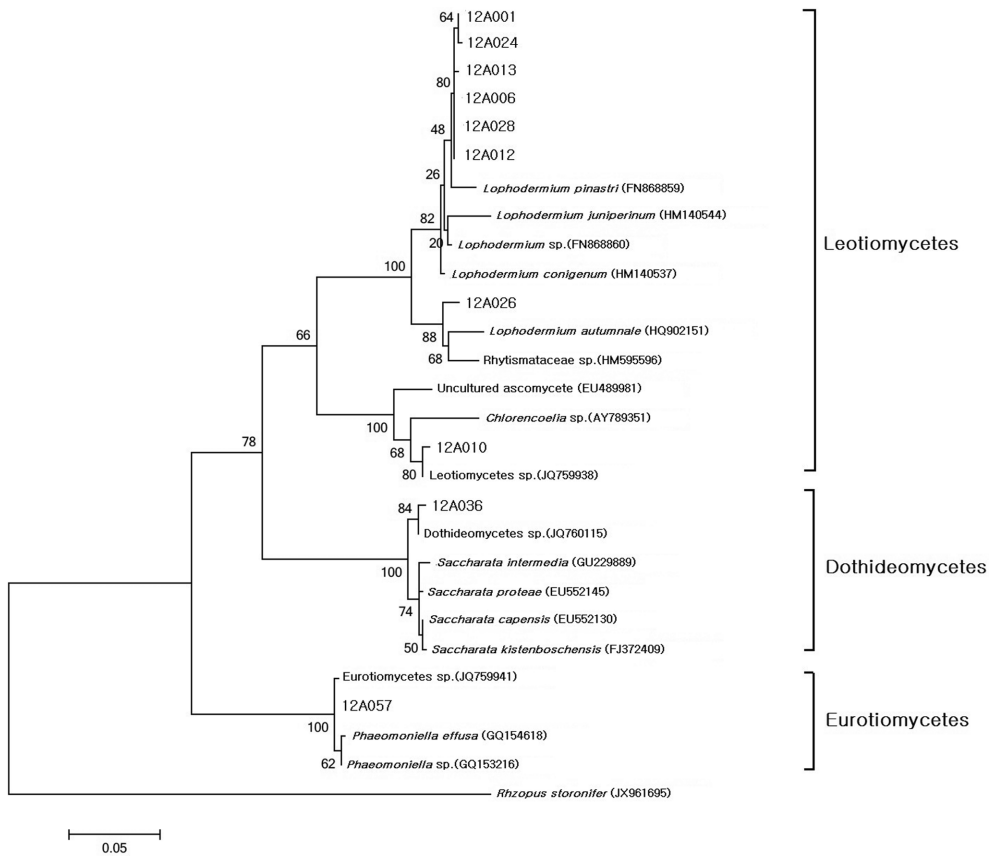


Fig. 2. Phylogenetic analysis based on partial large subunit rDNA sequences showing the relationships of endophytic fungi isolated from conifer leaves with reference taxa. The tree shown was derived using the neighbor joining method.

2). Isolates related to *Lophodermium* sp. based on the ITS sequence were classified as *L. conigenum* when the partial LSU rDNA sequence was used; therefore, we identified this organism as *L. conigenum*. Isolates 12A010 and 12A026 were closely related to fungal endophytes for ITS sequences (Table 1), but grouped with fungi that belonged to Leotiomyces and Rhytismataceae, respectively, based on their LSU sequences. Thus, these isolates as well as two other isolates, 12A057 and 12A062, could not be identified at the species or genus level.

Many studies have traditionally used sequence data from the ITS region to identify sterile cultures and evaluate morphotaxon boundaries [18, 19]. ITS data are considered useful for these purposes due to the rapid evolution of the ITS. However, most fungi are not represented in GenBank, and some GenBank records are misidentified or lack taxonomic information [6]. Therefore, BLAST and phylogenetic analyses of other genomic regions should be combined with those of the ITS region to improve the accuracy of identification. In this study, we selected representative isolates from morphotypes, and then conducted phylogenetic analysis based on ITS and partial LSU rDNA sequences. However, four out of six taxa could not be identified at the species level, indicating that these isolates might be new fungal endophytes. Accordingly, further taxonomic study of these organisms is necessary.

A total 58 endophytes were isolated from 132 leaf fragments in this study and the isolation frequency was 43.9%. Overall, 56 fungal endophytes were isolated from 72 leaf fragments of *P. densiflora*, while only two were isolated from 60 leaf fragments of *P. koraiensis* (Table 2), giving an isolation frequency of 77.8% for *P. densiflora* and 3.3% for *P. koraiensis*.

