RESEARCH ARTICLE

Pezizomycotina (Ascomycota) Fungi Isolated from Freshwater Environments of Korea: *Cladorrhinum australe*, *Curvularia muehlenbeckiae*, *Curvularia pseudobrachyspora*, and *Diaporthe longicolla*

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ABSTRACT

Fungi are ubiquitous and indispensable components of nearly all ecosystems on earth, including freshwater environments. A survey of fungal diversity in freshwater environments of Korea led to the identification of four unrecorded Pezizomycotina (Ascomycota) species in 2016 and 2017, based on morphology and molecular phylogeny; these included *Cladorrhinum australe*, *Curvularia muehlenbeckiae*, *Curvularia pseudobrachyspora* (Dothideomycetes), and *Diaporthe longicolla* (Sordariomycetes).

Keywords: Diversity, Dothideomycetes, Freshwater, ITS rDNA, Sordariomycetes

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INTRODUCTION

Although fungi mainly act as decomposers in the earth's ecosystems, they are also closely associated with other organisms as pathogens or symbionts. In aquatic ecosystems, fungi play essential roles in organic matter cycling and food web dynamics [1]; however, freshwater environments are not extensively studied as fungal habitats. Thus, there is urgent need to expand our knowledge on the diversity and ecology of aquatic fungi to better understand their roles in ecosystems and response to aquatic ecosystem shifts due to environmental pollution and climate change [2].

Advances in DNA sequencing technologies have stimulated a new wave of research on aquatic fungi, leading to the discovery of novel fungal biodiversity in the aquatic realm [1,3]. The most common taxonomic groups found in submerged substrates of aquatic habitats belong to the phyla Ascomycota, Basidiomycota, and Chytridiomycota (fungi), as well as the fungus-like group Saprolegniales in the phylum Oomycota (Straminipila). To date, the presence of approximately 3,000 fungal species and 138

saprolegnialean species have been reported in aquatic habitats [4]. Studies on the diversity and community structure of freshwater fungi in Korea over the past five years resulted in reports of about 30 new species of fungi [5-8] and Oomycota [9,10].

To understand the diversity, distribution, and ecological functions of freshwater fungi, we isolated diverse strains from freshwater streams of Korea, including five Ascomycota isolates isolated from freshwater environments: one *Cladorrhinum*, three *Curvularia*, and one *Diaporthe*. The genus *Curvularia* exhibits a high species diversity, comprising mostly phytopathogens or decomposers of plant organic matters [11]. They are characterized by unique conidia, which are mostly curved, asymmetrical in shape, and possess few internal septa. However, the morphologically similar genus, *Bipolaris*, does not form asymmetric conidia. The genus *Cladorrhinum* is known to be beneficial in agriculture and animal husbandry, with some species involved in the production of biocontrol agents, plant growth promoters, and useful enzymes [12]. The genus *Diaporthe* comprises phytopathologically important microfungi with diverse host associations and worldwide distribution [13].

Herein, we investigated the molecular phylogenetic and morphological characteristics of the Korean isolates of Pezizomycotina (Ascomycota) and reported them as novel to Korea.

MATERIALS AND METHODS

Isolation of fungal strains and culture conditions

In 2016 and 2017, fungal strains were collected from samples of decaying plant twigs, stems, litters, and water in freshwater streams of Korea. Each sample was diluted conventionally and then inoculated on potato dextrose agar (PDA; Difco, Detroit, MI, USA) with 100 μg L^{-1} streptomycin for 2 days at 25℃. When fungal hyphal growth was observed under a microscope, its tip was transferred to a new PDA plate for morphological examinations. The fungi were cultured at 25℃ for 7-20 days depending on the requirements for sporulation. Five isolates have been deposited to the Culture Collection of the Nakdonggang National Institute of Biological Resources (NNIBR, Sangju, Korea). Information on all isolates used in the present study is summarized in Table 1.

Table 1. Fungal isolates from freshwater environments.

