RESEARCH ARTICLE

# Four Endophytic Ascomycetes New to Korea: *Cladosporium anthropophilum*, *C. pseudocladosporioides*, *Daldinia eschscholtzii*, and *Nigrospora chinensis*

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## ABSTRACT

Ascomycota is the largest phylum of the Fungi, including approximately 6,600 genera. They are often isolated from soils, indoor air, and freshwater environments, but also from plants as pathogens or endophytes. In this study, four species of Ascomycota (two of *Cladosporium* and one of each *Daldinia* and *Nigrospora*) were collected from the leaves of four woody plants (*Camellia japonica, Ginkgo biloba, Quercus* sp., *Vitis vinifera*). Their cultural characteristics were investigated on five different media (PDA, V8A, CMA, MEA, CZA) at 3 days after incubation at 25°C in darkness. BLASTn search and phylogenetic analysis were performed using the internal transcribed spacer (ITS) rDNA sequences, in addition to *tef1* gene sequences for *Cladosporium* species. Based on the cultural, morphological, and phylogenetic data, the isolates were identified as *Cladosporium anthropophilum*, *Cladosporium pseudocladosporioides*, *Daldinia eschscholtzii*, and *Nigrospora chinensis*. Previously, some members of *Cladosporium* and *Nigrospora* have been recorded as endophytes inhabiting the leaves and stems of various plants, whereas *Daldinia eschscholtzii* is a wood-inhabiting endophyte or wood-decaying fungus. To our knowledge, this is the first report of these four ascomycetes in Korea.

**Keywords:** Ascomycota, Diversity, Endophyte, ITS rDNA, *tef1*



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## Introduction

Ascomycota is a phylum of the kingdom Fungi, and along with Basidiomycota, it belongs to the subkingdom Dikarya. Under the three subphyla of Ascomycota, *viz*. Pezizomycotina, Saccharomycotina, and Taphrinomycotina, approximately 6,600 genera have been listed to date [1]. Active and extensive surveys of fungal diversity over the past decade have accelerated the discovery of indigenous and unrecorded fungi in Korea. As a result, many previously unknown ascomycetes have been reported from various abiotic habitats including soils [2, 3], indoor air [4], freshwater environments [5-7], as well as from plants as endophytes [8, 9].

*Cladosporium* is one of the most cosmopolitan genera in distribution. They play a key role as primary agents of plant and animal diseases and have high environmental impacts such as allergy, decay, and deterioration [10-13]. *Cladosporium* species are frequently isolated from soil, food, textiles, and other organic matter [14], but are also known to be common endophytes [15, 16].

The genus *Nigrospora* is an important genus of Ascomycota, with a cosmopolitan distribution and a broad host range, e.g., *N. sphaerica*, *N. oryzae*, and *N. chinensis* [17]. *Nigrospora* species have often been isolated as endophytes from the leaves and stems of various plants [18-21], but are also commonly recorded as plant pathogens on many economic crops, fruits, and ornamentals [17, 22].

*Daldinia* is considered to comprise saprobes that cause white rot mainly on dead angiospermous woods [23]. As *Daldinia* species often colonize their host plants in apparently dormant stages without disease symptoms, early colonization relates to their endophytic lifestyle [24, 25].

In 2017 and 2018, fungal strains of *Cladosporium*, *Daldinia*, and *Nigrospora* were isolated from the leaves of grapevine and woody plants in Jeollabuk-do of Korea. To identify these fungal strains, we performed morphological and molecular phylogenetic analyses.

