RESEARCH ARTICLE

First Report of *Apinisia keratinophila* Isolated from Soil in Korea

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ABSTRACT

In 2022, a fungal isolate, designated KNUF-22-049, was obtained from a soil sample collected from a field in Sang-ju, Gyeongsangbuk-do, Korea. The strain exhibited cultural and morphological characteristics, including colony color and size and the shapes and sizes of conidiogenous cells and conidia, that were consistent with those of *Apinisia keratinophila*. A phylogenetic analysis using the sequences of the internal transcribed spacer regions and the large subunit of the nuclear ribosomal RNA gene confirmed that isolate KNUF-22-049 is most closely related to *A. keratinophila* CBS 947.73^{T} at a species level. Both the morphological observations and phylogenetic analysis indicated that KNUF-22-049 is indeed an *A. keratinophila* strain. This study represents the first documentation of *Apinisia keratinophila* in Korea.

Keywords: *Apinisia keratinophila*, Onygenales, Soil-inhabiting fungi, Taxonomy

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INTRODUCTION

The genus *Apinisia* was initially established by La Touche in 1969 within the family Gymnoascaceae, with the type species *A. graminicola* [1]. In 2007, the genus was transferred to the family Onygenaceae by Lumbsch and Huhndorf [2]. Recently, the novel family Apinisiaceae was established to accommodate the two genera, *Arachnotheca* and *Apinisia*, with the latter serving as the type genus [3]. Morphologically, the two genera differ in their conidiogenous cells and conidia. *Apinisia* produces monoblastic or polyblastic conidiogenous cells and globose, oval to pyriform conidia, while *Arachnotheca* is characterized by simple or branched conidiogenous cells and arthroconidia [3]. According to Apinis and Rees [4], *A. queenslandica* was initially considered as a second species of the genus *Apinisia*, but it was reclassified into the genus *Uncinocarpus* by Sigler et al. [5] in 1998, and later moved to the genus *Amauroascus* by Solé et al. [6]. Similar taxonomic status changes were observed with *A. racovitzae*, which was considered to be the third member of the genus *Apinisia* but is now regarded as a species of the genus *Kuehniella* [7-9]. Currently, the genus *Apinisia* consists of three species: *A. graminicola*, *A. pyriformis*, and *A. keratinophila*. Unlike the other two, the latter has undergone taxonomic reclassification. Initially isolated

from soil, it was described in 1978 as the type species of the novel genus *Myriodontium*, bearing the name *M. keratinophilum* [10]. Recent phylogenetic analyses performed by Li et al. [3] using internal transcribed spacer (ITS) and the 28S large subunit of the nrRNA gene (LSU) sequences revealed that five species, including *M. keratinophilum* (strains CBS 948.73^{T} and 947.73), clearly separated from all known families in the order Onygenales. This led to the establishment of the above-mentioned family Apinisiaceae, and *M. keratinophilum* was reclassified as a member of the genus *Apinisia*, now named *A. keratinophila*.

The aim of this study was to morphologically and molecularly characterize an *Apinisia* species previously unreported in Korea. We describe the identification of a soil-inhabiting fungal strain belonging to the genus *Apinisia*, isolated from soil near a deciduous forest, using cultural, morphological, and molecular phylogenetic approaches.

MATERIALS AND METHODS

Sample collection and fungal isolation

The fungal isolate used in this study, KNUF-22-049, was collected from a field soil sample in Sangju, Gyeongsangbuk-do, Korea (36°17'47.87"N 128°1'6.25"E). The soil sample was collected dried naturally, and then stored in a plastic bag at 4°C for subsequent analysis. The fungus was isolated using a conventional dilution planting technique. One gram of the soil sample was mixed with 10 mL of sterile distilled water, and the resulting suspension was vortexed before performing a serial dilution. Subsequently, 50-100 μL of each suspension was distributed onto potato dextrose agar (PDA; Difco, Detroit, MI, USA) in Petri dishes. To evaluate the growth rate of fungal colonies, Petri dishes underwent incubation at 25°C for 7 days. Growing colonies were then individually isolated by transferring them onto fresh plates, which were subsequently incubated under identical conditions. The pure cultures were systematically cultivated on PDA agar at a temperature of 25°C and four fungal strains sharing identical morphologies, namely KNUF-22-049, KNUF-22-050, KNUF-22-051, KNUF-22-052 were isolated. Subsequent sequencing of the ITS regions revealed that all isolates were identical. Therefore, one of these strains, KNUF-22-049, was selected for further investigation. The fungal strain was preserved as a glycerol suspension (20% w/v) at a temperature of -70°C. Stock culture of the isolate, namely, KNUF-22-049 (NIBRFGC000509836) was deposited in the National Institute of Biological Resources (NIBR).

