RESEARCH ARTICLE

Two Previously Unrecorded Endophytic Fungal Species in the Order Pleosporales from Korea

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ABSTRACT

We isolated endophytic fungi from the leaves of *Quercus acutissima* Carruth and the stems of *Rhododendron mucronulatum* Turcz. These fungi were characterized morphologically and subjected to molecular phylogenetic analyses using internal transcribed spacer, large subunit rDNA, translation elongation factor-1-α, the second largest subunit of RNA polymerase II, and β-tubulin sequences. We accordingly identified two endophytic fungi, *Didymella coffeae-arabicae* and *Austropleospora keteleeriae*, in the order Pleosporales, which have not previously been recorded in Korea. Herein, we described the morphological characteristics and molecular phylogenetic analyses of these two endophytic fungi, contributing to our understanding of fungal diversity in Korea. Our findings highlight the significance of endophytic fungi in ecological study and their potential utility as a source of novel bioactive compounds.

Keywords: *Austropleospora keteleeriae, Didymella coffeae-arabicae,* Endophytic fungus, Pleosporales

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INTRODUCTION

The fungal order Pleosporales is the largest within the class Dothideomycetes in the phylum Ascomycota, comprising approximately one-quarter of all known Dothideomycetes species [1]. This order is recognized for its notable diversity, with different species occupying a wide range of ecological niches, including as epiphytes on living leaves and stems, endophytes within plant tissues, and parasites [2, 3]. Additionally, some species function as hyperparasites of other fungi or insects, engage in symbiotic relationships with lichens, and thrive as saprobes on decaying plant material, including stems, leaves, and bark. Most Pleosporales species produce unicellular ascomata [4], and with the exception of those species in the families Diademaceae and Sporormiaceae, these ascomata are characterized by papillate and ostiolate structures [5]. Pseuparaphyses are present within the ascomata [6], and the asci are primarily bitunicate with a double-walled structure. Although generally cylindrical, clavate, or cylindro-clavate, a few species produce inversely obclavate or globose asci [5].

Endophytic fungi are those that colonize plant tissues without causing harm and can potentially confer

beneficial properties to the host plant [7], including promoting plant growth, enhancing resistance to diseases, and increasing tolerance to environmental stresses [8]. Additionally, endophytic fungi are a prolific source of secondary metabolites [9], many of which may have potential applications in medicine, agriculture, and industry [10]. Consequently, studies on these fungi can enhance our understanding of the ecological interactions between plants and fungi and provide a rich reservoir of natural compounds for biotechnological exploration.

In this study, we aimed to isolate and characterize previously unreported endophytic fungi in Korea and identified two fungal strains within the order Pleosporales, for which we performed morphological characterization and molecular phylogenetic analyses.

MATERIALS AND METHODS

Sample Collection and Fungal Isolation

In March 2023, stems of *Rhododendron mucronulatum* were collected from Samcheok, Gangwon-do, and in April of the same year, leaves of *Quercus acutissima* were collected from Gongju, Chungcheongnam-do. After confirming the absence of any diseases, the samples were transported to the laboratory within 24 h, where the leaves and stems were washed several times with distilled water and then sequentially surface-sterilized in 35% H_2O_2 for 1 min and 70% ethanol for 30 s. The sterilized samples were then cut into segments measuring 1 cm × 0.5 cm (leaves) and 1 cm (stems) and plated on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA). The plates were incubated at 25°C, and fungi growing from within the samples were subcultured to obtain pure cultures.