# MATERIALS AND METHODS

## Sampling and fungal isolates

Four fungal samples were collected from a leaf of grapevine (*Vitis vinifera*) at a plastic house in Namwon (N35°30'31", E127°36'37") and from the leaves of three woody plants (*Camellia japonica, Ginkgo biloba, Quercus* sp.) at Kunsan National University, Gunsan-si (N35°56'41", E127°40'52") in Jeollabuk-do of Korea. The information on all isolates used in the present study is summarized in Table 1. Each sample was collected into a 50 mL conical tube and transferred to the laboratory on frozen ice packs. Healthy leaves were washed with running tap water, surface-sterilized with 70% ethanol and 3% NaClO, and rinsed with sterilized distilled water. After drying in a clean bench, the leaves were cut into 1 $cm^2$ pieces and inoculated on potato dextrose agar (PDA; Difco, Detroit, MI, USA) with 100 μg/L streptomycin for three days at 25°C in the dark. When fungal hyphal growth was observed under an Olympus SZ6045 microscope (Olympus, Tokyo, Japan), its tip was transferred to a new PDA plate for morphological examination and genomic DNA extraction. These were incubated at 25°C for 3–20 days depending upon the requirements for sporulation.

**Table 1.** Information on four fungal species isolated from the leaves of grapevine (*Vitis vinifera*) and woody plants (*Camellia japonica*, *Ginkgo biloba*, *Quercus* sp.) in Korea

| Species | Substrate | Strain no. | Sequence ID | Collection date | Geographic origin | GenBank No. ITS / *tef1α* |
|---|---|---|---|---|---|---|
| *Cladosporium anthropophilum* | *Vitis vinifera* | NIBRFG0000503205 | P136 | 10 Sep 2018 | Jeollabuk-do; Namwon | MN267562/MN284912 |
| *C. pseudocladosporioides* | *Quercus* sp. | NIBRFG0000503206 | P047 | 24 Nov 2017 | Jeollabuk-do; Gunsan | MN267563/MN284911 |
| *Daldinia eschscholtzii* | *Camellia japonica* | NIBRFG0000503204 | P053 | 24 Nov 2017 | Jeollabuk-do; Gunsan | MN267558/ - |
| *Nigrospora chinensis* | *Ginkgo biloba* | NIBRFG0000503203 | P052 | 24 Nov 2017 | Jeollabuk-do; Gunsan | MN267561/ - |

### Cultural and morphological characteristics

Cultural characteristics were investigated at 3 days after incubation at 25°C in darkness, on five different media: potato dextrose agar (PDA; Difco), V8 agar (V8A; 50 mL clarified V8 juice, 2 g $CaCO_3$, 15 g agar, 950 mL deionized water), corn meal agar (CMA; Difco), malt extract agar (MEA; Difco), and Czapek dox agar (CZA; MBcell, Seoul, Korea). For microscopy, fungal structures formed on the different media were transferred to a drop of distilled water on a microscope slide and covered with a coverslip. The slides were then examined and photographed using an Olympus BX53F microscope (Olympus, Tokyo, Japan) equipped with a DigiRetina 16M digital camera (Tucsen, Fuzhou, China).

### Phylogenetic analysis

In total, 5–10 mg of mycelia were ground in a mixer mill (MM2; Retsch, Hann, Germany) for 15 min, using about 300 mg of glass beads (Bio Spec Products, Bartlesville, OK, USA) with 1 mm diameter. Genomic DNA was extracted using the MagListo 5M plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) rDNA region was performed with the primer set, ITS1 and ITS4 [26], and for *Cladosporium* species, an additional marker, *tef1*, was amplified with the primers EF1-728F and EF1-986R [27]. Amplicons were visualized on 2% agarose gel, purified using an AccuPrep PCR Purification Kit (Bioneer), and were sequenced by Macrogen (Seoul, Korea), using the same primers used for amplification. The ITS rDNA sequences were edited using DNAStar software package version 5.05 (DNASTAR, Madison, WI, USA). Alignment was performed using MAFFT 6 [28] with the Q-INS-i algorithm [29]. To construct a global phylogeny of fungal species, previously published sequences of the type or authentic strains of *Cladosporium*, *Daldinia*, and *Nigrospora* were retrieved from the National Center for Biotechnology Information (NCBI) GenBank. A minimum evolution (ME) tree was constructed with MEGA 6.0 [30], using the Kimura 2-parameter model substitution model with 1,000 bootstrap replicates. All other parameters were set to default.

