

Two Endophytic *Diaporthe* Species Isolated from the Leaves of *Astragalus membranaceus* in Korea

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Abstract We characterized two endophyte fungi from the leaves of *Astragalus membranaceus* in Korea. The isolated strains were identified on the basis of the morphological characters and sequences analysis of the internal transcribed spacer and large subunit regions of the rDNA and β -tubulin gene. To the best of our knowledge, this is the first report of *Diaporthe oncostoma* and *Diaporthe infecunda* in Korea, and we have provided descriptions and figures.

Keywords *Astragalus membranaceus*, *Diaporthe infecunda*, *Diaporthe oncostoma*, Endophytic fungi

Diaporthe Nitschke, including the asexual state of *Phomopsis* (Sacc.) Bubak, is the one of the largest genera of Ascomycota and is composed of up to 2,000 species names [1]. *Diaporthe* species have been reported to be endophytes and saprobes [2], but also, they are economically important plant pathogens with a wide range of host plants worldwide [3]. *Diaporthe* species are ubiquitous endophytes of many host plants [4]. Endophytic fungi colonize plant tissues, without causing any apparent disease symptoms [5, 6]. Although the fungi are potentially pathogenic, their pathogenicity was suppressed during the host plant is healthy [5]. They form a symbiotic relationship with a wide range of host plants worldwide and provide benefits to the host plants [7]. They help in seed germination [8], provide pathogen resistance to the host plants, and deter herbivory by producing various enzymes and secondary metabolites [6, 9, 10].

Astragalus membranaceus Bunge is a perennial herb that belongs to the family Fabaceae and is mainly grown in China and Korea [11, 12]. Its roots contain various biologically active substances that has been used as a cardiogenic, diuretic,

and vasodilator [13, 14]. Recently, substances extracted from *A. membranaceus* have shown anticancer effects [15] and boosted the immune system [16]. We isolated endophytic fungi colonizing *A. membranaceus* and identified them on the basis of morphological characteristics and rDNA and β -tubulin gene sequence analysis. In this study, we have reported two *Diaporthe* species isolated from the roots of *A. membranaceus* that have not been reported in Korea.

Sampling. Leaves of *A. membranaceus* were collected from a cultivated field in Jecheon, Korea (37°15' N, 128°18' E). Healthy leaves without any disease symptoms were selected and transported to the laboratory in polyethylene bags within 24 hr of sampling.

Isolation of endophytic fungi. The leaf samples were cleaned with tap water and surface-sterilized with 30% H₂O₂ solution. The leaf samples were cut into 5 × 5-mm squares and placed in petri dishes containing potato dextrose agar (PDA). The petri dishes were sealed and incubated at 25°C and examined periodically. The hyphae growing out from the root fragments were transferred to new petri dishes with PDA and incubated at 25°C. The isolates were stored in 20% glycerol at -80°C at the Mycology Laboratory of Korea National University of Education, Cheongju, Korea, and deposited as a glycerol stock at the Culture Collection of National Institute of Biological Resources, Incheon, Korea.

Morphological characterization. The growth characteristics of the fungal colonies, such as color and diameter, were recorded after incubation on PDA at 25°C under dark conditions for 7 days. The conidiophores and conidia were examined under a light microscope (AXIO Imager A1; Carl Zeiss, Oberkochen, Germany).

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Phylogenetic analysis. Genomic DNA was extracted from the fungal mycelia by using the Exgene Plant SV mini kit (GeneAll, Seoul, Korea), according to the manufacturer's protocols. The internal transcribed spacer (ITS) region of rDNA was amplified using primers ITS1F and ITF4 [17] and the large subunit (LSU) region of rDNA was amplified using primers LR0R and LR16 [18]. In addition, primers Bt2a and Bt2b was used to amplify β -tubulin (*TUB*) gene [19]. The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C (ITS), 44°C (LSU) or 58°C (*TUB*) for 30 sec, 72°C for 1 min, and final extension at 72°C for 5 min. The amplicons were sequenced by SolGent (Daejeon, Korea). The sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST. Phylogenetic analysis was conducted using the neighbor-joining method in MEGA6 [20].