Morphological and Molecular Analyses

The isolated fungi were cultured on PDA and malt extract agar (MEA; Kisan bio, Seoul, Korea) at 25°C for 7 days in the dark for subsequent examination of colony characteristics. The microscopic structures of the cultured fungi were observed using an Axio Imager A2 optical microscope (Carl Zeiss, Overkochen, Germany). DNA was extracted from the fungal mycelia using a HiGene Genomic DNA Prep Kit (BioFACT, Daejeon, Korea) according to the protocol provided by the manufacturer and was used as a template for polymerase chain reaction (PCR) amplification of five different sequence regions for molecular identification. The internal transcribed spacer (ITS) was amplified using ITS1F/ITS4 primers [11], large subunit ribosomal DNA (LSU) using LR0R/LR16 primers [12], β-tubulin (*TUB*) using Bt2a/Bt2b primers [13], the second largest subunit of RNA polymerase II (*RPB2*) using fRPB2-5f/fRPB2-7cR primers [14], and translation elongation factor-1-α (*TEF*) using EF1-983F/EF1-2218R primers [15]. The PCR products obtained were electrophoresed on 1.5% agarose gels for 20 min to confirm the size of the amplified DNA, followed by Sanger sequencing (SolGent, Daejeon, Korea). The DNA sequences were analyzed for similarity with database accessions using the Basic Local Alignment Search Tool of the National Center

for Biotechnology Information (NCBI). Phylogenetic trees were constructed using the neighbor-joining method with the MEGA11 program and were validated by 1000 bootstrap replicates. Information on the newly recorded fungal species has been deposited at the National Institute of Biological Resources (NIBR), and the DNA sequences have been deposited in the NCBI database.

RESULTS AND DISCUSSION

Didymella coffeae-arabicae (Aveskamp, Verkley & Gruyter) Qian Chen & L. Cai, Stud. Mycol. 82: 175 (2015) [MB#814098]

Morphological characteristics: Colonies were grown on PDA and MEA media at 25 °C for 7 days, and their characteristics were observed. On PDA, the colonies were circular, flat, and measured 60–66 mm in diameter with a filamentous margin. The center was olive-green in color, gradually fading to an ivory-colored margin. The reverse side of the colony was dark brown to black in the center and ivory-colored at the margin (Fig. 1, Table 1). On MEA, the colonies were circular, flat, and measured 65–74 mm in diameter with a filamentous margin. The center was a beige to yellowish ochre color, and the margin was ivory-colored. The color of the reverse side was similar to that of the front side. Conidia were oval to elliptical in shape, hyaline, and measured 5.45 (4.12–7.4) × 3.67 (2.49–4.75) μm (n=20). Chlamydospores had thick brown cell walls, are multicellulares and intercalares. Conidiomata exude black to light apricot or beige exudates, typically solitary, with variable sizes and shapes. They are mostly solitary, glabrous, papillate, or possess an elongated neck.

Specimen examined: Gongju, Chungcheongnam-do, Korea, 36°20′9.7″N, 127°11′14.64″E, April 28, 2023, *Didymella coffeae-arabicae,* isolated from the leaves of *Quercus acutissima* Carruth, strain KNUE23N733, NIBRFGC000520715, GenBank No. PQ199317 (ITS), PQ199319 (LSU), PQ227105 (TUB), and PQ227103 (RPB2)

Notes: *Didymella coffeae-arabicae* was first reported as *Phoma coffeae-arabicae* in 2009 [16], but *Phoma* is phylogenetically polyphyletic, requiring reclassification [17]. Based on phylogenetic analysis of *Phoma* species using a combination of multiple DNA gene regions, the species was later placed within the genus *Didymella* in 2015 [18]. *Didymella coffeae-arabicae* has been reported as an endophyte of various plant species and has been shown to inhibit the pathogen *Fusarium oxysporum* in the medicinal plant *Hydrocotyle verticillata* Thunb. [19]. The ITS sequence of the new isolate showed 100% identity with *D. coffeae-arabicae* CBS 123380 (MH863293), and *TUB* sequence showed 100% identity with *D. coffeae-arabicae* CBS 123380 (FJ427104). The LSU sequence showed 100% identity with *D. coffeae-arabicae* C461A (MK348029), and *RPB2* sequence showed 100% identity with *D. coffeae-arabicae* CBS 123380 (KT389603). A phylogenetic tree constructed using the neighbor-joining method based on a combined analysis of ITS, LSU, *TUB*, and *RPB2* sequences revealed that the new isolate formed a monophyletic group with *D. coffeae-arabicae* CBS 123380 (Fig. 2).

