RESEARCH ARTICLE

Morphological and Phylogenetic Analysis of a New Record of *Paraconiothyrium kelleni* from Soil in Korea

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ABSTRACT

A fungal strain designated KNUF-21-66Q1 was isolated from soil in Chungcheongbuk Province, Korea. Moderate growth of colonies was observed on potato dextrose agar, oatmeal agar (OA), malt extract agar, and cornmeal agar media at 25°C, and the detailed morphology was examined on OA medium. The colonies on OA medium were flat, had entire margin, hyaline, and yellow concentric rings in 3-4 weeks. Conidiomata were pycnidial, solitary or clustered, globose to subglobose, black-brown, and 300-500 µm in diameter. Conidiogenous cells were smooth, hyaline, globose to ampulliform, and 6.0-9.0×3.0-6.0 µm in size (n=15). Conidia were hyaline to pale brown, slightly golden, obovoid to slightly ellipsoidal, smooth, guttulate, and 3.0-4.7×2.1-3.3 µm in size (n=100). The strain was confirmed based on phylogenetic analysis using internal transcribed spacer regions, the partial 28S rDNA of large subunit, and β-tubulin gene sequences. The morphological observations and phylogenetic analysis revealed that the strain KNUF-21-66Q1 was similar to the previously described *Paraconiothyrium kelleni*. To our knowledge, this is the first report of *P. kelleni* in Korea.

Keywords: Morphology, *Paraconiothyrium kelleni*, Phylogeny, Soil-inhabiting fungi

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INTRODUCTION

The genus *Paraconiothyrium* belongs to the family Didymosphaeriaceae, which was introduced from the asexual morphs grouping with *Paraphaeosphaeria*, and the following four new taxa were described: *Paraconiothyrium estuarinum* (type species), *P. brasiliense*, *P. cyclothyrioides*, and *P. fungicola* [1]. The species of *Paraconiothyrium* are polyphyletic within Didymosphaeriaceae and separated from the genera *Coniothyrium*, *Paraphaeosphaeria*, *Alloconiothyrium*, and *Dendrothyrium* as a paraphyletic group [2,3]. According to the 2022 version of the outline of fungi and fungus-like taxa, Didymosphaeriaceae contains 33 genera and approximately 254 species [4]. Currently, the total 32 described *Paraconiothyrium* species belong to Didymosphaeriaceae in the Index Fungorum database (https://www.indexfungorum.org/names/

Names.asp: accessed on February 27, 2023). The morphological characteristics of *Paraconiothyrium* species compromise with the presence of simple or complex conidiomata having a pycnidial or eustromatic type. Conidiogenous cells are discrete or integrated with a percurrent or phialidic nature. Similarly, conidia are primarily aseptate or single septate, thin walled and smooth, and during liberation hyaline, which later turns brown [1]. However, the genus *Paraconiothyrium* is considered cosmopolitan in nature with diverse host habitats and geographical distributions [5]. *Paraconiothyrium* species have been most often isolated from warm regions such as Brazil, Italy, Papua New Guinea, South Africa, and Turkey [6]. Similarly, it has also been reported that *Paraconiothyrium* species are diversely available and found in different sources such as soil, pathogenic fungus, plant endophytic fungi, and marine [7].

The objective of this study was to screen the native fungal species from soil in Korea. Based on morphological and cultural characteristics along with their molecular phylogenetic analysis, the isolated fungus was an unreported species of the genus *Paraconiothyrium*. Hence, to our knowledge, this is the first report of its isolation and identification in Korea.

MATERIALS AND METHODS

Sample collection and fungal isolation

In 2021, soil samples were collected from Chungcheongbuk Province (37°01′37″N, 127°16′45″E), Korea, using a sterile spatula and stored in an air-dried plastic bag at 4°C. The isolation was performed using the dilution plating technique, as described previously [8]. A soil sample (1 g) was added to 10 mL of sterile distilled water, and the soil suspension was diluted serially and spread on potato dextrose agar plates (PDA; Difco, Detroit, MI, USA). The plates were incubated for 3-4 days at 25°C until single colonies were observed. Next, the single colonies were transferred to new PDA plates and incubated at 25°C. The strain KNUF-21-66Q1 was selected based on cultural, morphological, and phylogenetic analyses. The fungal strain was maintained in 20% glycerol at -80°C for further investigation. Stock cultures of the strain KNUF-21-66Q1 (NIBRFGC000510192) were deposited at the National Institute of Biological Resources (NIBR) as metabolically inactive cultures.