Species	Strain No.	Seq. ID	Source	Date isolated	Location (GPS)	GenBank Acc. No. (ITS/*gpd*)
Cladorrhinum australe	CNFG 2023	W80	A decaying plant stem	April 20 2016	Gyeongnam, Changnyeong-gun, Yeongbang-myeon, Changyeongbo (35°22'55"N 128°27'54"E)	MN893924/-
Curvularia muehlenbeckiae	CNFG 2024	W192	A decaying twig	May 20 2016	Kyungsangbuk-do, Sangju-si, Dongnum-dong, Sangjubo (36°26'07"N 128°13'18"E)	MN893921/ N895399
Curvularia muehlenbeckiae	CNFG 3205	W547	Plant litter	Aug 25 2017	Chungcheongnam-do, Gongju-Si, Geum River (36°27'47"N 127°6'3"E)	MN893923/ MN895397
Curvularia pseudobrachyspora	CNFG 2027	W263	A decaying plant stem	Jun 29 2016	Jeollanam-do; Naju-si; Dasi-myeon; Juksan-ri, in Juksan reservoir (34°58'17"N 126°37'34"E)	MN893922/ MN895398
Diaporthe longicolla	CNFG 2025	W159	Freshwater	May 20 2016	Gyeongsangbuk-do; Sangju-si; Donam-dong (36°26'07"N 128°13'19"E)	MN893920/-

ITS: internal transcribed spacer.

Cultural and morphological characteristics

Cultural characteristics were investigated three days after inoculation at 28℃ in the dark on the following three different media: PDA, V8 agar (V8A; 8% V8 juice [v/v] and 1.5% agar [w/v] adjusted to pH 6.0), and corn meal agar (CMA; Difco, Detroit, MI, USA). Fungal structures formed on the three different media were mounted on a drop of distilled water on a microscope slide and covered with a coverslip. The slides were examined and imaged using an Olympus BX53F microscope (Olympus, Tokyo, Japan) equipped with a DigiRetina 16M digital camera (Tucsen, Fuzhou, China). At least 50 units for each structure were measured.

Phylogenetic analysis

In total, 5-10 mg of mycelia were ground in a mixer mill (MM2, Retsch, Germany), using 170 mg of 1 mm glass beads (Bio Spec Products Inc., Bartlesville, USA) per sample. Genomic DNA was extracted using the MagListo 5M Plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), following the manufacturer's instructions. For all fungal isolates, PCR amplification of the complete the internal transcribed spacer (ITS) rDNA region was performed with the primer set, ITS1 and ITS4 [14]. For the *Curvularia* isolates, glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene was additionally amplified [15]. Amplicons were visualized on 1.2% agarose gel, purified using an AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea), and sequenced by a DNA sequencing service (Macrogen Inc., Seoul, Korea). The ITS rDNA sequences were analyzed using the DNAStar software package version 5.05 (DNAStar, Inc., Madison, Wis., USA) and aligned using MAFFT 7 [16] with the Q-INS-i algorithm [17]. The minimum evolution tree was inferred in MEGA6.0 [18], using the Tamura-Nei substitution model and by performing 1000 bootstrap replicates. All other parameters were set to default values.

RESULTS AND DISCUSSION

Based on the cultural and morphological characteristics of the five isolates (W80, W159, W192, W263, W547), it was confirmed that the two isolates W80 and W159 belonged to *Cladorrhinum* (Sordariales) and *Diaporthe* (Diaporthales), respectively, of the class Sordariomycetes. In comparison, the remaining three isolates W192, W263, and W547 belonged to *Curvularia* (Pleosporales) of the class Dothideomycetes. Molecular sequence analysis was performed to confirm this classification.

A BLASTn search for the ITS sequence of the *Cladorrhinum* isolate W80 revealed that it is identical to *Cladorrhinum australe* KT321051.1. In the ITS phylogenetic tree (Fig. 1A), it grouped with other reference sequences of *C. australe* available in GenBank, with the maximum supporting value. Interestingly, there were two subclades within this clade, and the Korean isolate formed a subclade with the *C. australe* strain INTA-AR 21, isolated from a corn crop in Argentina [12].