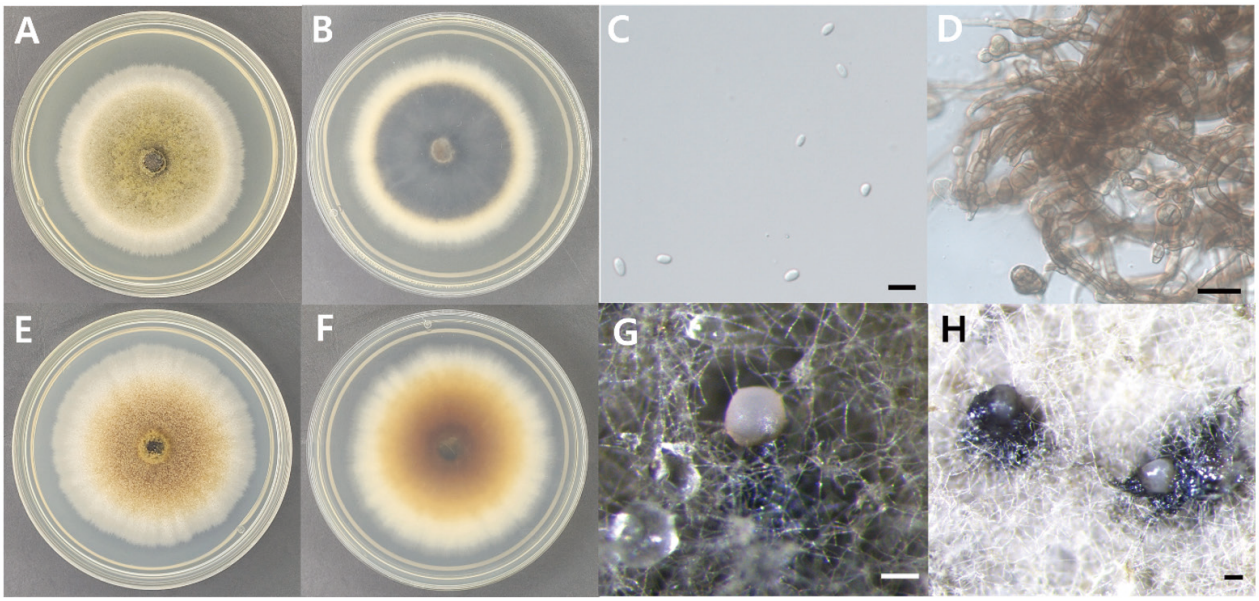

Fig. 1. Morphology of *D. coffeae-arabicae* KNUE 23N733. A: Colony grown for 7 days on potato dextrose agar (PDA), front. B: Colony grown for 7 days on PDA, reverse. C: Conidia. D: Chlamydospores. E: Colony grown for 7 days on malt extract agar (MEA), front. F: Colony grown for 7 days on MEA, reverse. G, H: Conidiomata. Scale bars: C=10 μm, D=20 μm, G and H=100 μm.

Table 1. Morphological characteristics of the KNUE 23N733 and CBS 123380 strains of *Didymella coffeae-arabicae*