***Diaporthe oncostoma* (Duby) Fuckel, Jahrb. Nassau. Ver. Naturkd. 23–24: 205 (1870) (Fig. 1).**

The colony grown on PDA at 25°C for 14 days was 35–40 mm in diameter (Fig. 1A, Table 1). The surface of the colony

was flat and smooth with no exudate. Dense mycelial growth was observed, with aerial growth at the margin. The color of the colony was white at the center and reddish brown at the margin. The reverse side was yellowish green at the center and reddish brown at the margin. The colony on malt extract agar (MEA) for 7 days was 30–35 mm (Fig. 1B). The surface of the colony was convexly raised at the center. The conidium was hyaline, glassy cylindrical with a septum, and was bent in a half-moon shape (Fig. 1C). Its size was (8–32) × (3–5) μ m.

Specimen examined: Korea, Chungcheongbuk-do, Jecheon-si, 37°15' N, 128°18' E, 14 Aug 2015, isolated from healthy leaves of *Astragalus membranaceus*, strain 15C507 (GenBank No. MF547406, NIBR No. NIBRFG0000499911).

Note: Fuckel [21] has simply characterized the ascospores of this species in the original description. Gomes *et al.* [1] described *D. oncostoma* specimens isolated from dead branches or leaves of *Robinia pseudoacacia* in France, Germany, and Russia and reported two types of conidia, bent oblong alpha conidia and fusiform beta conidia; however, only alpha conidia were found. This species has

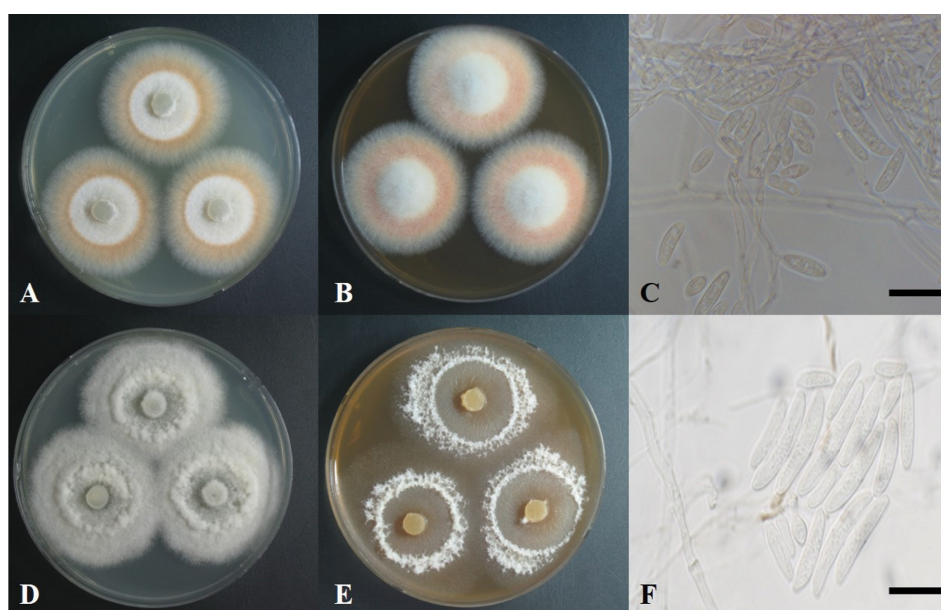


Fig. 1. Colonies of *Diaporthe oncostoma* grown on potato dextrose agar (PDA) (A), malt extract agar (MEA) medium (B), conidia (C); *Diaporthe infecunda* grown on PDA (C), MEA medium (E), conidia (F) (scale bars: C, F = 20 μ m).