## RESULTS AND DISCUSSION

For two *Cladosporium* isolates (P046 and P136), the sequences of two markers, ITS and *tef1α*, were employed to compare with the reference sequences available in GenBank and to reconstruct a phylogenetic tree. The result of NCBI BLASTn for the ITS sequences failed to determine the two isolates at a species level (data not shown), as the region exhibited too low-resolution power to distinguish among closely related *Cladosporium* species. On the contrary, the *tef1* sequences displayed more informative characters useful for their identification in the BLASTn searches; P136 was identical to *Cladosporium anthropophilum* (MF473352.1, MF473351.1), and P046 was identical to *C. pseudocladosporioides* (HM148406.1, HM148405.1). In a phylogenetic tree inferred using the *tef1α* sequences of *Cladosporium* species available in GenBank (Fig 1), P136 grouped with the reference sequences of *C. anthropophilum* (bootstrapping value of 98%), whereas P046 grouped with *C. pseudocladosporioides* (91%), consistent with the results of

sequence similarity-based BLASTn searches.

For the *Daldinia* isolate (P053), the NCBI BLASTn showed that the complete ITS rDNA sequence shares a similarity of 99.80% (1 out of 505 characters was different) with *Daldinia eschscholtzii* (KY432354.1). The ITS sequence was also employed to reconstruct a phylogenetic relationship between *Daldinia* species (Fig. 2A). The isolate P053 was contained in a group of *Daldinia eschscholtzii* sequences, with a high supporting value of 95%.

A BLASTn search for the ITS sequence of the *Nigrospora* isolate (P052) revealed that it matches *Nigrospora chinensis* (MK371770.1, NR153475.1), with a sequence similarity of 100%. In the ITS tree (Fig. 2B), it formed a separate clade with other reference sequences of *N. chinensis* available in GenBank, and the grouping was supported by a high supporting value of 97%.