Characteristics	*D. coffeae-arabicae* KNUE 23N733	*D. coffeae-arabicae* CBS 123380 [16]
Colony	PDA, MEA, 25°C, 7 days.	MEA, OA, CHA, 25°C, 7 days.
Color	PDA: Front, greenish olivaceous near the center, with ivory edges; reverse, fuscous-black near center, ivory edges. MEA: Front, beige to yellow ochre near the center, with ivory edges; reverse concolorous.	MEA: Front, aerial mycelium white with rosy-vinaceous tinges; agar surface iron-gray; reverse, fulvous to amber, leaden-black in zones with abundant pycnidia. OA: Aerial mycelium white; immersed mycelium hyaline or greenish olivaceous, fuscous-black near center; reverse concolorous.
Size	PDA: 60–66 mm in diameter. MEA: 65–74 mm in diameter.	PDA: No observations. MEA: 57–70 mm in diameter. OA: 61–66 mm in diameter. CHA: Similar growth rate to that on MEA
Shape	PDA: Circular, flat, with a filamentous margin. MEA: Circular, flat, with a filamentous margin.	MEA: Entire, smooth, sharp margin with aerial mycelium condensed. OA: Entire, smooth margins with sparse or absent tufted aerial mycelium. CHA: Aerial mycelium compact or tufted, primrose to citrine-green, pale greenish glaucous near center, leaden-gray near margin; reverse leaden-black.
Conidia	Ellipsoidal to ovoid, hyaline, 5.45 (4.12–7.4) × 3.67 (2.49–4.75) μm.	Ellipsoidal to ovoid, hyaline, eguttulate, or with 1-4 small polar droplets, 4.5–6 (4–7) × 3–4 (2.5–4.5) μm.
Conidiomata	Black to light apricot to beige exudate, mostly solitary, variable in shape and size, glabrous, papillate or with an elongated neck.	Mostly solitary or in chains, variable in shape and size; mostly ovoid, but also (sub)globose or elongated, glabrous, papillate, or with an elongated neck; mostly uni- or bi-ostiolate, 150–310 (100–310) × 110–200 (100–240) μm.
Chlamydospores	Thick-cell walled, brown, multicellulares, intercalares.	Multicellular, immersed, dictyosporous, pseudosclerotioid, intercalary, or solitary, 40–100 (23–190) × 15–30 (11–30) μm.

PDA: potato dextrose agar; MEA: malt extract agar; OA: oatmeal agar; CHA: cherry decoction agar.