Table 1. Comparison of morphological characteristics of *Diaporthe oncostoma* 15C507 with original descriptions

Characteristics	<i>Diaporthe oncostoma</i> 15C507	<i>Diaporthe oncostoma</i> [1, 21]
Colony	PDA, 25°C, 14 days	PDA, 25°C, 14 days
Color	Surface white with aerial mycelium, reverse core yellowish green, periphery umber	Surface dirty white with aerial mycelium, reverse umber
Size	30–35 mm in diam	35–40 mm in diam
Shape	The edges are uneven radial, mycelium dense	Sparse aerial mycelium
Conidia	Hyaline, 1-septate, bent cylindrical, (8–32) × (3–5) μ m	Alpha conidia: hyaline, fusoid to ellipsoidal, straight to slightly curved, (7.5–)9–11(–12) × (2–)3(–4) μ m

PDA, potato dextrose agar.

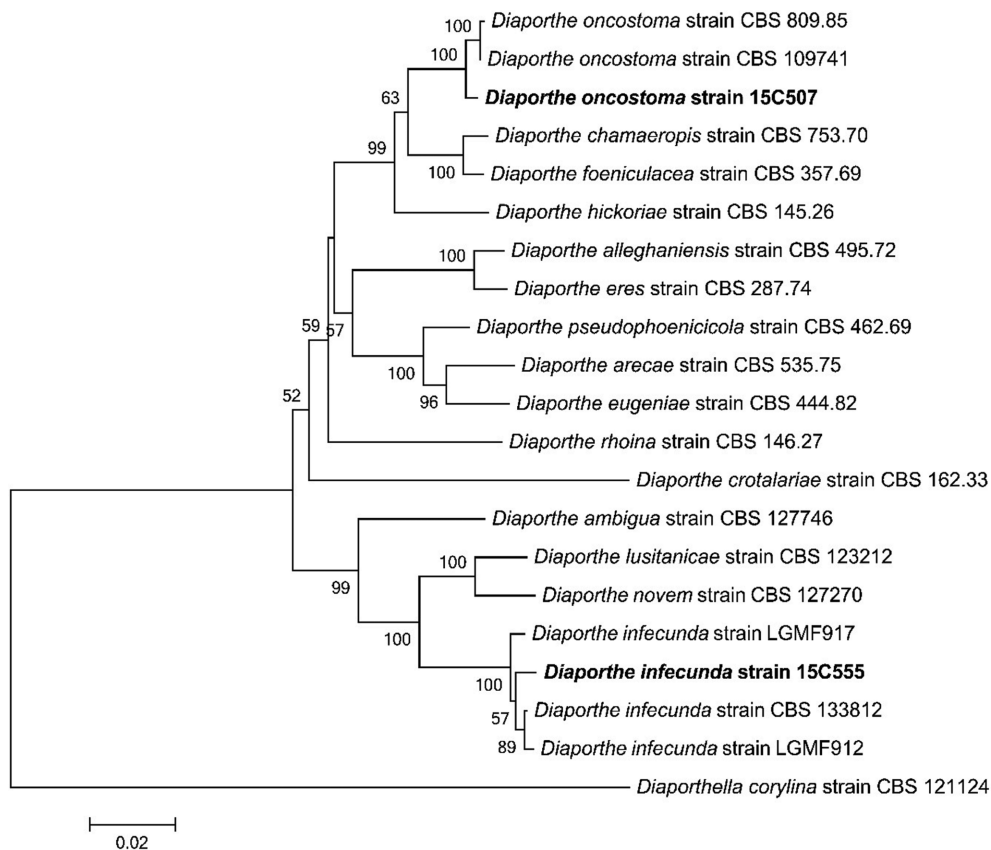


Fig. 2. Neighbor-joining tree of *Diaporthe oncostoma*, *Diaporthe infecunda* isolates based on a combined alignment of sequences of internal transcribed spacer, large subunit region of rDNA, and β -tubulin. *Diaporthella corylina* was used as an outgroup. Bootstrap values > 50% are provided above branches. The scale bar indicates the expected number of substitutions per nucleotide.

