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Research Article

Diversity and Seasonal Variation of Endophytic Fungi Isolated from Three Conifers in Mt. Taehwa, Korea

Chang-Kyun Kim, Ju-Kyeong Eo and Ahn-Heum Eom*

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

Abstract The needled leaves of three conifer species were collected in Mt. Taehwa during different seasons of the year. Total 59 isolates and 19 species of endophytic fungi were isolated from the leaves and identified using morphological and molecular characteristics. As a result, Shannon index was different in its host plant; *Larix kaempferi* had a highest value of species diversity. According to the sampling season, 9 species of 19 species were isolated during fall season. The results suggest that the existing of host plant and sampling season are major factors of distribution of endophytic fungi.

Keywords Conifers, Endophytes, Internal transcribed spacer, *Larix kaempferi*, Species diversity

Coniferous forests are considered to be in decline worldwide. Several factors are hypothesized to contribute to this decline, such as the geographical isolation of conifer species distribution areas, the destruction of conifer forests by humans, and polluted air conditions due to acid rain and climate change [1, 2]. Moreover, the decrease in conifer forest cover represents a global situation, with these trees and associated ecosystems being extremely important from the perspective of biological resources.

Endophytes are fungi that grow in the living tissues of plants, without causing any apparent disease [3]. Currently, our understanding about these organisms remains limited as it is difficult for researchers to isolate these organisms from host plants. Arnold *et al.* [4] analyzed endophytic fungi from *Pinus taeda* L. by using both the culture media method and PCR-cloning, with the latter technique proving more powerful. The results indicated that many species of endophytic fungi could not be isolated from their host plant; therefore, greater effort is required to detect their presence in plants, in parallel with a more taxonomical approach to validate their existence [4].

Only a few studies on the endophytic fungi of plants exist in Korea, several of which have been conducted on woody plants, including *Lindera obtusiloba* [5], *Pinus densiflora* [6, 7], and *Pinus koraiensis* [8]. In this study, we isolated endophytic fungi from 3 species of conifers growing on Mt. Taehwa in Korea, in addition to analyzing the biodiversity and seasonal variation in the numbers of these fungi.

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

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MATERIALS AND METHODS

Plant materials. The sampling site was in Mt. Taehwa, Danyang-gun, Chungcheongbuk-do, Korea (N 37°07'01.42", E 128°29'05.11"). Healthy leaves were collected from conifer species growing at the site, including 3 juniper trees (*Juniperus rigida* Siebold et Zucc.), 4 Japanese larch trees [*Larix kaempferi* (Lamb.) Carr.], and 5 pine trees (*Pinus densiflora* Siebold et Zucc.). Each tree was growing at a distance of at least 100 m from the other sampled trees between 400~800 m in altitude, and all trees were sampled using a GPS, an aluminium tag, and a tagging tape: April (spring), July (summer), and November (autumn).

Isolation of endophytic fungi. Samples were treated within 48 hr after collection. All the 2-yr-old leaves were washed with tap water and then placed for 3 min in 1% NaOCl solution, 2 min in 70% ethanol, and finally washed twice with distilled water [9]. These surface-sterilized leaves were cut into 4 segments that were 5 mm in length and then placed into 3 types of culture medium: potato