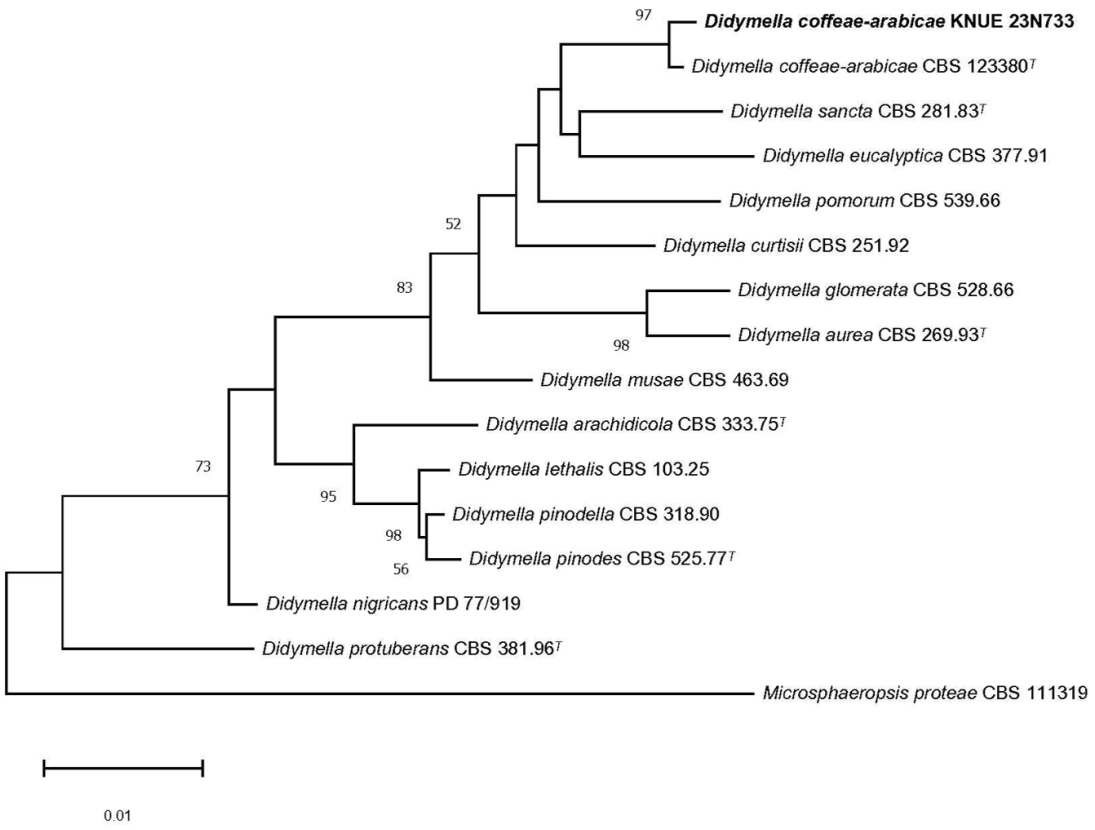

Fig. 2. Neighbor-joining phylogenetic tree of *D. coffeae-arabicae* KNUE 23N733 based on concatenated sequences of the internal transcribed spacer (ITS), large subunit ribosomal DNA (LSU) regions, β-tubulin (*TUB*), and the second largest subunit of RNA polymerase II (*RPB2*). *Microsphaeropsis proteae* serves as the outgroup. The numbers at the nodes represent bootstrap values greater than 50% (1,000 replicates). Ex-type strains are indicated by a superscript "T" following the strain reference number. The strain isolated in this study is in a bold.

Austropleospora keteleeriae Jayasiri, E.B.G. Jones & K.D. Hyde, Mycosphere 10 (1): 65 (2019) [MB#555541]

Morphological characteristics: Colonies were cultivated on PDA and MEA media at 25 °C for 7 days, and their characteristics were observed. On PDA, the colonies were circular, flat, and measured 35–41 mm in diameter with a soft texture. The center of the colonies was beige to light yellow in color, gradually fading toward an ivory-colored margin. The reverse side of the colonies was golden yellow to yellow at the center and light yellow to beige at the margin (Fig. 3, Table 2). On MEA, the colonies were circular, flat, and measured 43–46 mm in diameter with a soft texture. The center was ochre to light yellow, gradually fading toward a white to ivory-colored margin. The reverse side was golden yellow to deep yellow at the center and light yellow to beige at the margin. The conidia are globose to ellipsoid, hyaline or dark brown, and measure 6.26 (5.25–7.38) × 3.47 (2.37–4.67) μm (n=20). Conidiomata are dark brown to black, ellipsoidal or elongated ovoid, and glabrous.

Specimen examined: Samcheok-si, Gangwon-do, Korea, 37°12′3.2″N 129°2′11.2″E, March 31,

2023, *Austropleospora keteleeriae*, isolated from the stems of *Rhododendron mucronulatum* Turcz, strain KNUE23N307, NIBRFGC000518068, GenBank No. PQ199398 (ITS), PQ199399 (LSU), and PQ227104 (*TEF*).

Notes: The genus *Austropleospora* was first reported in 2010 with the single species *A. osteospermi* [20]. In 2015, *Paraconiothyrium archidendri* was reclassified as a species of *Austropleospora* [21]. Additional species, including *A. keteleeriae* in 2012 and *A. ochracea* in 2019, were subsequently reported, bringing the total to four known species within this genus [22, 23]. *A. archidendri* has spore-forming cells similar in shape and size to those of *A. keteleeriae*, but with hyaline, thin-walled spore-forming cells attached to the spore-forming cells. Comparative analysis of the ITS sequences of these two species revealed nine (1.9%) nucleotide differences, identifying *A. keteleeriae* as a distinct species [24]. The ITS sequence of the new isolated strain showed 99.7% identity with *A. keteleeriae* ZHKU 22-0209 (OP297802), LSU sequence showed 100% identity with *A. keteleeriae* ZHKU 22-0209 (OP297772), and TEF sequence showed 99.8% identity with *A. keteleeriae* MFLUCC 18-1551 (MK360045). A phylogenetic tree constructed using the neighbor-joining method, based on the combined analyses of ITS, LSU, and *TEF* sequences, revealed that the new isolate formed a monophyletic group with *A. keteleeriae* MFLUCC 18-1551 (Fig. 4).

In this study, we successfully identified two endophytic fungal species, *D. coffeae-arabicae* and *A. keteleeriae*, previously unrecorded in Korea. Both fungi, members of the order Pleosporales, were isolated from different host plants, *Q. acutissima* and *R. mucronulatum*, respectively. Morphological and molecular analyses revealed clear distinctions between these fungi and their respective closely related species, confirming their presence in Korea for the first time. The findings of this research substantially contribute to our understanding of fungal biodiversity in Korea and highlight the importance of continued exploration and documentation of endophytic fungi.