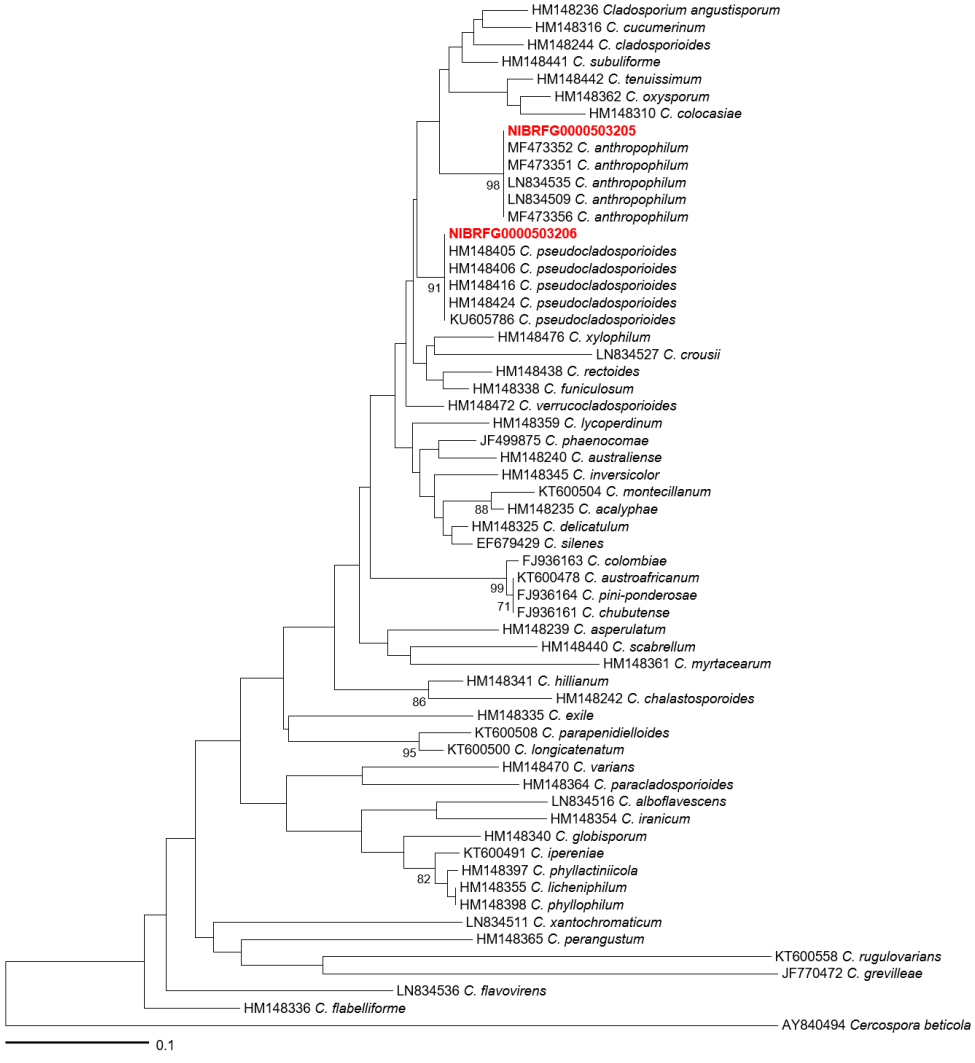


**Fig. 1.** Phylogenetic tree of *Cladosporium anthropophilum, Cladosporium pseudocladosporioides,* and closely related species, inferred from translation elongation factor 1 (*tef1*α) sequences. The numbers at the nodes are the bootstrap values obtained from 1,000 replications. The Korean isolate presented in this study is indicated in red. The scale bar equals the number of nucleotide substitutions per site.