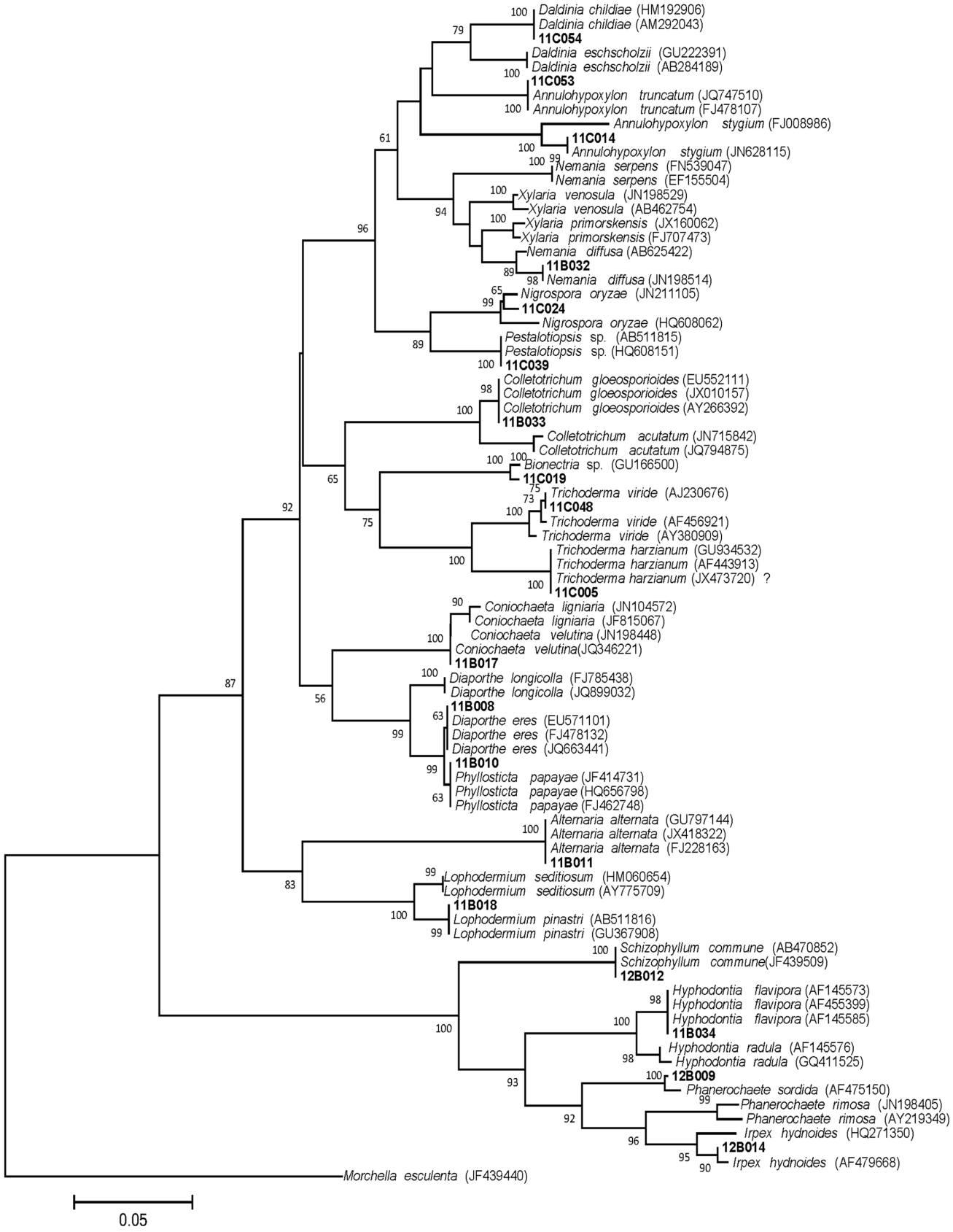

Fig. 1. Neighbor-joining phylogenetic tree showing relationship between endophytic fungi (bolds) from the present study and related fungi based on internal transcribed spacer sequences. *Morchella esculenta* was used as an outgroup and bootstrap values > 50% (1,000 replicates) are shown at the branches.

dextrose agar (PDA), malt extract agar, and water agar. Finally, the samples were cultivated in an incubator at 25°C and in darkness. Any hyphae that extended from the leaf fragments were used in a successive culture with PDA.

DNA extraction and data analysis. All isolates were grouped into morphotypes on the colony shape, height, and color of the aerial hyphae, in addition to the base color, growth rate, margin characteristics, surface texture, and depth of growth into the medium. One or 2 isolates of each morphotype were selected for molecular identification. DNA was extracted according to the protocol of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and PCR was performed to amplify the internal transcribed spacer (ITS) region, including 5.8S rDNA, by using the primers ITS1F and ITS4 [10] 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 a final extension at 72°C for 5 min. The PCR products were sequenced and compared with reference sequences on NCBI by using BLAST. MEGA5 [11] was used to construct the phylogenic tree with neighbor-joining analysis.

RESULTS AND DISCUSSION

A total of 59 morphotypes were isolated from the host plants (Table 1). Only morphotypes with a ≥ 97% similarity value [12] were used for analysis (Fig. 1). It was not possible to identify *Bionectria* spp. and *Pestalotiopsis* spp. to the species level based on sequence. Depending on host plants, 6 species were identified among the 12 morphotypes from the juniper trees, 8 species among 21 morphotypes from the Japanese larch, and 11 species among 26 morphotypes from the pine trees. One species was isolated from both the juniper tree and the Japanese larch, 3 species were isolated from both the Japanese larch and the pine tree, and 2 species were isolated from both the pine tree and the juniper tree. However, no species was isolated from all 3 host plant species. In addition, *N. diffusa* was the most abundant species in the juniper tree; *P. papaya*, the Japanese larch; and *L. pinastri*, the pine tree (Table 1).