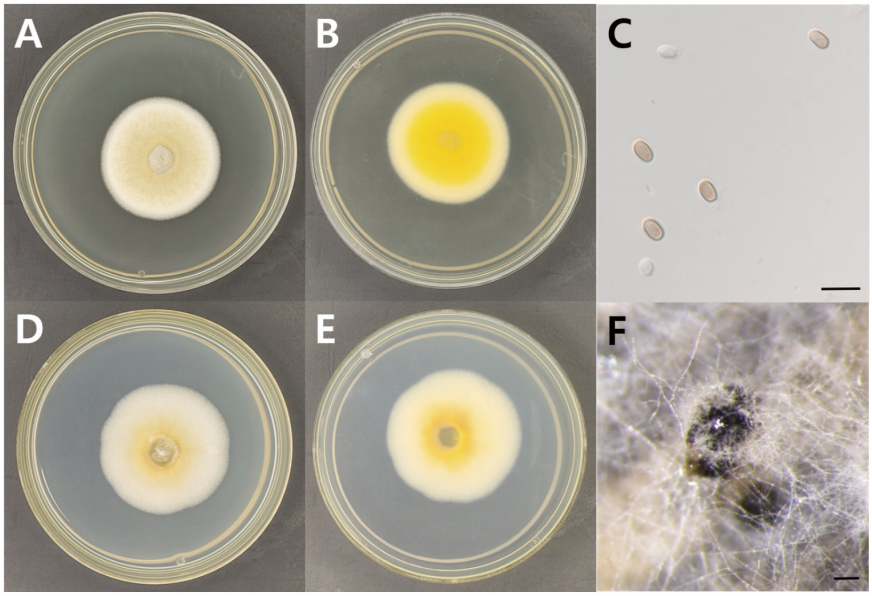

Fig. 3. Morphology of *A. keteleeriae* KNUE 23N307. A: Colony grown for 7 days on potato dextrose agar (PDA), front. B: Colony grown for 7 days on PDA, reverse. C: Conidia. D: Colony grown for 7 days on malt extract agar (MEA), front. E: Colony grown for 7 days on MEA, reverse. F: Conidiomata. Scale bars: C=10 μm, F=100 μm.

Table 2. Morphological characteristics of the KNUE 23N307 and MFLUCC 18–1551 strains of *Austropleospora keteleeriae*

Characteristics	*A. keteleeriae* KNUE 23N307	*A. keteleeriae* MFLUCC 18–1551[23]
Colony	PDA and MEA, 25°C, 7 days.	MEA, 18°C, 7 days.
Color	PDA: Front, pale gold-yellow near the center, with ivory-colored edges; reverse, gold to yellow near the center, ivory-colored edges. MEA: Light yellow to yellow ochre near the center, with ivory to white edges; reverse, gold to dark yellow near the center, ivory to light yellow edges.	MEA: Front, colonies grow in four layers, from the center to margins, showing gray, brown, pinkish gray, and dark brown, respectively; reverse, center brown, middle off-white, and dark brown at margin.
Size	PDA: 35–41 mm in diameter. MEA: 43–46 mm in diameter.	MEA: 25–30 mm in diameter.
Shape	PDA: Circular, flat, with a filamentous margin, downy. MEA: Circular, flat, with a filamentous margin, downy.	MEA: Circular, with an irregular margin.
Conidia	Globose to ellipsoid, hyaline or dark brown, 6.26 (5.25–7.38) × 3.47 (2.37–4.67) µm.	Solitary, hyaline when young, becoming dark brown at maturity, globose to obovate, single-celled, thick, and smooth-walled, 5 (4–5.5) × 5.5 (5–6) µm.
Conidiophores	No observations.	Conidiophores reduced to conidiogenous cells, arising from the base and sides of the conidioma; phialidic, enteroblastic, determinate, ampulliform, lining the inner wall layer of the pycnidium; hyaline, smooth, thin-walled, 6.2 (5–7) × 4.5 (4–5) µm.
Conidiomata	Dark brown to black, ellipsoidal or elongated ovoid, glabrous.	210–240 µm high × 220–255 µm in diameter ($\bar{x}$=228 × 242 µm, n=30); pycnidial, solitary, immersed, globose to obpyriform, unilocular, centrally ostiolate. Wall 33–55 µm wide ($\bar{x}$=44 µm; n=20), thick, 5 or 6 layered, composed of an outer 4 or 5 layers, brown, and inner 1 or 2 layers of hyaline, thin-walled cells of textura angularis.