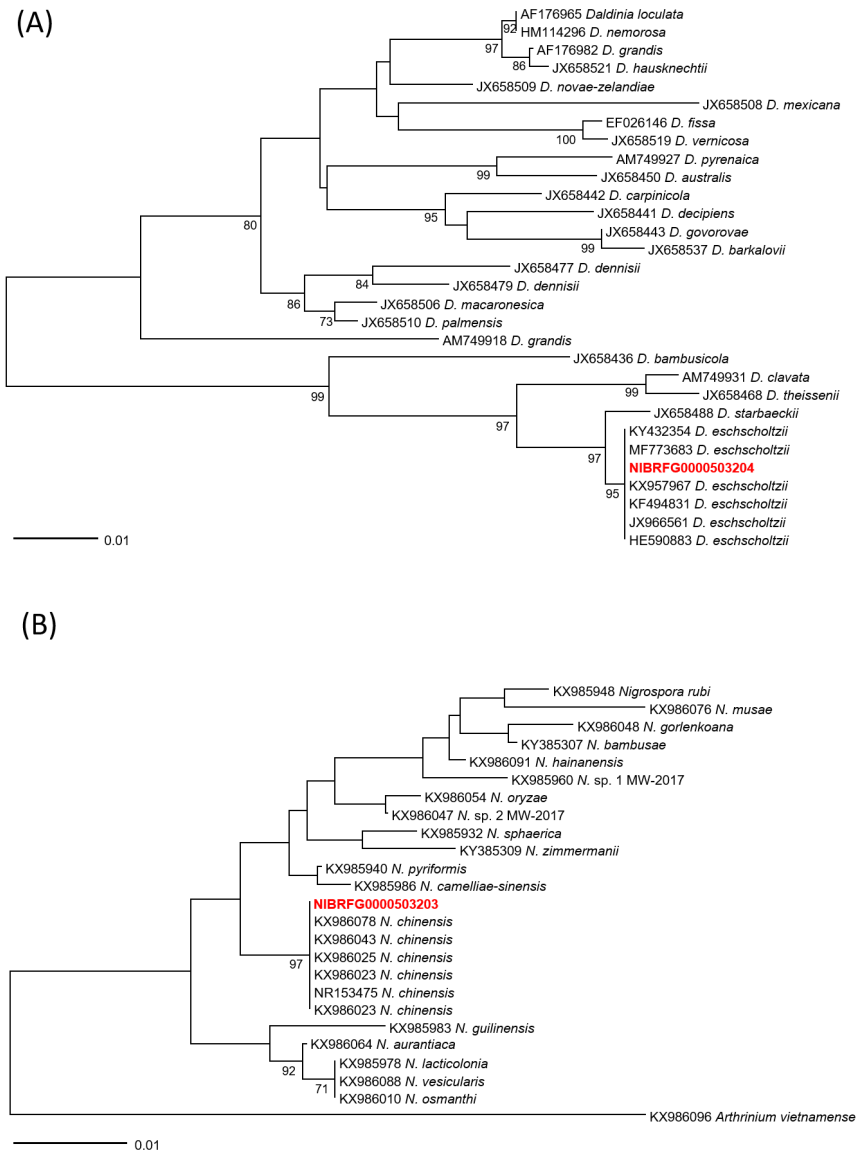


**Fig. 2.** Phylogenetic trees of *Daldinia* (A) and *Nigrospora* (B) species, inferred from the internal transcribed spacer (ITS) rDNA sequences. The numbers at the nodes are the bootstrap values obtained from 1,000 replications. The Korean isolate presented in this study is indicated in red. The scale bar equals the number of nucleotide substitutions per site.

## Morphological description

***Cladosporium anthropophilum*** Sandoval-Denis, Gené & Wiederhold, Persoonia 36: 290 (2016) [MB#815334]

**Description:** Colony grey-green to deep green, flat or folded, velvety to dusty or granular; 15–25 mm diameter on PDA, 10–20 mm on V8A, 10–15 mm on CMA, 10–15 mm on MEA, and 8–12 mm on CZA. On MEA and CZA, they grow slower than on the other media and are not dense. Mycelium immersed, superficial, subhyaline to pale green. Conidiophores erect, cylindrical, septate, often branched, pale green to

brown, 200–500 μm in length. Conidiogenous cells terminal or intercalary, (sub-) cylindrical, 12–50 × 2–5 μm. Ramoconidia aseptate, cylindrical, 20–40 × 2–4.5 μm, pale green, smooth, thickened, and darkened. Conidia forming short branched chains, aseptate; terminal conidia small, oval to ellipsoidal, 3–10 × 2–3 μm, subhyaline; intercalary conidia ellipsoidal, 4–10 × 2–3 μm, light green to brown; secondary ramoconidia ellipsoidal to cylindrical, 6–30 × 2–5 μm.

**Isolate examined:** Republic of Korea; Jeollabuk-do; Namwon-si; Ayeong-myeon, Cheonggye-ri, in a plastic house of a vineyard (N35°30'31", E127°36'37"), endophytic to *Vitis vinifera*, 10 Sep. 2018, D.J. Lee & Y.-J. Choi, NIBRFG0000503205 (P136).

**Note:** *Cladosporium anthropophilum* has been introduced as a saprobic or a clinically relevant species [12]. Along with *Cladosporium halotolerans*, this species is considered the most prevalent human pathogen [12, 13], though the present Korean sample was isolated from a leaf of a grapevine. The long conidiophores and oval to ellipsoidal conidia, as well as the molecular phylogenetic position, are in line with the original description of this species. This is the first report of *C. anthropophilum* in Korea.