Morphological characterization

For its cultural and morphological analysis, isolate KNUF-22-049 was cultivated on PDA and then incubated at 25°C in darkness for 14 days, and the characteristics of the colonies, including color, size and shape, were meticulously recorded. Additionally, growth was evaluated on phytone extract agar (PYE; Sigma-Aldrich, St. Louis, USA), Sabouraud dextrose agar (SDA; MBcell, Seoul, Korea), and yeast-starch agar (YpSs). Observations, measurements, and photographs of colony, conidia, and conidiogenous cell

characteristics were collected after the 14-day incubation period using a light microscope (BX-50, Olympus, Tokyo, Japan).

DNA extraction, PCR and sequencing

A phylogenetic analysis was conducted using the internal transcribed spacer (ITS) regions and the 28S large subunit (LSU) of the ribosomal DNA. The total genomic DNA of strain KNUF-22-049 was extracted from fungal mycelia cultivated on PDA using the HiGene Genomic DNA Prep Kit (Biofact, Daejeon, Korea) following the manufacturer's instructions. Subsequently, the ITS regions and LSU were amplified using the primer pairs ITS1F and ITS4 [11] and LROR [12] and LR5 [13]. The amplified PCR products were purified using the ExoSAP-IT PCR Product Cleaning Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Sanger sequencing was conducted by Bioneer (Daejeon, Korea). Phylogenetic neighbors were identified, and similarities with closely related sequences were calculated using the BLAST search program available on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis

The ITS and LSU sequences were aligned using the Clustal X program [14]. Separate phylogenetic trees were built by using the LSU sequences and concatenated partial ITS and LSU sequences, with the expectation that incorporating longer sequences would enhance both the resolution and reliability of the analysis. Nineteen related taxa were retrieved from GenBank and refined using the BioEdit program [15]; comprehensive information regarding these strains is outlined in Table 1. Multiple sequence alignments were performed using the Clustal X program [14]. Gaps and the 5' and 3' ends of the alignments were manually adjusted in BioEdit [15]. Evolutionary distance matrices were produced following the methodology outlined by Kimura [16]. Subsequently, phylogenetic trees were created using the neighbor-joining algorithm [17] in the MEGA7 program [18], and bootstrap values were computed from 1,000 replications.

Table 1. Isolates used in this study and their GenBank accession numbers

Species	Strain	GenBank accession numbers	
		ITS	LSU
Apinisia graminicola	CBS 721.68^{T}	–	NG_056945
Apinisia keratinophila	CBS 947.73^{T}	KT155969	KT155314
Apinisia keratinophila	**KNUF-22-049**	**LC802117**	**LC802120**
Apinisia pyriformis	CGMCC 3.22427^{T}	OQ798931	OQ758128
Apinisia pyriformis	CGMCC 3.22430	OQ798927	OQ758124
Amauroascus albicans	NRRL 5141^{T}	NR_111883	NG_057151
Amauroascus albicans	FMR 18031	ON720231	ON720770
Arachnotheca glomerata	CBS 348.71^{T}	NR_111884	NG_056931
Arachnotheca glomerata	CBS 349.71	MH860158	MH871926
Arachnotheca pulvereum	CGMCC 3.22341^{T}	OQ798932	OQ758129
Arachnotheca pulvereum	CGMCC 3.22347	OQ798933	OQ758130
Arthroderma ciferrii	CBS 625.79^{T}	KT155948	KT155293
Arthropsis hispanica	CBS 351.92^{T}	HE965758	HE965759
Arthropsis hispanica	FMR 19035	ON720233	ON720772
Chrsosporium pallidum	GCMCC 3.19575^{T}	MK329090	MK328995
Chrysosporium carmichaelii	CBS 643.79^{T}	NR_077133	NG_058857
Chrysosporium sulfureum	CBS 634.79^{T}	KT155953	KT155298
Chrysosporium undulatum	CBS 964.97^{T}	KT155970	KT155315
Harorepupu aotearoa	PDD 105262^{T}	KP683350	NG_059544
Kuehniella racovitzae	CBS 156.77^{T}	MZ435253	AB040696

ITS: Internal transcribed spacer regions; LSU: 28S large subunit of the nrRNA gene.
^{T} Type strain.
The newly generated sequence is indicated in bold.

