한 국 균 학 회 지 The Korean Journal of Mycology Research Note

A Note on a Dark Septate Endophyte *Phialocephala piceae* Isolated from Needle Leaves of *Thuja koraiensis* in Korea

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ABSTRACT : A dark septate endophytic fungal strain 13E043 was isolated from the needle leaves of *Thuja koraiensis* from a forest in Korea. Morphological characteristics of conidia and phialids. Along with a molecular phylogenetic analysis based on the internal transcribed spacer region of rDNA, the isolate was identified as *Phialocephala piceae* (also known as *Phaeomollisia piceae*). This is the first report of a dark septate endophyte isolated from the foliage of conifer trees in Korea.

KEYWORDS : Conifer, Dark septate endophyte (DSE), *Phialocephala piceae*, *Thuja koraiensis*

Phialocephala piceae Rossman is a fungus belonging to the order Helotiales of the phylum Ascomycota. It is found in Canada, Lithuania, Sweden and Switzerland, in association with the living foliage of *Picea abies*, *P. glauca*, *P. mariana*, *P. rubens*, *Pinus strobus* and decomposing branches or wood of *Acer saccharum* [1]. *Phialocephala piceae* was previously classified under the genus *Phaeomollisia*, erected by Grünig et al. [2], but recently moved to *Phialocephala piceae*, mainly based on the phylogenetic placement within a clade of *Phialocephala* [3, 4].

Many species of *Phialocephala* consist of an unique endophyte group with dark septate hyphae, namely dark septate endophytes (DSEs), which are ubiquitous colonizers of healthy plant tissues, mostly plant roots without inducing disease symptoms [1]. DSEs exhibit a worldwide distribution, but are especially abundant in stressed environments, suggesting a symbiotic association with their host plants. It has been demonstrated that *Phialocephala* species are the most dominant DSEs in roots and above ground tissues of conifers in heathland, forest and alpine ecosystems across northern temperate and boreal regions [5]. The interactions between *Phialocepala* species and their host plants range from potential pathogenic, to neutral or mutualistic association [6].

Most studies on endophytic fungi inhabiting conifers have been conducted in boreal and temperate regions of Europe and North America since 1970s [7]. However, in Korea, there is no report, to the best of our knowledge, for the dark septate fungi, although a few studies have reported diverse endophytic fungi to date [8-11]. Current climate changes, especially global warming, have resulted in the gradual decline of coniferous forest. In addition, potential symbionts of conifers such as endophytes and mycorrhizal fungi have declined [12, 13]. Therefore, it is important to study the biodiversity of endophytes and the relationship with their hosts for the conservation of the coniferous forest. In this study, we isolated a DSE from the needle leaves of *Thuja koraiensis* Nakai in a forest of Mt. Hwaak, Korea and identified the isolate as *Phialocephala piceae*. Here, we have described the morphological and phylogenetic characteristics of this isolate.

Kor. J. Mycol. 2016 December, **44**(4): 338-341
https://doi.org/10.4489/KJM.2016.44.4.338
pISSN 0253-651X • eISSN 2383-5249

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Received September 21, 2016
Revised September 23, 2016
Accepted September 26, 2016

Isolation of the fungal strain

Needle leaves were harvested from *T. koraiensis* (altitude 1,468 m; N 37° 59′, E 127° 25′) in a mixed forest. The healthy leaves of the host plants were collected, packed into polyethylene bags and transported to the laboratory. The surface of each needle 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 leaves were then cut into 1 cm sections and four segments per plate were cultured in potato dextrose agar (PDA). Plate media were sealed and incubated at 25°C for up to 8 wk, during which time they were checked every day for hyphal growth. The mycelia were transferred to PDA for isolation and axenic culturing. Isolates were stored in 20% glycerol at -80°C at the Mycology Laboratory of Korea National University of Education (Strain 13E043), Cheongju, Korea and deposited as glycerol stock at the Culture Collection of National Institute of Biological Resources (NIBR), Incheon, Korea, with an accession number of NIBRGR 0000194910.

Morphological characterization

Fungal isolates were cultured on each of two different media, PDA and malt extract agar (MEA), to determine morphological characteristics. Growth characteristics of fungal colonies, such as color, size, and diameter, were determined after incubation at 25°C under dark conditions for 7 days. Fungal structures of conidia, phialides and hyphae were examined under a light microscope (AXIO Imager A1; Carl Zeiss, Oberkochen, Germany).