***Cladosporium pseudocladosporioides*** Bensch, Crous & U. Braun, Studies in Mycology 67: 71 (2010) [MB#517087]

**Description:** Colony smoke-gray, felty-floccose, flat or folded, velvety to dusty or granular; 10–15 mm diameter on PDA, 10–15 mm on V8A, 9–14 mm on CMA, 8–11 mm on MEA, and 8–10 mm on CZA. On CMA, mycelium dirty-grey and not dense. On MEA and CZA, they grow slower than on the other media. Mycelium immersed and superficial. Conidiophores erect, cylindrical, solitary, mostly straight. Conidiogenous cells mostly terminal but sometimes intercalary, slightly attenuated, cylindrical or oblong, 10–30 μm long. Ramoconidia cylindrical or oblong, 20–50 × 3–5 μm, 0–2(–3)-septate, pale olivaceous, smooth, base 2–3 μm wide. Conidia forming in chains numerous, catenate; terminal conidia small, obovoid, ovoid or ellipsoid, 3–6 × 1–3 μm, intercalary conidia ovoid, ellipsoid or sub-cylindrical, 4–20 × 2–3 μm, 0(–1)-septate, secondary ramoconidia ellipsoid, ovoid or cylindrical, 6–30 × 2–4 μm, 0–1(–2)-septate, olivaceous to pale brown.

**Isolate examined:** Republic of Korea; Jeollabuk-do; Gunsan-si; Miryong-dong; at Kunsan National University (N35°56'41", E127°40'52"), endophytic to *Quercus* sp., 24 Nov. 2017, D.J. Lee & Y.-J. Choi, NIBRFG0000503206 (P047).

**Note:** This cosmopolitan species is found globally in air, soil, water, food, fungal fruiting bodies, and plant materials [11]. Before the introduction of *Cladosporium pseudocladosporioides* [11], this species was classified under the morphologically close *C. cladosporioides* complex. Although it was quite difficult to distinguish *C. pseudocladosporioides* and *C. cladosporioides* based on their morphological features, phylogenetic analysis using multigene sequences facilitated this distinction [10]. Similarly, they occupied distant positions in the present phylogenetic tree. Previously, two Korean isolates were included in a specimen list of *C. pseudocladosporioides* [10, 11]. This is the first official report of this species in Korea, with its detailed characteristics.