Cultural and morphological characterization

The strain KNUF-21-66Q1 was cultured and incubated on different media to investigate its cultural and morphological characteristics. The strain was transferred onto PDA, oatmeal agar (OA; Difco, Detroit, MI, USA), malt extract agar (MEA; Difco, Detroit, MI, USA), and cornmeal agar (CMA; Difco, Detroit, MI, USA) and incubated at 25°C for 10-21 days [3,9]. Different parameters such as colony color, size, shape, and growth were measured and recorded on each different media. The morphological characteristics were observed under a light microscope (BX-50, Olympus, Tokyo, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

To obtain genomic DNA for molecular analysis, fresh fungal mycelium grown on PDA for 1 week at 25°C was extracted using the protocol of the HiGene™ Genomic DNA prep kit (BIOFACT, Daejeon, Korea) and its manufacturer's instructions. For DNA amplification, polymerase chain reactions were performed using internal transcribed spacer (ITS) regions, 28S rDNA of large subunit (LSU), and β-tubulin (*TUB2*) [10]. The primer pairs ITS1F/ITS4 [11,12], LROR/LR5 [13,14], and T1/Bt2b [15,16] were used for the amplification of ITS regions, LSU, and *TUB2*, respectively. The PCR products were confirmed on 1% agarose gel electrophoresis stained with ethidium bromide. The amplified PCR products were then purified using EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Macrogen (Daejeon, Korea).

Molecular phylogenetic analyses

The obtained sequences were compared with the reference sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI) (Table 1). Alignments of each gene were performed, the sequences were combined to reveal the position of the isolate, and evolutionary distance matrices for the neighbor-joining algorithm were generated using Kimura's two-parameter model [17]. Phylogenetic analysis was performed using the MEGA 7.0 program with the bootstrap values based on 1000 replicates [18].

Table 1. GenBank accession numbers used for the phylogenetic analyses in this study

Species	Strain numbers	Country	GenBank accession numbers		
			ITS	LSU	*TUB2*
Paraconiothyrium kelleni	**KNUF-21-66Q1**	**South Korea**	**OQ799075**	**OQ799076**	**OQ818867**
P. kelleni	CBS 149289	Chile	OP348922.1	OP348925.1	OP328918.1
P. kelleni	CBS 149290^{T}	Chile	OP348920.1	OP348923.1	OP328916.1
P. kelleni	CBS 149291	Chile	OP348921.1	OP348924.1	OP328917.1
P. fuckelii	CBS 797.95^{T}	The Netherlands	JX496113.1	JX496226.1	JX496452.1
P. fuckelii	CBS 764.71B	The Netherlands	JX496112.1	JX496225.1	JX496451.1
P. fuckelii	CBS 508.94	Italy	JX496096.1	JX496209.1	JX496435.1
P. archidendri	CBS 168.77^{T}	Myanmar	JX496049.1	JX496162.1	JX496388.1
P. variabile	CBS 121754	South Africa	JX496031.1	JX496144.1	JX496370.1
P. hakeae	CBS 142521^{T}	Australia	KY979754.1	KY979809.1	KY979920.1
P. brasiliense	CBS 395.87	Italy	JX496083.1	JX496196.1	JX496422.1
P. brasiliense	CBS 115.92	Italy	JX496022.1	JX496135.1	JX496361.1
P. cyclothyrioides	CBS 972.95^{T}	Papua New Guinea	JX496119.1	JX496232.1	JX496458.1
P. cyclothyrioides	CBS 432.75	Sri Lanka	JX496088.1	JX496201.1	JX496427.1
P. estuarinum	CBS 109850^{T}	Argentina	JX496016.1	JX496129.1	JX496355.1
P. fungicola	CBS 113269^{T}	Albania	JX496020.1	JX496133.1	JX496359.1
P. africanum	CBS 121166^{T}	South Africa	NR154294.1	JX496142.1	JX496368.1
Darksidea alpha	CBS 135656	Hungary	KP183971.1	KP184007.1	KP184220.1

The newly generated sequences are indicated in bold.
ITS: internal transcribed spacer regions of rDNA; LSU: 28S rDNA of large subunit; *TUB2*: β-tubulin.
^{T}Type strain.