The Shannon index (H′) [13] was used to assess the species diversity of the endophytic fungi (Table 1). In the juniper tree, the total H′ was 1.47, and the highest H′ (1.00) was observed in April. In the Japanese larch, the H′ was 1.74, and the highest H′ (1.33) was observed in November. In the pine tree, H' was 1.58, and the highest H′ (1.43) was observed in November. The Japanese larch showed the highest species diversity.

Depending on sampling season, 7 species were identified among the 9 morphotypes in April, 8 species among 21 morphotypes in July, and 9 species among 29 morphotypes in November. One species was isolated during both April and July, 4 species were isolated during both July and November, and 1 species was isolated during both April and November (Table 1).

More than 600,000 species of endophytic fungi are theorized to exist worldwide [14], and various scientific

Table 1. Relative frequencies of endophytic fungi identified depending on host species and sampling season

Strain No.	Closest taxa in Genbank (accession No.)	Relative frequencies (%)								
		Juniperus rigida			*Larix kaempferi*			*Pinus densiflora*		
		Apr	Jul	Nov	Apr	Jul	Nov	Apr	Jul	Nov
11B034	*H. flavipora* (FJ228163)	-	100	-	-	-	-	-	-	-
12B009	*P. sordida* (FJ478107)	-	-	-	-	-	-	33.3	-	-
12B014	*I. hydnoides* (FJ008986)	-	-	-	-	-	-	33.3	-	-
12B012	*S. commune* (GU166500)	-	-	-	-	-	-	33.3	-	-
11B011	*A. alternate* (AY266391)	-	-	-	-	12.5	-	-	-	-
11B010	*P. papayae* (JQ346221)	-	-	-	-	50.0	-	-	-	-
11B018	*L. pinastri* (HM192904)	-	-	-	-	-	-	-	25.0	42.1
11B033	*C. gloeosporioides* (FJ478132)	-	-	-	-	6.2	-	-	25.0	-
11B017	*C. velutina* (AF455399)	11.1	-	-	-	-	-	-	25.0	-
11B008	*D. eres* (FJ462768)	-	-	-	-	31.3	-	-	-	5.3
11B032	*N. diffusa* (GU367908)	66.7	-	-	-	-	-	-	25.0	10.5
11C014	*A. stygium* (AB625435)	-	-	-	-	-	-	-	-	5.3
11C053	*A. truncatum* (EU918714)	11.1	-	-	-	-	-	-	-	-
11C054	*D. childiae* (AB511815)	11.1	-	-	-	-	-	-	-	-
11C039	*Pestalotiopsis* sp. (JN253600)	-	-	-	-	-	40.0	-	-	31.6
11C019	*Bionectria* sp. (FJ462748)	-	-	-	-	-	20.0	-	-	-
11C005	*T. harzianum* (AB470852)	-	-	100	-	-	20.0	-	-	-
11C048	*T. viride* (GU934533)	-	-	-	-	-	20.0	-	-	-
11C024	*N. oryzae* (AJ230676)	-	-	-	-	-	-	-	-	5.3
Total isolates		9	1	2	0	16	5	3	4	19
Shannon diversity index (H')		1.00	0	0	0	1.14	1.33	1.09	1.38	1.43
Species richness		4	1	1	0	4	4	3	4	6

approaches have been used to detect endophytic fungi to date. In the present study, H′ was the highest in the Japanese larch, with the greatest number of endophytes being isolated during July and November. These results indicate that endophytes do not only exist in the leaf and/or do not use horizontal transfer; instead, endophytes might extend from the plant tissue like a branchlet. During July and August, the climate in Korea is warm and humid; hence, climate probably affects endophytic dispersal [15]. These results also indicate that the number of morphotypes belonging to endophytic fungi increases across the season. Therefore, it is likely that a combination of these factors enhanced the species diversity of the Japanese larch.

J. rigida is mainly distributed in lower altitude forests, while *L. kaempferi* is primarily distributed in middle altitude forests. However, *P. densiflora* is found at higher altitudes, where the forest conditions are cooler and drier in the Korean Peninsula. Thus, most endophytic fungi were obtained from lower to middle altitudes, with only one species of endophytic fungi being discovered at an altitude above 800 m. This observation indicates that endophyte distribution is influenced by the distribution of host plants. Surveys for endophytic fungi have been conducted at all altitudes, with specimens being found at all sites; however, endophytic fungi have an ability to adapt to variations in abiotic and biotic conditions along the altitudinal gradient [16]. Thus, standpoints of host specificity and adaptation ability are required, which is only possible through the collection and study of endophytes as ecological components and biological resources.

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