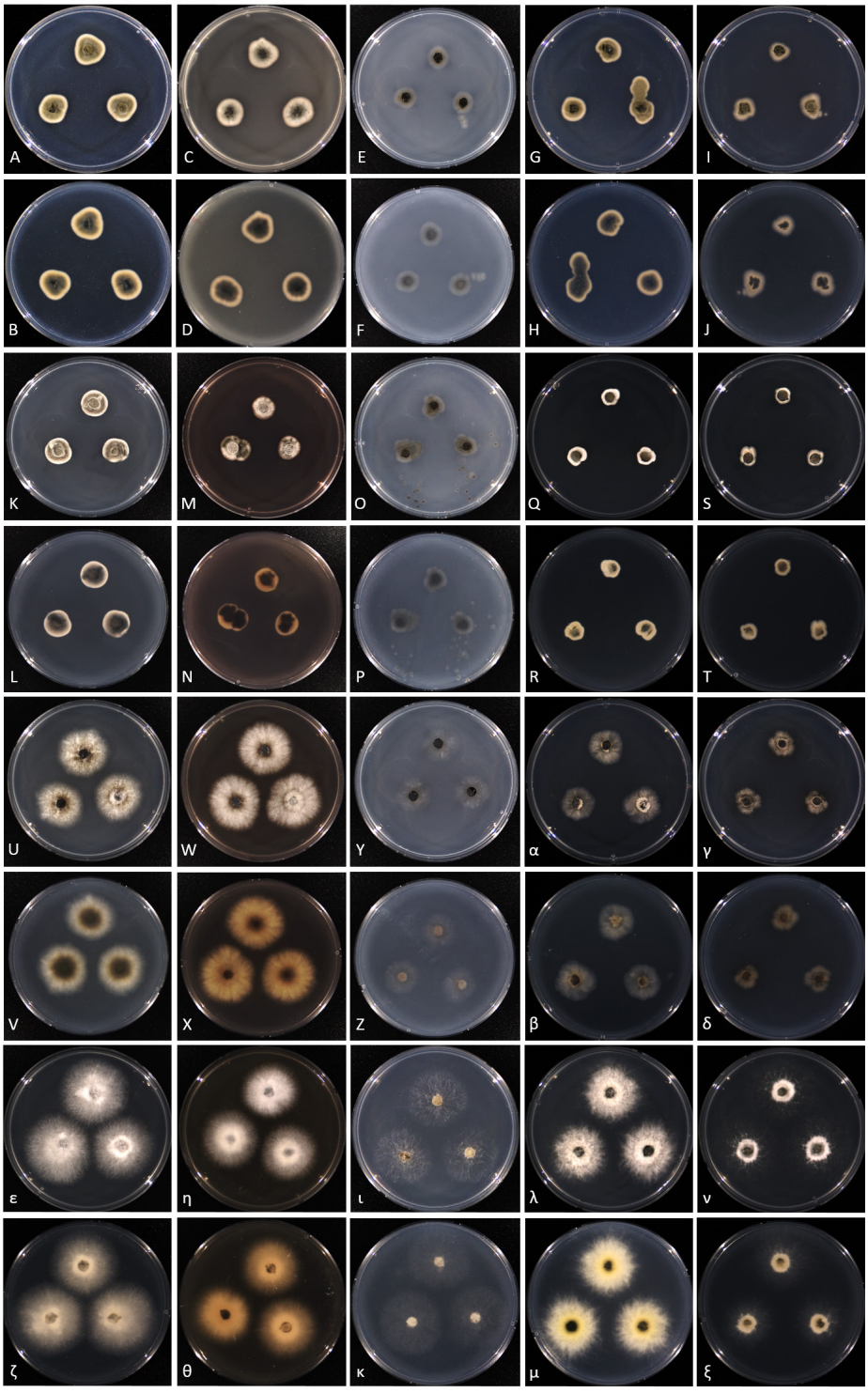


**Fig. 3.** Cultural features of *Cladosporium anthropophilum* NIBRFG0000503205 (A–J), *Cladosporium pseudocladosporioides* NIBRFG0000503206 (K–T), *Daldinia eschscholtzii* NIBRFG0000503204 (U–δ), and *Nigrospora chinensis* NIBRFG0000503203 (ε–ξ). Colonies on potato dextrose agar at the top (A, K, U, ε) and bottom view (B, L, V, ζ). Colonies on V8 agar at the top (C, M, W, η) and bottom view (D, N, X, θ), Colonies on corn meal agar at the top (E, O, Y, ι) and bottom view (F, P, Z, κ). Colonies on malt extract agar at the top (G, Q, α, λ) and bottom view (H, R, β, μ). Colonies on Czapek dox agar at the top (I, S, γ, ν) and bottom view (J, T, δ, ξ).

***Daldinia eschscholtzii*** (Ehrenb.) Rehm, Annales Mycologici 2(2): 175 (1904) [MB#544992]

**Description:** Colony initially white, turning smoke-grey with age, and reverse appears black in color; 60 mm diameter on PDA, 60 mm on V8A, 25 mm on CMA, 20 mm on MEA, 10 mm on CZA. On PDA, CMA, MEA, and CZA, cottony and dense in the center with aerial hyphae, but on V8A, felty to fluffy, azonate. Hyphae septate, initially hyaline, then melanized with age, with a thin- to thick-wall. Conidiophores septate, hyaline to melanized, mononematously, dichotomous or trichotomous, bearing one to three terminal conidiogenous cells, 80–150 × 2–3 µm. Conidiogenous cells hyaline, cylindrical, bearing conidia on its apical region, 8–30 × 1–2 µm. Conidia solitary, ellipsoid, aseptate, hyaline, with attenuated base, 4–7 × 1.5–2.0 µm.

**Isolate examined:** Republic of Korea; Jeollabuk-do; Gunsan-si; Miryong-dong; at Kunsan National University (N35°56'41", E127°40'52"), endophytic to *Camellia japonica*, 24 Nov. 2017, D.J. Lee & Y.-J. Choi, NIBRFG0000503204 (P053).