RESULTS AND DISCUSSION

Paraconiothyrium kelleni Santelices, Campos-Quiroz, Carrasco-Fernández, M. Guerra & J.F. Castro, Persoonia 49: 305 (2022) [MB#845530]

Specimen collection: Bibongsan, Jecheon, Chungcheongbuk-do, Korea (37°01′37″N, 127° 16′45″E), isolated from soil.

Morphology of KNUF-21-66Q1

Cultural characteristics: The growth of the colony of strain KNUF-21-66Q1 was moderate on PDA, OA, MEA, and CMA after 10 days of incubation at 25°C (Fig. 1). Its cultural and morphological characteristics were compared with those of the previously described strain *P. kelleni* (Table 2). Colonies grown on PDA were 31-33 mm in diameter in 10 days and had an even, pale orange, white margin followed by light to dark brown showing a concentric and radiating pattern. The reverse side showed buff margin and white to gray circular zones toward the center (Fig. 1A). Colonies grown on MEA were 34-36 mm in diameter in 10 days showing white, creamy white near the edge and pale yellow to dark brown toward the center. The reverse side showed an umber brown in center surrounded by pale fulvous (Fig. 1B). Similarly, on CMA, the colony reached 50-51 mm in diameter in 10 days and showed a transparent white color, flat mycelium, with the reverse side also showing the same features (Fig. 1C). On OA, the colonies reached 48-49 mm in diameter in 10 days and exhibited flat, entire margin, areas of sparse hyaline mycelium, and yellow concentric rings in old culture. The reverse side showed a colorless margin with white wooly floccose and fluffy aerial mycelium (Fig. 1D).

Morphological characteristics: Conidiomata were asexual morphs that were pycnidial, discrete, mostly submerged in the media, and soft, solitary, or clustered, ostiolate, measuring 300–500 μm in diameter with a shape of globose to subglobose, black-brown in color (Fig. 1E). Pycnidium was observed after 5–6 weeks, showing a thick wall yellowish in color (Figs. 1F and 1G). Conidiogenous cells were smooth, hyaline, and flat at the base and tapered considerably toward the tip with phialidic nature and globose to ampulliform, and the size ranged from 6.0-9.0×3.0-6.0 μm (n=15). Conidiophores were not observed separately and reduced to conidiogenous cells (Fig. 1H). Conidia were hyaline and later turned pale brown, slightly golden when matured, obovoid to slightly ellipsoidal, aseptate, smooth, and guttulate, although non-guttulate conidia were also observed. The conidia size was 3.0-4.7×2.1-3.3 μm (n=100) with an average length-to-width (L/W) ratio of 1.6 (Fig. 1I).