For the *Diaporthe* isolate W159, the NCBI BLASTn search revealed that it matches several reference sequences of *Diaporthe longicolla* available in GenBank, with a sequence similarity of 100%. In the

ITS phylogenetic tree (Fig. 1B), this isolate formed a clade with the ex-type sequence of *D. longicolla* (NR144924), and the grouping was supported by a high bootstrapping (BS) value of 100%.

For the three *Curvularia* isolates W192, W263, and W547, the sequences of two markers, ITS and *gpd*, were compared with reference sequences available in GenBank to reconstruct a phylogenetic tree. NCBI BLASTn search of the ITS sequences of the two isolates W192 and W547 failed to determine their species, as the region exhibited very low resolution for distinction among closely related *Curvularia* species. On the other hand, the *gpd* sequences provided more informative characters for species identification of these isolates, which matched *Curvularia muehlenbeckiae* (LT715806, HG779108). The BLASTn search for the isolate W263 revealed a sequence similarity of 100% with *C. pseudobrachyspora* in both ITS (MH516132, MH819562) and *gpd* (MG656404, MF490841, MH822837) regions.

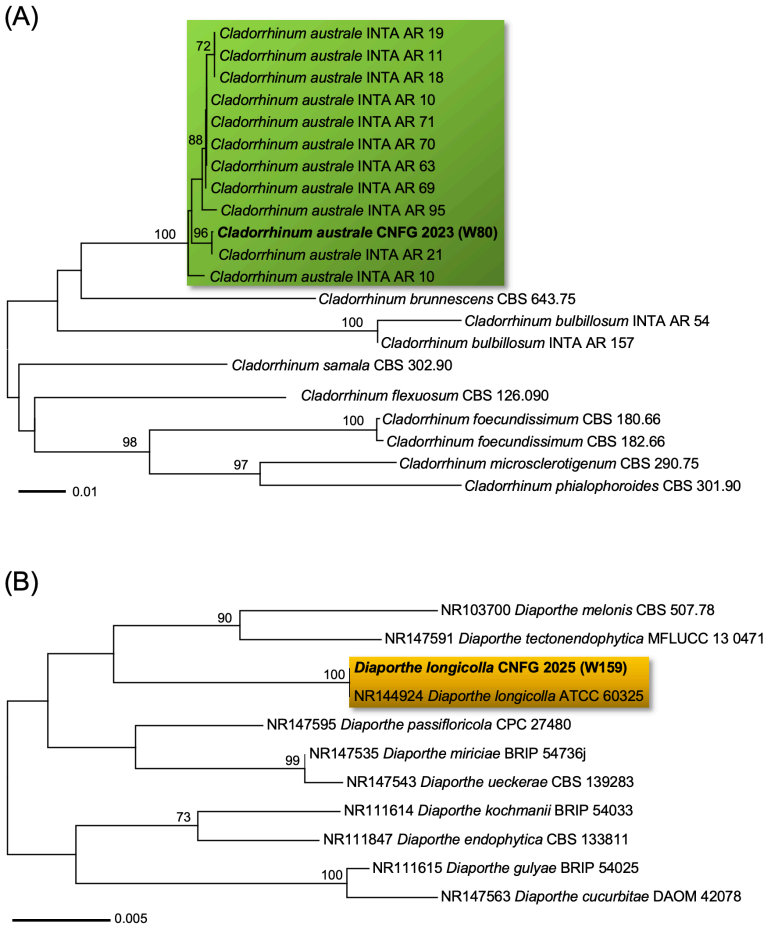

Fig. 1. Minimum evolution tree of *Cladorrhinum* (A) and *Diaporthe* species (B) based on the internal transcribed spacer (ITS) rDNA sequences. Bootstrapping support values higher than 70% are given above the branches. The specimens collected in Korea are shown in bold. The scale bar equals the number of nucleotide substitutions per site.