Taxonomy of isolate 13E043

Phialocephala piceae (T.N. Sieber & C.R. Grünig) Rossman, IMA Fungus 5:104. 2014

≡ *Phaeomollisia piceae* T.N. Sieber & C.R. Grünig, Mycol. Res. 113:215. 2009 (Basionym)

Morphological characteristics were examined on PDA (Fig. 1A, 1B) and MEA (Fig. 1C, 1D) after 7 days of incubation at 25°C and compared with original description by Grünig et al. [2]. The diameters of the colonies was 32~33 mm on PDA and 26 mm on MEA. Colony color was olive in the center, pale olive at the margin, and the reverse was dark olive on PDA. Colonies on MEA were olive and the reverse was black. Aerial mycelium was dense. Colony color was slightly different from original description by Grünig et al. [2] due to the different growth conditions (Table 1). Conidiophores were dark brown and

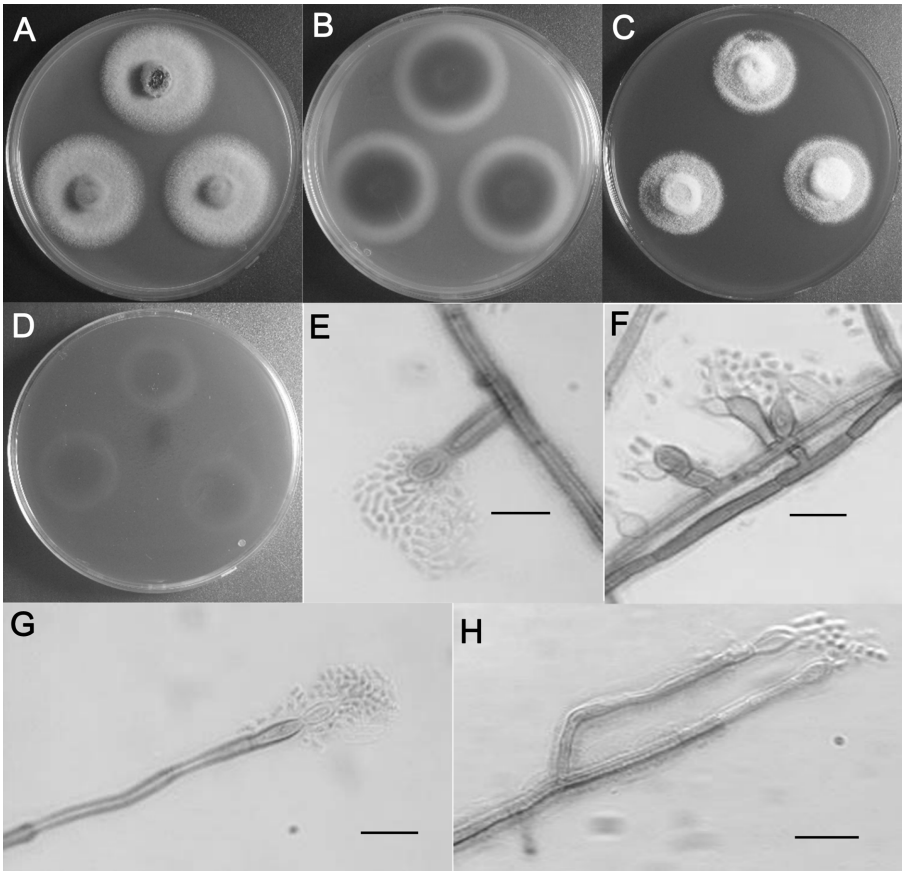

Fig. 1. Morphological characteristics of *Phialocephala piceae* 13E043 grown for 7 days at 25°C. A, colonies on PDA; B, colonies reverse on PDA; C, colonies on MEA; D, colonies reverse on MEA; E~H, conidia, phialides and conidiophores; PDA, potato dextrose agar; MEA, malt extract agar (scale bar = 10 um).

Table 1. Morphological characteristics of *Phialocephala piceae*

Characteristics	*Phialocephala piceae* strain 13E043	*Phialocephala piceae*[a]
Colony	MEA, 25°C, 7 days	MEA, 20°C, 21 days
Color	olive green, reverse of colony black	grey to olive, dark grey in center, reverse of colony black-olive
Size	26 mm in diam	50~60 mm in diam
Shape	aerial mycelium dense, cottony, convex	aerial mycelium dense, 2~3 mm high, lanose, felty
Conidiophores	dark brown, 41~60 um in length	unbranched or branched
Phialide	light brown, 5.7~9.3 × 2.6~7.2 μm including 1.7~3.5 μm long collarettes; long slender, 14.6~16.8 × 2.4~3.9 μm, including 2 × 1.4~2 μm collarettes	light brown, short thick, 7~13 × 3.5~4.5 μm, including 1.5~3.5 μm long collarettes; long slender, 17~22 × 2.5 μm, including collarettes 3~4 × 2~2.5 μm
Conidia	spherical, hyaline, 0.8~2.4 um in diam; conical, hyaline, 1.6~3 μm	spherical, hyaline, 1.5~2 μm in diam; conical with a truncate base, hyaline, 3.7 ~2 μm

MEA, malt extract agar.
[a]Original Description by Grünig et al. [2].