**Note:** Like many filamentous fungi, morphological examination alone cannot precisely distinguish the morphologically similar isolates of *Daldinia* at the species level. The advent of molecular phylogenetic analysis has laid the foundations for the study of a precise species concept and diversity among *Daldinia* species [23]. *D. eschscholtzii i*s widespread in warm tropical climates and is commonly isolated from dead woody plants such as dicotyledonous crops, trees and occasionally, marine algae [23]. Its preference for wood substrates is consistent with that of the present isolate from a woody plant, *Camellia japonica*, in Korea.

***Nigrospora chinensis*** Mei Wang & L. Cai, Persoonia 39: 118–142 (2017) [MB820732]

**Description:** Colony cottony and dense in the center with aerial hyphae, initially white, becoming black with age; 40 mm diameter on PDA, 30 mm on V8A, 25 mm on CMA, 35 mm on MEA, 30 mm on CZA. Hyphae hyaline, smooth, branched, septate, 2–5 µm in diameter. Conidiogenous cells hyaline, solitary,

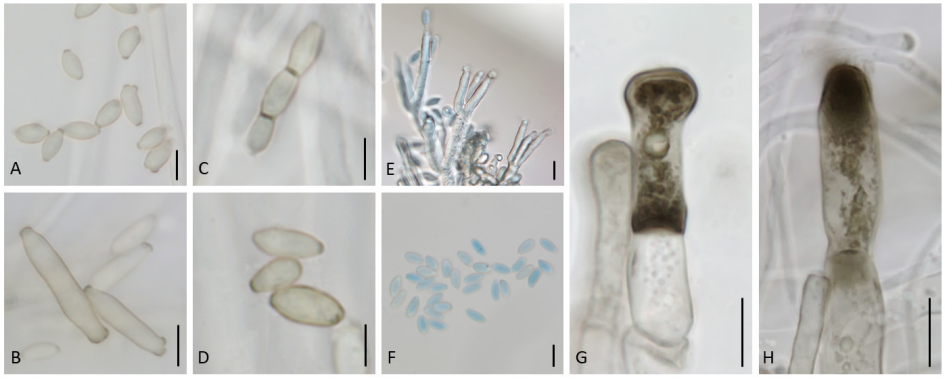


**Fig. 4.** Morphological features of *Cladosporium anthropophilum* NIBRFG0000503205 (A–B), *Cladosporium pseudocladosporioides* NIBRFG0000503206 (C–D), *Daldinia eschscholtzii* NIBRFG0000503204 (E–F), and *Nigrospora chinensis* NIBRFG0000503203 (G–H). A, D, F: Conidia, B: Secondary ramoconidia, C: Conidia in a chain; E, Conidiophores; G & H, Conidiogenous cells giving rise to conidia. *D. eschscholtzii* (E–F) mounted on cotton blue. (Scale bars = 5 µm).

determinate, straight, subglobose or ampulliform, 5–10 × 4–6 μm, with a rounded apex. Sterile cells terminal, brown to dark brown, ellipsoidal or clavate, lobed or slightly curved, 20–40 × 5–11 μm. Conidia solitary, aseptate, globose, subglobose or ellipsoidal, blackish, 9–14 × 7–10 μm, with a smooth wall.

**Isolate examined:** Republic of Korea; Jeollabuk-do; Gunsan-si; Miryong-dong; at Kunsan National University (N35°56'41", E127°40'52"), endophytic to *Ginkgo biloba*, 24 Nov. 2017, D.J. Lee & Y.-J. Choi, NIBRFG0000503203 (P052).

**Note:** *Nigrospora chinensis* has been recently described by Wang et al. [17]. They introduced this species as one of the three most ubiquitous species of *Nigrospora*, with a wide geographic distribution and a broad host spectrum in China. For the Korean isolate, the cultural characteristic of being initially white and gradually turning black, morphological features of sterile cells and conidia, and the phylogenetic placement matched those in the original description of *N. chinensis* [17], although it originated from a so-far unknown host plant, *Ginkgo biloba*. This is the first report of *N. chinensis* in Korea, and this species seems to be common at least in East Asia.

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