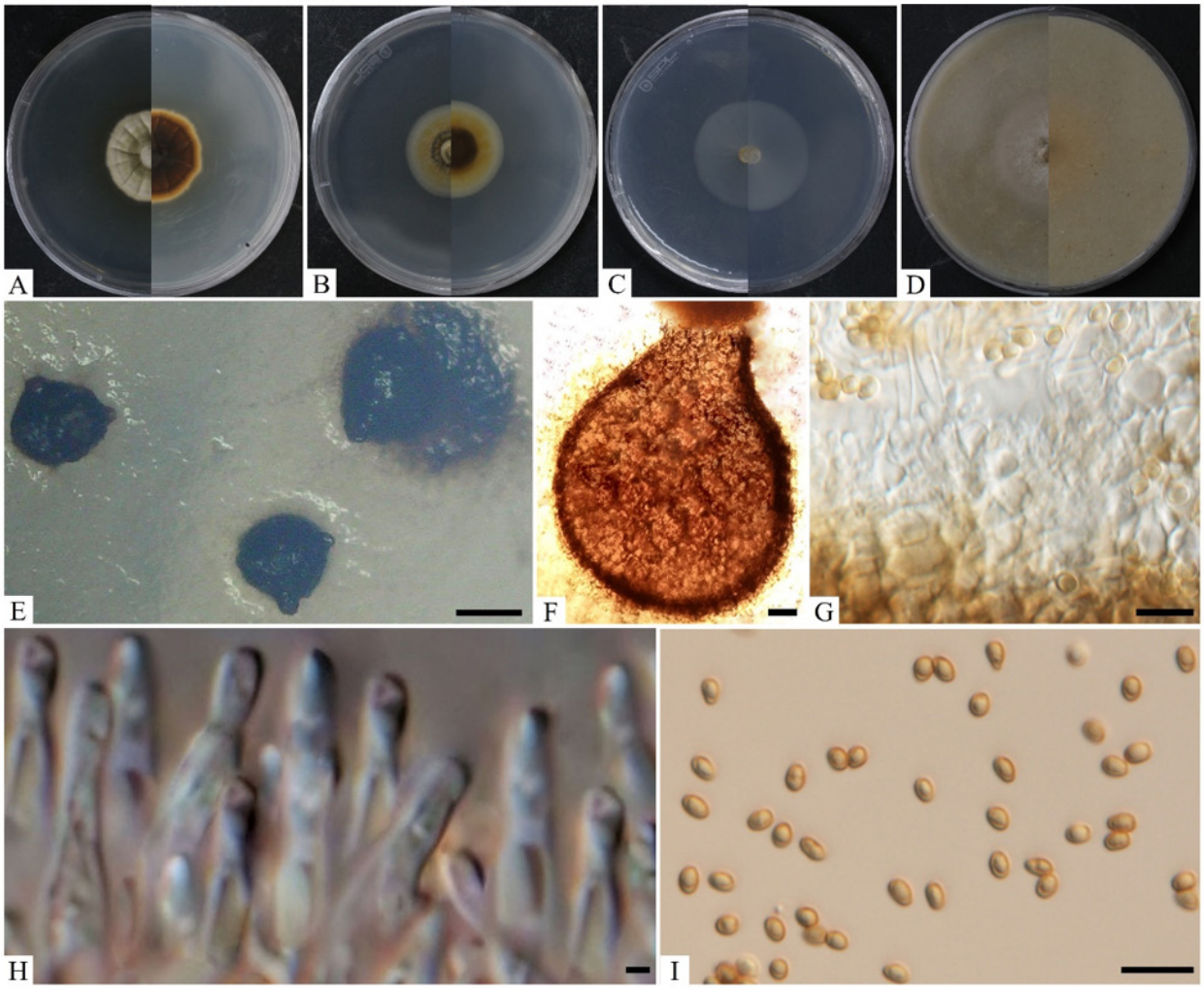

Fig. 1. Cultural and morphological characteristics of KNUF-21-66Q1. Colonies on potato dextrose agar (PDA) (A), malt extract agar (MEA) (B), cornmeal agar (CMA) (C), and oatmeal agar (OA) (D), front and reverse, respectively, after 10 days at 25℃. Conidiomata after 21 days at 25℃ from OA (E), pycnidium (F), pycnidial wall (G), conidiogenous cells (H), and conidia (I). Scale bars: E=200 μm, F=20 μm; G-I=10 μm.

