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 mebranaceus, Diaporthe infecunda, Diaporthe oncostoma, Endophytic fungi

Diaporthe Nitschke, including the asexual state of Phomopsis (Sacc.) Bubak, is 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 cardiotonic, 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).
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) μm.

**Specimen examined:** Korea, Chungcheongbuk-do, Jecheon-si, 37°15’ N, 128°18’ E, 14 Aug 2015, isolated from healty 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
Kim et al. have 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* 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).

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

| Characteristics | *Diaporthe infecunda* strain 15C555 | *Diaporthe infecunda* [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) μm | Conidia was not confirmed |

*PDA,* potato dextrose agar.

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**Diaporthe infecunda** R. R. Gomes, C. Glienke & Crous, *Persoonia* 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) μm.
Specimen examined: Korea, Chuncheongbuk-do, Jcheon-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 endophytic strains LGMP917 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* KGMP917 (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 record 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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