In a phylogenetic tree inferred using the ITS sequences of *Curvularia* species available in GenBank (Fig 2A), W192 and W547 grouped with the reference sequences of *C. hominis* and *C. muehlenbeckiae* (BS value of 85%), whereas W263 grouped with *C. pseudobrachyspora* (77%). In the phylogenetic tree constructed using *gpd* sequences (Fig. 2B), the two isolates (W192 and W547) grouped with *C. muehlenbeckiae* (BS value of 100%) but were clearly distinct from *C. hominis*. The isolate W263 formed a well-supported clade (98%) with three sequences of *C. pseudobrachyspora*. The phylogeny-based identification is consistent with the results of the sequence similarity-based BLASTn searches.

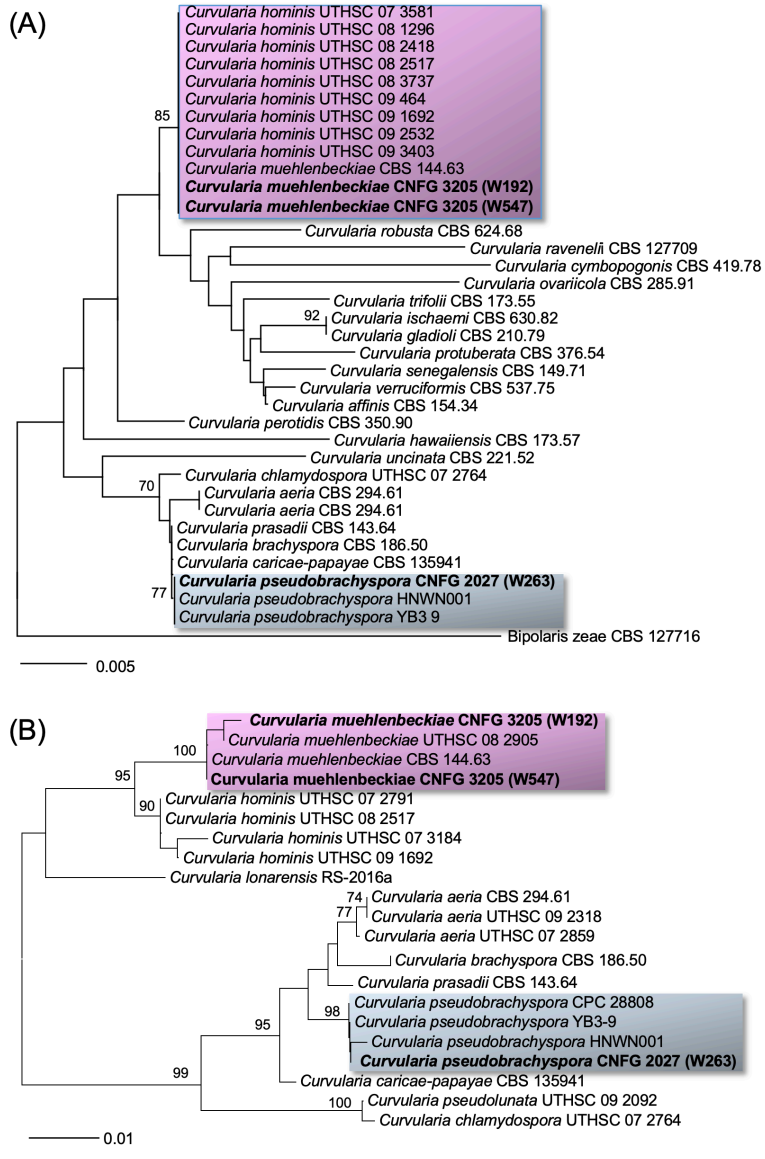

Fig. 2. Minimum evolution trees of *Curvularia* species based on (A) the internal transcribed spacer (ITS) rDNA sequences and (B) glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene sequences. Bootstrapping support values higher than 50% are given above the branches. The specimens collected in Korea are shown in bold. The scale bar equals the number of nucleotide substitutions per site.

Taxonomy

Based on the molecular phylogenetic and morphological data, we identified four species of Pezizomycotina (Ascomycota) that were previously unrecorded in Korea; these included *Cladorrhinum australe*, *Curvularia muehlenbeckiae*, *Curvularia pseudobrachyspora*, and *Diaporthe longicolla*.