Table 2. Morphological comparison of KNUF-21-66Q1 with the reference and closest species of *Paraconiothyrium*

Characteristics	KNUF-21-66-Q1[a]	*P. kelleni* CBS 149290[Tb]	*P. fuckelli* CBS 797.95[Tc]
Colony	OA: flat, entire margin, hyaline, and yellow concentric rings in old culture.	OA: flat, entire margin, hyaline mycelium, and yellow concentric rings.	OA: glabrous, colorless margin, immersed, center faintly hazel or ocherous, pure white.
Conidiomata	Pycnidial, globose to subglobose, black-brown, 300-500 μm.	Pycnidial, globose to subglobose, black-brown, 130-336 μm.	Pycnidial, glabrous, black, 300-400 μm.
Condiogenous cells	Smooth, hyaline, phialidic nature, globose to ampulliform, 6.0-9.0×3.0-6.0 μm.	Globose to ampulliform, mucronate apex, hyaline, smooth, (3.2-) 3.9-4.4 (-5.6)×(2.4-) 3.2-3.7 (-4.9) μm.	Broadly ampulliform to globose, holoblastic, often annellidic, hyaline, 4-10 (-13)×3-5 μm.
Conidia	Obovoid to ellipsoidal, aseptate, smooth, guttulate, hyaline to brown, slightly golden, 3.0-4.7×2.1-3.3 μm.	Ellipsoidal, smooth, aseptate, guttulate, hyaline to pale brown, (3.2-) 3.6-3.8 (-4.2)×(2.2-) 2.4-2.6 (-3.0) μm.	Subglobose to ellipsoid or obovoid, hyaline, olivaceous-brown, smooth, aseptate, 3-4×2-3 (-3.5) μm.

OA: oatmeal agar.
[a]Fungal strain investigated in this research, [b,c]Sources of descriptions [9,3], [T]Type strain.

Molecular phylogeny of KNUF-21-66Q1

The NCBI database from the BLAST search showed that the ITS (569 bp) regions sequences of the strain KNUF-21-66Q1 exhibited a high similarity of 99.8% with the different strains of *P. kelleni* (CBS 149289, CBS 149290^{T}, and CBS 149291), followed by maximum similarities of 95.0-95.8% with *P. variabile* (JAC12363, 18EPLE022, CBS 121163, and CBS 680.83). The LSU sequences (815 bp) of the strain shared 100% similarity with the strains of *P. kelleni* (CBS 149289, CBS 149290^{T}, and CBS 149291), 99.8-99.9% similarities with several strains of *P. fuckelii* (T235I1, CBS 797.95, CBS 764.71B, and CBS 653.85), and *P. rosae* MFLU 15-1115. *TUB2* (620 bp) exhibited the highest similarity of 99.3-99.8% with the strains of *P. kelleni* (CBS 149289, CBS 149290^{T}, and CBS 149291), followed by maximum similarities of 89.5-91.7% with the strains of *P. fuckelii* (CVG482, JZB320004, CBS 653.85, and CBS 797.95). Concatenated datasets of ITS, LSU, and *TUB2* sequences were used to determine the molecular relationship between the isolated Korean strain and closely related *Paraconiothyrium* species (Fig. 2). The tree topology of the neighbor-joining method in the phylogenetic analysis confirmed that the strain KNUF-21-66Q1 was closely clustered with the previously identified *P. kelleni*. Hence, KNUF-21-66Q1 was identified as *P. kelleni*, which was isolated from soil in Korea.