RESULTS AND DISCUSSION

Cultural and morphological characteristics

To observe the morphological characteristics, the strain KNUF-22-049 was cultured on PDA, PYE, SDA and YpSs at 25°C for 14 days, producing colony diameters of 41.1-42.9, 24.4-29.2, 31.4-31.7, and 35.2-36.4 mm, respectively (Fig. 1A-D).

White to yellowish-white colonies formed on all media, and PDA was used to observe the morphological characteristics. The fungal colonies formed wavy mycelia at the edges and had cottony structures overall. They formed peridial hyphae that were differentiated, septate branched, anastomosed, densely interwoven, smooth, and thin-walled, measuring 1.7-2.5 μm wide. Conidiogenous cells were intercalary or terminal, more or less cylindrical, and 10-30 μm in length×2.5-5 μm in diameter (Fig. 1E). Conidia were solitary, continuous, subglobose to dacryoid, hyaline, smooth, and 2.5-3.7×2.1-3.0 μm in diameter (Fig. 1F). These cultural and morphological features of strain KNUF-22-049 share the typical characteristics of *A. keratinophila*. In contrast, they distinguish strain KNUF-22-049 from its relative, *A. pyriformis.* In particular, the isolate exhibited a different color on the reverse side of the colony and produced a differently shaped colony. Additionally, KNUF-22-049 differed from *A. pyriformis* by its larger conidia (2.5-3.7×2.1-3.0

μm vs 2.0-2.5×2.0-2.5 μm). Thus, the cultural and morphological characteristics of strain KNUF-22-049 indicate its affiliation with *Apinisia keratinophila* (Table 2).

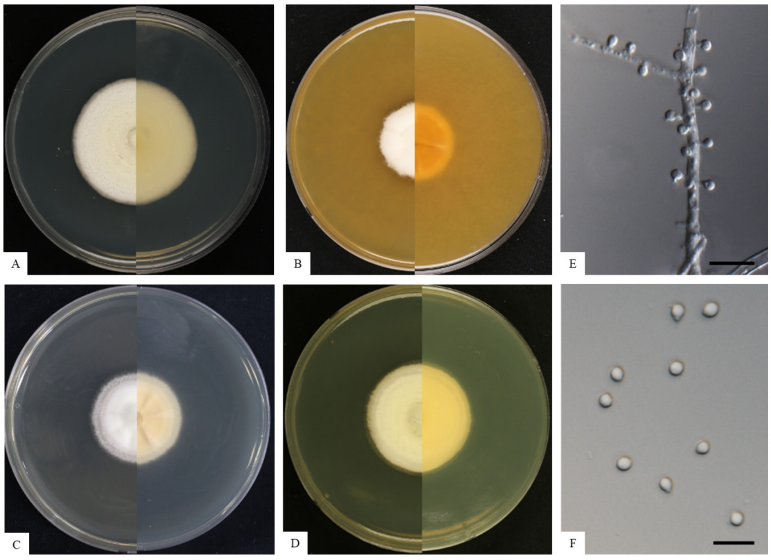

Fig. 1. Culture and morphological characteristics of *Apinisia keratinophila* KNUF-22-049. A, Colony on potato dextrose agar; B, Colony on phytone extract agar; C, Colony on sabouraud dextrose agar; D, Colony on yeast-starch agar; E, Mature conidiogenous cells and conidia; F, Mature conidia (scale bars: E, F=10 μm).

Table 2. Morphological characteristics of the strain isolated in this study, KNUF-22-049, and those of previously reported *Apinisia* isolates for comparison

Characteristics		KNUF-22-049	*Apinisia keratinophila*[a, b] CBS947.73[T]	*Apinisia pyriformis*[c] CGMCC 3.22427[T]
Colony	Color	PDA: White, reverse uncolored; PYE: White, reverse greyish-orange; YpSs: Yellowish-white, reverse uncolored	PDA: White to yellowish-white, reverse uncolored to greyish-orange; PYE: White to yellowish-white, reverse reddish-orange; YpSs: White, reverse uncolored	PDA: White, reverse white to yellowish brown
	Size (mm)	Mean daily colony spread of 1.9-2.5 mm on PDA, 2.4-2.4 mm on PYE, 1.7-2.4 mm on YpSs.	Mean daily colony spread of 1.8-2.3 mm on PDA, 2.0-2.7 mm on PYE, 1.7-2.2 mm on YpSs.	67-69 mm diam after 3 weeks on PDA
	Shape	Cottony, with concentric zones, wavy mycelia at the edges	Cottony, with concentric zones, white patches at the margin	Felty to pulverulent, annular, margin undulate
Conidiogenous cells	Size (μm)	10-30×2.5-5	11-25×2.3-4.5	13-34×1.5-3.0
	Shape	Conidiogenous cells intercalary or terminal, more or less cylindrical	Conidiogenous cells intercalary or terminal, hyaline, more or less cylindrical	Conidiogenous cells phialidic, monoblastic, terminal, hyaline, smooth- and thin-walled, determinate, discrete, cylindrical.
Conidia	Size (μm)	2.5-3.7×2.1-3.0	2.0×3.0	2.0-2.5×2.0-2.5
	Shape	Conidia solitary, continuous, subglobose to dacryoid, hyaline, smooth	Conidia solitary, formed more or less simultaneously, dry, continuous, subglobose to dacryoid, hyaline, smooth	Conidia oval to pyriform, globose, smooth and thin-walled, hyaline, aseptate