been considered a saprotrophic or weak parasitic fungus, but also, reported as a pathogen that causes stem canker in *R. pseudoacacia* in Russia and Greece [22]. Based on a Blast search of NCBI GenBank database, the closest matches for the ITS, LSU, and TUB sequences of *D. oncostoma* 15C507 were a fungal strain CBS 100454 [1] isolated from leaf spot of *R. pseudoacacia* in Germany (KC343160 with 97% similarity, KC343160 with 98% similarity and KC344128 with 98% similarity, respectively). The association with *Diaporthe* was confirmed by the phylogenetic inference of combined sequences of ITS, LSU, and TUB. The isolate 15C507 was clustered together with *D. oncostoma* CBS100454 (Fig. 2).

***Diaporthe infecunda* R. R. Gomes, C. Glienke & Crous, *Personia* 31: 1 (2013) (Fig. 1).**

The colony grown on PDA at 25°C for 14 days was 40–45 mm in diameter (Fig. 1D, Table 2). The surface of the colony was rough with no exudate. Loose mycelial growth was observed. The colony was white and convexly raised at the center. The reverse side was hyaline at the center and dark cream or beige at the margin. The colony grown on MEA for 7 days was 30–35 mm in diameter (Fig. 1E). The surface of the colony was flat, hyaline at the center, and white at the margin. The conidia was hyaline, glassy oblong with a septum (Fig. 1F) and its size was (9–32) × (2–6) μ m.

Table 2. Comparison of morphological characteristics of *Diaporthe infecunda* 15C555 with original descriptions

Characteristics	<i>Diaporthe infecunda</i> strain 15C555	<i>Diaporthe infecunda</i> [1]
Colony	PDA, 25°C, 14 days	PDA, 25°C, 14 days
Color	Mycelium white on the whole, reverse dirty cream to beige	Surface umber with patches of white, reverse chestnut
Size	30–35 mm in diam	35–40 mm in diam
Shape	The edges are uneven, rough surface, arranged in concentric rings	Arranged in concentric rings, richly sporulating on the aerial mycelium
Conidia	Hyaline, ellipse, 1-septate, (9–32) × (2–6) μ m	Conidia was not confirmed

PDA, potato dextrose agar.

Specimen examined: Korea, Chungcheongbuk-do, Jecheon-si, 37°15' N, 128°18' E, 8 Aug 2014, isolated from healthy leaves of *Astragalus membranaceus*, 15C555 (GenBank No. MF547407, NIBR No. NIBRFG0000499912).

Note: Gomes *et al.* [1] first described this species isolated from the leaves of *Schinus terebinthifolius* and *Maytenus ilicifolia*, which are medicinal plants found in Brazil. This fungus has been reported as an endophyte and is sterile. Based on a BLAST search of NCBI GenBank database, the closest matches for the ITS and LSU and TUB sequences of *D. infecunda* 15C507 were an endophytic strain LGMF917 for ITS and LSU and CBS133812 for TUB [1] isolated from leaf of *Schinus terebinthifolius* in Brazil (KC343129 with 99% similarity, KC343126 with 99% similarity, and KC344094 with 98% similarity, respectively). The association with *Diaporthe* was confirmed by the phylogenetic affinity of combined sequences of ITS, LSU, and TUB. The isolate 15C555 was clustered together with *D. infecunda* KGMF917 (Fig. 2).

A. membranaceus is an important medicinal plant in Korea. However, endophytic fungi for this plant have not been studied in Korea. In addition, some studies report that endophytic fungi produce secondary metabolites with anti-inflammatory activities. Therefore, two *Diaporthe* species isolated from *A. membranaceus* and described as new records can contribute to the knowledge of diversity of endophytic fungi in Korea and further efforts to identify endophytic fungi in medicinal plants will allow a better understanding of the relationship between medicinal plants and their endophytic fungi.

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