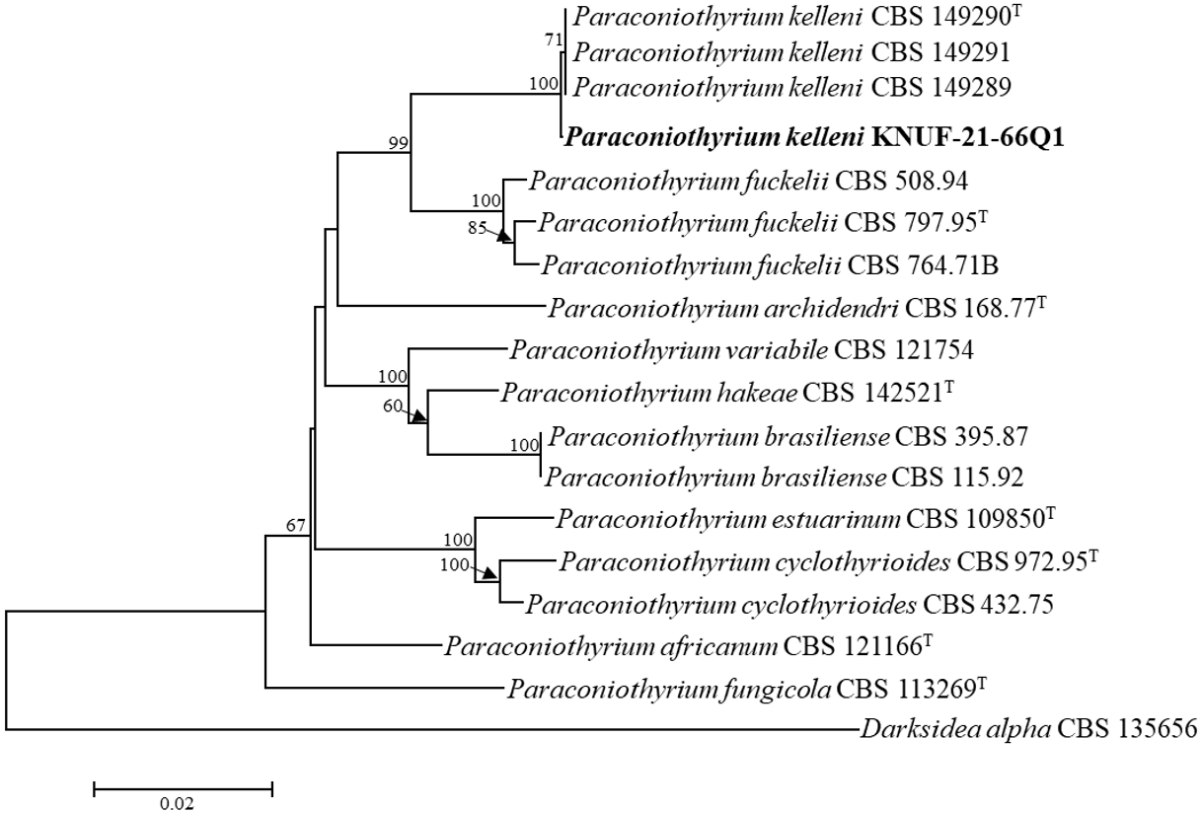

Fig. 2. Neighbor-joining phylogenetic analysis of KNUF-21-66Q1 based on combined sequence data of β-tubulin (*TUB2*), 28S rDNA of large subunit (LSU), and internal transcribed spacer (ITS) regions showing the phylogenetic position among the closest species of *Paraconiothyrium*. The strain isolated in this study is in bold, and the bootstrap values (>50%) are based on 1000 replicates. *Darksidea alpha* CBS 135656 was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Paraconiothyrium species are found to be associated with wilting of grapes, trunk rots of vines, pear, and apple trees, and cane blight of raspberry [19-21]. Apart from plant pathogens, species such as *P. cyclothyrioides* are associated with the causative agent of human cutaneous phaeohyphomycosis [22]. Three unreported *Paraconiothyrium* species have already been recorded from Korea, viz., *P. brasiliense* from Chinese maple leaf [23], *P. archidendri* from plant litter in freshwater, and *P. fuckelii* from the wild plant *Rubus oldhamii* [24,25]. However, some of the *Paraconiothyrium* species are rationally utilized for biocontrol, bioremediaton, and antibiotic production. Pathogens such as *Phytophthora* spp. and *Colletotrichum* spp. have been found to exert an antagonistic effect with *P. brasiliense* [26]. Moreover, various bioactive compounds were isolated from *Paraconiothyrium* species, such as the antitumour drug "Paclitaxel," also known as taxol, produced by endophytic fungi *P. brasiliense* and *P. variabile* and used to treat various cancers [7,27,28]. In total, 150 secondary metabolites have been isolated from *Paraconiothyrium* species, including novel diterpenes, sesquiterpenes, polyketides, and aromatic compounds [5].

In conclusion, further research is required to investigate the etiology, pathogenicity of *P. kelleni* and the sources of secondary metabolites based on the ecological and environmental conditions in Korea. To the best of our knowledge, this is the first record of *P. kelleni* in Korea.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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