PDA: Potato dextrose agar; PYE: Phytone extract agar; YpSs: Yeast-starch agar.
[a] Source of the description [20].
[b] Source of the description [10].
[c] Source of the description [3].

Phylogenetic relationships of KNUF-22-049

To genetically identify the isolated fungal strain, nucleotide sequences of the ITS regions and LSU were acquired (865 bp and 617 bp, respectively) and compared with selected sequences from the GenBank database using BLAST. The ITS sequence of KNUF-22-049 exhibited a 98.7% similarity with that of *Apinisia keratinophila* OTU_303 (MN957308) but was relatively distant from its other close neighbors: *Kuehniella racovitzae* (former *A. racovitzae*) CBS 156.77 (MZ435253), *Chrysosporium carmichaelii* E00083342 (KC923439), and *C. pallidum* CGMCC 3.19575^{T} (NR_172829), which showed only 90.5, 89.3, and 88.6% identities, respectively. Based on the sequence of the LSU gene, the isolate showed 100% similarity with *A. keratinophila* CBS 947.73^{T} (NG_063938), 99.4% with *Apinisia pyriformis* CGMCC 3.22431 (OQ758125), 98.5% with *Apinisia graminicola* CBS 721.68^{T} (NG_056945), 98.5% with *C. carmichaelii* CBS 643.79^{T} (NG_058857), and 96.7% with *Arachnotheca glomerata* CBS 349.71 (MH871926). Notably, *A. keratinophila* emerged as the closest phylogenetic relative in both cases.

However, these findings unequivocally demonstrate that none of the gene sequences provided precise identification of strain KNUF-22-049 at the species level. Therefore, a multilocus analysis was conducted using concatenated ITS and LSU sequences. As depicted in Fig. 2, the topology of the resulting neighbor-joining phylogenetic tree revealed that the isolate clustered with the type strain of *A. keratinophila*, indicating a close association consistent with a species level relationship. Unfortunately, the ITS sequence was not available for the type species of the genus *Apinisia*, *A. graminicola*. Therefore, an additional NJ phylogenetic tree was constructed using only the LSU sequences to clarify the phylogenetic relationship between the isolate and *A. graminicola*. The position of KNUF-22-049 in the NJ tree clearly demonstrates that it is distinct from *A. graminicola* but again supported a close, species level relationship with *A. keratinophila* (Fig. 3). Collectively, the morphological and molecular analyses demonstrated that strain KNUF-22-049 belongs to *A. keratinophila*. To the best of our knowledge, our study represents the first report of this fungal species in Korea.

Apinisia keratinophila (formerly *Myriodontium keratinophilum*) has been isolated from soil and river sediments in various regions worldwide, including India [19], Spain [20], Italy, Germany, and California [21]. Our study confirms that soil is a primary ecological niche for this fungal species. *Apinisia keratinophila* exhibits keratinolytic properties and has also been isolated from various keratinous materials, such as nails, hair, and feathers [21]. While *A. keratinophila* is primarily associated with the degradation of such keratinaceous materials, there is limited information available on its pathogenicity toward humans [21,22]. This fungal species is not commonly considered a primary pathogen in humans. However, it can be a potential opportunistic pathogen, especially in immunocompromised individuals or in those with pre-existing skin conditions, indicating that further research is needed to better understand its pathogenicity. As a keratinophilic fungus, *A keratinophila* primarily occupies an ecological niche in soil and environments rich in keratin-containing materials and it is not typically associated with plant diseases. The closely related species *A. graminicola,* is known to cause a leaf spot disease in the grasses of *Miscanthus giganteus* and *Brachypodium distachyon* [23], emphasizing the need for further research to determine whether *A*

keratinophila also possesses plant pathogenicity.