PDA: potato dextrose agar; MEA: malt extract agar.

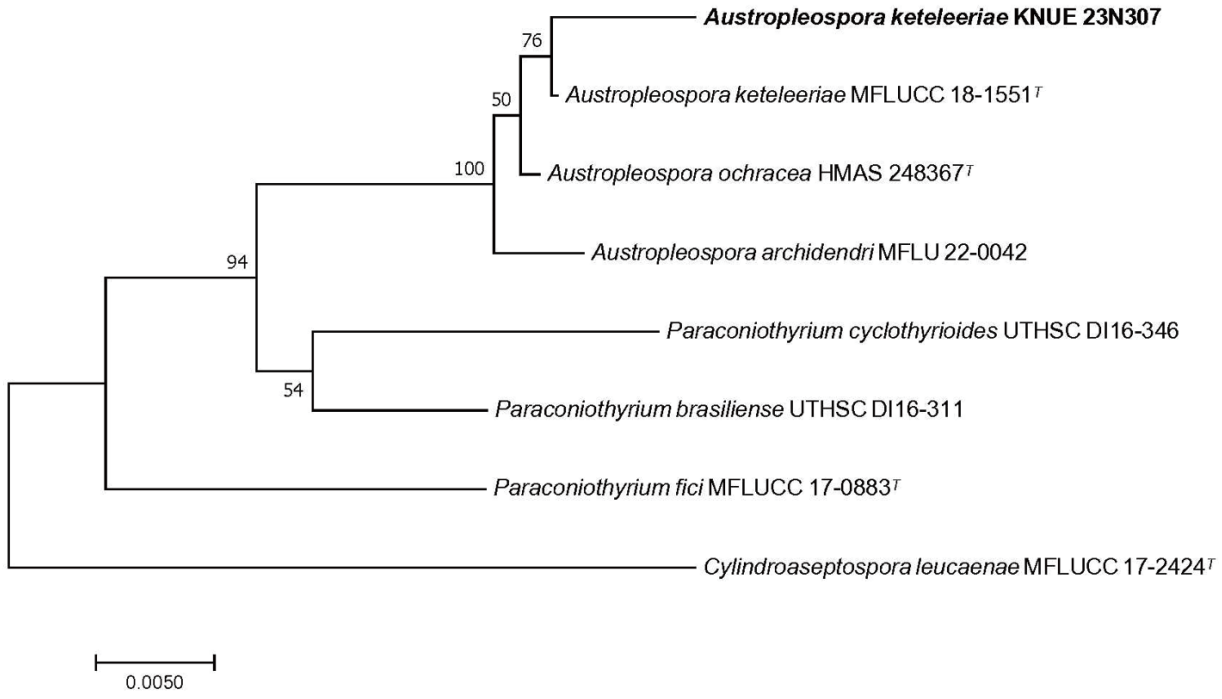

Fig. 4. Neighbor-joining phylogenetic tree of *A. keteleeriae* KNUE 23N307 based on concatenated sequences of the internal transcribed spacer (ITS), large subunit ribosomal DNA (LSU) regions, and translation elongation factor-1-α (*TEF*). *Cylindroaseptospora leucaenae* serves as the outgroup. The numbers at the nodes represent bootstrap values greater than 50% (1,000 replicates). Ex-type strains are indicated by a superscript "T" following the strain reference number. The strain isolated in this study is in a bold.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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