Four taxa were isolated from *P. densiflora*; *L. conigenum*, *Septoria pini-thunbergii*, Leotiomyces sp., and Polyporales sp., while two taxa, Rhytismataceae sp. and Eurotiomyces sp., were isolated from *P. koraiensis*. *L. conigenum* and *S. pini-thunbergii* were most dominant on the leaves of *P. densiflora*, and these taxa accounted for the largest proportion of fungal endophytes in the study site. Furthermore, these

organisms showed high affinity to *P. densiflora*, regardless of altitude. These findings are in accordance with the fact that coniferous endophytes are generally transmitted among hosts via contagious spread [6, 16]. Fungal endophytes isolated from *P. densiflora* were more diverse than those isolated from *P. koraiensis*, which suggests specificity between host plants and fungal endophytes.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2011-0014236).

References

- Petrini O. Fungal endophytes of tree leaves. In: Adrews JH, Hirano SS, editors. Microbial ecology of leaves. New York: Springer-Verlag; 1991. p. 179-97.
- Carroll GC, Carroll FE. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. Can J Bot 1978;56:3034-43.
- Stone JK, Bacon CW, White JF Jr. An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF Jr, editors. Microbial endophytes. New York: Marcel Dekker; 2000. p. 3-30.
- Kriel WM, Swart WJ, Crous PW. Foliar endophytes and their interactions with host plants, with specific reference to the gymnospermae. Adv Bot Res 2000;33:1-34.
- Müller MM, Valjakka R, Suokko A, Hantula J. Diversity of endophytic fungi of single Norway spruce needles and their role as pioneer decomposers. Mol Ecol 2001;10:1801-10.
- Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 2007;99:185-206.
- Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. J Nat Prod 2004;67:257-68.
- Gao XX, Zhou H, Xu DY, Yu CH, Chen YQ, Qu LH. High diversity of endophytic fungi from the pharmaceutical plant, *Heterosmilax japonica* Kunth revealed by cultivation-independent approach. FEMS Microbiol Lett 2005;249:255-66.
- Kil YJ, Eo JK, Eom AH. Molecular identification and diversity of endophytic fungi isolated from *Pinus densiflora* in Boeun, Korea. Kor J Mycol 2009;37:130-3.
- Kim CK, Eo JK, Eom AH. Diversity of foliar endophytic fungi isolated from *Lindera obtusiloba* in Korea. Kor J Mycol 2012;40:136-40.
- Edelstein L, Hadar Y, Chet I, Henis Y, Segel LA. A model for fungal colony growth applied to *Sclerotium rolfsii*. J Gen Microb 1983;129:1873-81.
- Photita W, Taylor PW, Ford R, Hyde KD, Lumyong S. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Divers 2005;18:117-33.
- White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors.

Table 2. Endophytic fungi isolated from needles of *Pinus densiflora* and *Pinus koraiensis* in Korea

Species	No. of isolates from the host plants		Relative abundance (%)
	<i>Pinus densiflora</i>	<i>Pinus koraiensis</i>	
Eurotiomyces sp.	–	1	1.7
Leotiomyces sp.	1	–	1.7
<i>Lophodermium conigenum</i>	41	–	70.7
Polyporales sp.	1	–	1.7
Rhytismataceae sp.	–	1	1.7
<i>Septoria pini-thunbergii</i>	13	–	22.4

- PCR protocols: a guide to methods and applications. New York: Academic Press; 1990. p. 315-22.
14. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28:2731-9.
 15. Nei M, Kumar S. *Molecular evolution and phylogenetics*. New York: Oxford University Press; 2000.
 16. Stone JK. *Foliar endophytes of Pseudotsuga menziesii (Mirb.) Franco. Cytology and physiology of the host-endophyte relationship [dissertation]*. Eugene: University of Oregon; 1986.
 17. Petrini O. *Taxonomy of endophytic fungi of aerial plant tissues*. In: Fokkema NJ, van den Heuvel J, editors. *Microbiology of the Phyllosphere*. Cambridge: Cambridge University Press; 1986. p. 175-87.
 18. Arnold A. *Neotropical fungal endophytes: diversity and ecology [dissertation]*. Tucson: University of Arizona; 2002.
 19. Lacap DC, Hyde KD, Liew EC. An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. *Fungal Divers* 2003;12:53-66.