Cladorrhinum australe Carmarán, C.C., Berretta, M., Martínez, S. *et al*. Mycol Progress 14: 94. (2015) [MB#813037] (Fig. 3, A-H)

Description: On PDA colonies rapidly expanding, initially hyaline, with local patches of sporulation, then blackening from the margin due to the formation of sclerotia masses. Reverse greenish-black. Optimum growth at 30℃. Hyphae hyaline, broad, with narrow side branches. Fertile hyphae arising in clusters, repeatedly branched at right angles, (sub-) hyaline, 2-4 μm wide, each cell bearing a single, distinct phialidic collarette near the middle on a neck 2-2.5 μm long. Conidia single-celled, hyaline, sub-spherical to broadly ellipsoidal, 2.5-3.5×2.0-2.5 μm.

Note: All morphological characteristics of the present isolate are consistent with the original descriptions of *C. australe* [12]. This species is morphologically close to *C. samala* but differs in the optimal growth temperature (30℃ for the former) and conidia (often basally truncate). The distinctiveness of these two species has been supported by molecular phylogenetic analysis (Fig. 1A). Since *C. australe* has recently been reported as a new species in Argentina [12], little was known about the geographical distribution of this species. This is the first report of *C. australe* outside Argentina.

Curvularia muehlenbeckiae Madrid, Da Cunha, Gené Guarro & Crous, Persoonia 33: 56 (2014) [MB#806055] (Fig. 4, I-P)

Description: Colonies on PDA attaining 34 mm diameter, on CMA attaining 30 mm diameter, and on V8A attaining 55 mm in 3 days at 25℃, cottony to funiculose, pale grey at the center, olive towards the periphery, with a fimbriate margin; reverse olivaceous-black, dark olive with a slightly fimbriate margin on V8A. Conidiophores mononematous, septate, simple or branched, straight to flexuous, geniculate towards the apex, subhyaline to dark brown, with cell walls often thicker than the vegetative hyphae, 15-300×2-4 μm. Conidiogenous cells terminal and intercalary, sub-cylindrical, proliferating sympodially; intercalary conidiogenous cells 4-17 μm long, terminal conidiogenous cells 4.5-20.0 μm long. Conidia mostly four-celled, asymmetrical to curved at the basal cells, 15-25×8-11 μm; intermediate cells dark brown, usually verruculose; end cells paler, less ornamented than central cells. Chlamydospores and sexual morph not observed.

Note: Cultural and morphological characteristics are in agreement with the descriptions of *Curvularia muehlenbeckiae* [11]. This species is close to *C. hominis* but differs in the conidial size; 15-25×8-11 μm in the Korean isolate vs 18-30×7-14 μm in *C. hominis.* This is the first report of *C. muehlenbeckiae* in Korea. Previously, this species has been recorded as a plant pathogen parasitic to *Muehlenbeckia* in India, *Oryza* sp.

in Australia, and *Sorghum* spp. in Japan and the USA [19]. Considering that the Korean strains were isolated from decaying plant twigs and litter, even in freshwater environments, it is speculated that *C. muehlenbeckiae* exhibits a high preference for plant substrates.