41~60 μm in length (Fig. 1). Two types of phialides were observed. Short thick phialides were 5.7~9.3 × 2.6~7.2 μm, including 1.7~3.5 μm long collarettes. Long slender phialides were 14.6~16.8 × 2.4~3.9 μm, including 2 × 1.4~2 μm collarettes. Conidia were also produced in two different types; spherical conidia were hyaline, 0.8~2.4 μm in diameter and conical conidia were hyaline and 1.6~3 μm in diameter.

Phylogenetic analysis

Fungal DNA was extracted using Exgene Plant SV mini kit (GeneAll, Seoul, Korea) according to the manufacturer's protocols. PCR was performed to amplify the internal transcribed spacer (ITS) region of rDNA, including the 5.8S region, with the primers ITS1F and ITS4 [14] 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. PCR products were sequenced by Solgent (Daejeon, Korea). The sequence was deposited in NCBI GenBank (accession number KX868076) and compared with those available in GenBank via the BLAST search function.

Phylogenetic analysis was conducted using the maximum likelihood (ML) method in MEGA7, with the Tam-

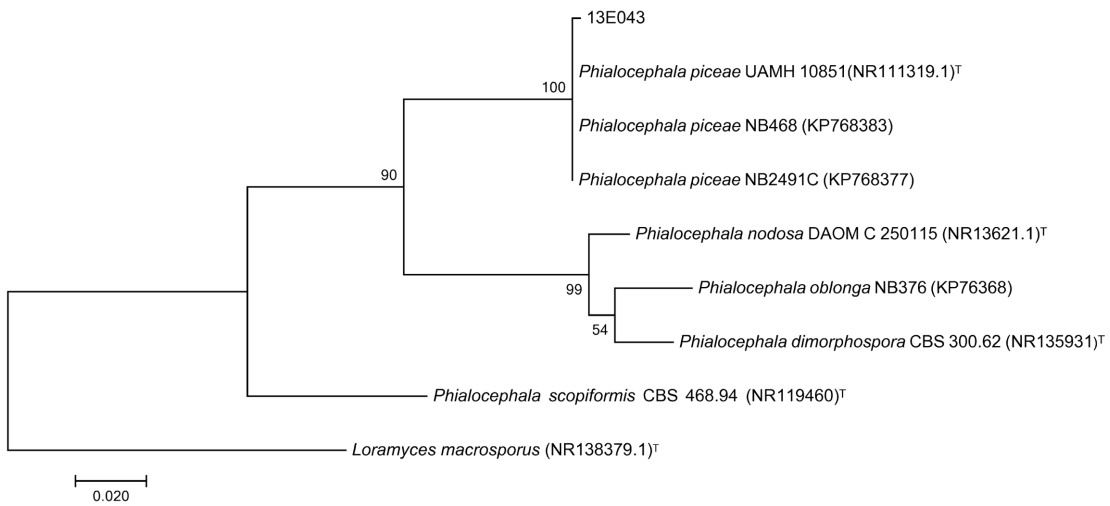

Fig. 2. Maximum likelihood tree of *Phialocephala piceae* 13E043 isolated from needle leaves of *Thuja koraiensis*. Internal transcribed spacer and 5.8S rDNA region were used to confirm the topological appropriation of the strain. Genbank accession numbers are followed by the species name in parenthesis and T indicates ex-type. *Loramyces macrosporus* was used as an outgroup and bootstrap values are shown at the branches (1,000 replicates).

ura-Nei substitution model [15]. All characters were equally weighted and unordered. Gaps and missing data were treated as complete deletions. Support for specific nodes on the ML tree was estimated by bootstrapping 1,000 replications. The sequence for *Loramyces macrosporus* was used as an outgroup. BLAST result showed that the sequence is identical with *P. piceae* strain UAMH 10851 (NR111319, exo-type strain 100% simailarity). The ML tree revealed that the Korean isolate 13E043 was positioned within the clade *P. piceae* with a bootstrap value of 100% (Fig. 2).

In this study, we isolated *P. piceae* (13E043), a member of DSE from the needle leaves of *T. koraiensis*. Although there were minor morphological differences between the Koran isolate and the original description of this species, they were within the normal variation range, considering different geographic origins and host plants. Although this species were isolated from the leave surface of *Diospyros kaki* in Korea, this is the first report of the DSE colonizing the needle leaves of coniferous tree [16], *T. koraiensis* in Korea and it will benefit further conservation studies and applied ecology in Korean forest.

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