In conclusion, our findings expand our understanding of the fungal diversity within Korea. Confirming the presence of *A. keratinophila* highlights its potential ecological significance and calls for further investigation into its distribution and ecological roles. Additionally, the combined morphological and phylogenetic characterization approach employed in this study serves as a valuable framework for accurate fungal identification, particularly in cases where morphological traits alone may not be conclusive. These findings contribute to the broader body of knowledge concerning fungal taxonomy and biodiversity, with implications for various fields, including agriculture, ecology, and human health. Moreover, further research endeavors exploring the ecological niche and potential pathogenicity of *A. keratinophila* in Korean environments, as well as its interactions with host organisms and surrounding ecosystems, are warranted.

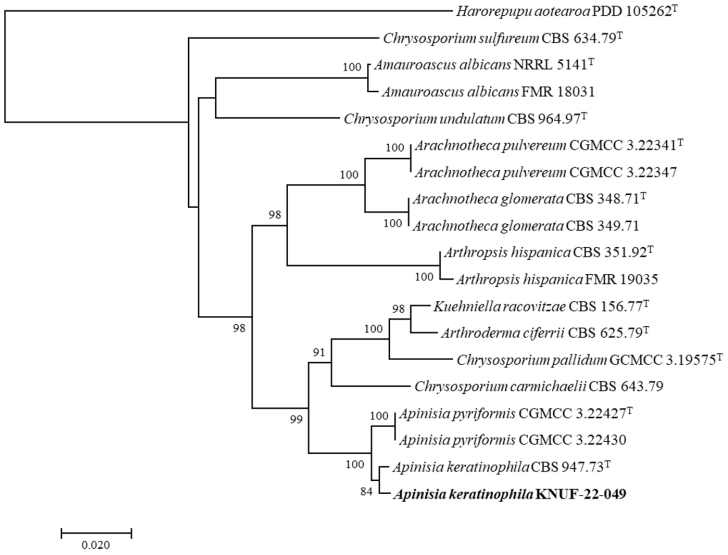

Fig. 2. Neighbor-joining phylogenetic tree based on the concatenated sequences of the 28S large subunit of the nrRNA gene (LSU) gene and internal transcribed spacer regions (ITS) regions. The phylogenic analysis shows the position of *Apinisia keratinophila* KNUF-22-049 among related strains. Bootstrap values (based on 1,000 replications) greater than 70% are shown at the branch points. The tree was rooted using *Harorepupu aotearoa* PDD 105262^{T} as the outgroup. The scale bar represents 0.02 substitutions per nucleotide position. 'T' indicates type strain.

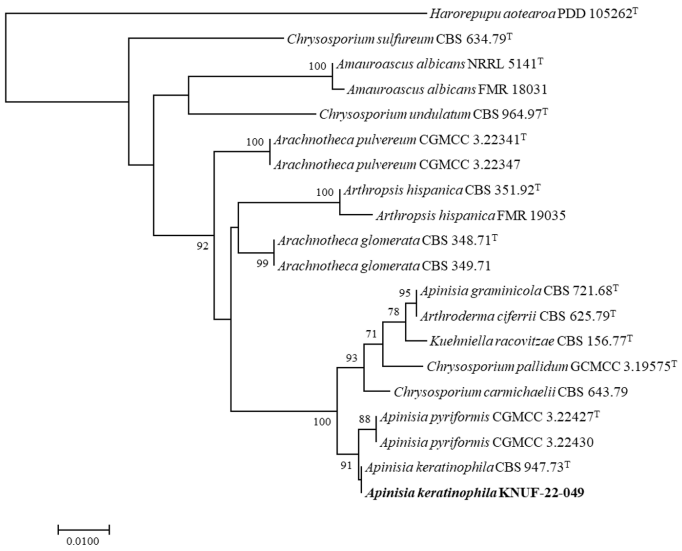

Fig. 3. Neighbor-joining phylogenetic tree based on 28S large subunit of the nrRNA gene (LSU) gene sequences showing the position of *Apinisia keratinophila* KNUF-22-049 among related strains. Bootstrap values (based on 1,000 replications) greater than 70% are shown at the branch points. The tree was rooted using *Harorepupu aotearoa* PDD 105262^{T} as the outgroup. The scale bar represents 0.01 substitutions per nucleotide position. 'T' indicates type strain.

CONFLICT OF INTERESTS

The authors declare that they have no potential conflicts of interest.

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