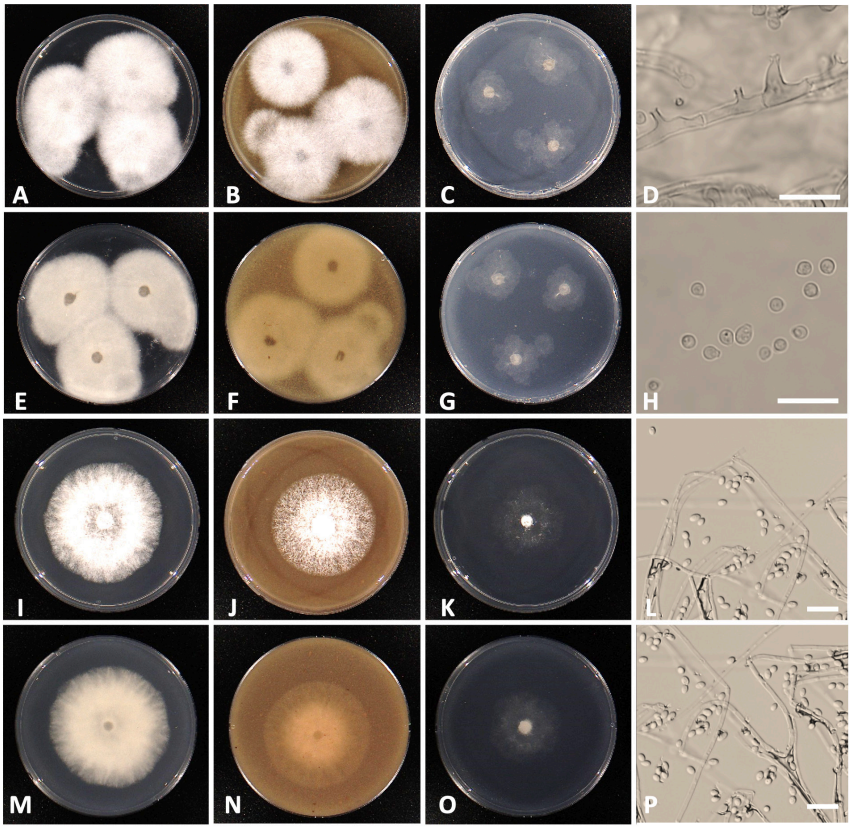

Fig. 3. Morphology of *Cladorrhinum australe* CNFG 2023 (W80) (A-H) and *Diaporthe longicolla* CNFG 2025 (W159) (I-P). First row: colonies on potato dextrose agar (PDA) (upper: observe view, lower: reverse view), Second row: colonies on V8A, Third row: colonies on CMA, Fourth row: conidiogenous cells and conidia. Scale bars=10 μm.

Curvularia pseudobrachyspora Y. Marín, Cheew. & Crous, Mycosphere 8 (9): 1569 (2017) [MB#822085] (Fig. 4, A-H)

Description: On PDA, at 25℃, colonies dark brown-to-black and usually zonate; reverse dark olivaceous with the margins transparent. Conidiophores borne singly or in groups, dark brown, unbranched but rarely branched, septate, sympodial, and geniculate at the apical region with rachis conidial ontogeny and up to 800 μm long, 100-390×3-7 μm. Conidiogenous cells terminal or intercalary, proliferating sympodially, pale brown, sub-cylindrical, 5-25×3.5-6.0 μm. Conidia dark brown to brown, verruculose, mostly curved, ellipsoidal to obovoid, cymbiform with three to four septa, with the middle cells broader and darker than the two end cells, 17.0 to 30.0×9.0 to 13.0 (av. 21.5×11.0) μm.

Note: This is the first report of *Curvularia pseudobrachyspora* in Korea. Both cultural and morphological characteristics of the Korean isolate resemble those in the original descriptions [20]. Since *C. pseudobrachyspora* was first described as a pathogenic fungus of *Eleusine indica* in Thailand in 2017 [20], it was reported as the pathogens of *Acorus calamus* in India [21] and *Areca catechu* in China [22] in 2019. Considering its existence in Korea, it is most likely that *C. pseudobrachyspora* is a Southeast Asian species.

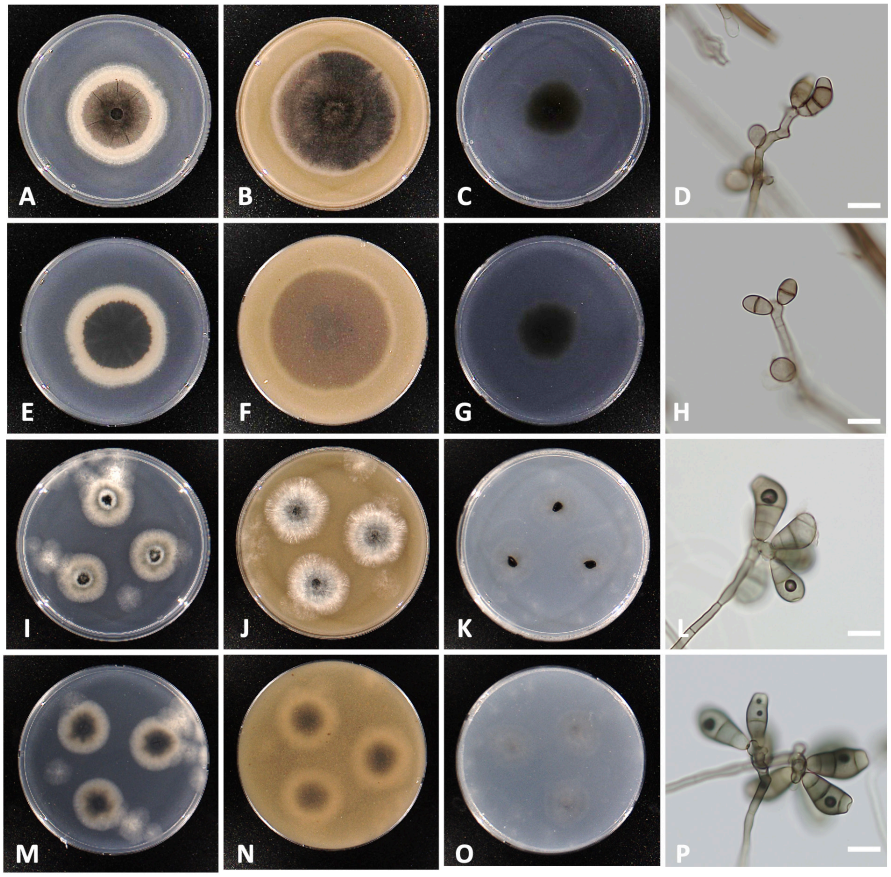

Fig. 4. Morphology of *Curvularia pseudobrachyspora* CNFG 2027 (W263) (A-H) and *Curvularia muehlenbeckiae* CNFG 3205 (W547) (I-P). First row: colonies on potato dextrose agar (PDA) (upper: observe view, lower: reverse view), Second row: colonies on V8A, Third row: colonies on CMA, Fourth row: Conidiophores and conidia. Scale bars=10 μm.

Diaporthe longicolla (Hobbs) J.M. Santos, Vrandecic & A.J.L. Phillips, Persoonia 27: 13 (2011) [MB#563213] (Fig. 3, I-P)

Description: Three days after inoculation on PDA, colonies floccose, dense, and white with occasional green-yellow areas, but after a week, producing abundant, black clusters of stroma with elongated necks at maturity. Reverse initially colorless and blackish with age. Conidiogenous cells hyaline, cylindrical, straight, unbranched, 5-11 μm long. Conidia hyaline, ellipsoidal to fusiform, guttulate. Conidia unicellular, hyaline, ellipsoid to ovoid, 4.5-8.0×1.7-4.5 (av. 6.0×2.6) μm.

Note: In addition to producing only alpha-conidia, the morphological characteristics of the Korean isolate closely resembled those of *Diaporthe longicolla* [23], although the conidia were somewhat wider: 1.7-4.5 μm in the present study vs (1.8-)2.1-2.2(-2.4) μm in the previous study [23]. This species was mainly known as a seed-borne pathogen but was also found in soybean plants [24]. Although this species is the most closely related to *Diaporthe sojae*, another common species associated with soybean, recent phylogenetic studies using authentic isolates and infection strategy, have distinguished between *D. longicolla* and *D. sojae* [23, 24]. Previously, *Phomopsis longicolla* was found to be the most common and virulent pathogen of soybeans in Korea [25]. The *Phomopsis* isolates used in this study seemed to be identical to *Diaporthe longicolla*, despite the taxonomic and nomenclatural confusion between *Diaporthe*/*Phomopsis sojae* and *D./P. longicolla* [24]. Although *D. longicolla* is not an absolute plant pathogen, the result of the present study suggests that this species can be isolated in freshwater environments, which is quite exceptional and interesting.

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