RESEARCH ARTICLE

Morphological and Molecular Characterization of the Newly Reported *Penicillium pimiteouiense* from Field Soil in Korea

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ABSTRACT

Penicillium pimiteouiense was discovered in South Korea during an investigation of fungal communities in soil collected from the Gyeongsangbuk-do province. In this study, we performed molecular analysis of this fungal isolate using internal transcribed spacer rDNA, β-tubulin, and Calmodulin gene sequences. We also performed morphological analysis using five agar media, potato dextrose, oatmeal, malt extract, czapek yeast extract, and yeast extract sucrose. In this study, the molecular and morphological analyses of *P. pimiteouiense* with detailed descriptions and figures has been carried out.

Keywords: β-tubulin, Calmodulin, Discovery, *Penicillium*, Phylogeny

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INTRODUCTION

Penicillium is a genus of Ascomycetous fungi belonging to the Aspergillaceae family, which plays an important role in the environment, and food and drug production [1]. *Penicillium* is a large and diverse genus that currently comprises 354 known species [2]. It has a cosmopolitan distribution and huge economic impact on human life [3]. Its main role in nature is decomposing organic materials, such as the devastating rotting caused by pathogenic species in pre- and post-harvest food crops [3]. Some species have positive effects on the food industry, such as in the production of cheeses, like Camembert, and fermented sausages [4].

Morphological identification is the traditional method used for the identification of *Penicillium* species [5]. Recently, along with morphological identification, molecular methods are being used extensively to study phylogenetic relationships among *Penicillium* species [6, 7]. In addition, fungal species can be identified using morphological, biochemical, and molecular markers [8]. Molecular tools are required

for the identification of *Penicillium* species, but the choice of accurate markers for use in the genus is challenging [8]. rDNA internal transcribed spacer (ITS), β-tubulin (tub2/BenA), and calmodulin (C*aM*) are the most commonly used markers for the identification of fungal species [2]. Using these morphological and molecular characterization tools, we report for the first time new *P. pimiteouiense* fungal isolate from soil samples in South Korea. *P. pimiteouiense* was first isolated from polycystic kidney cultures [9], and has also been isolated from sandy beach soil samples in Penang Island, Peninsular Malaysia [10]. This study aimed to (i) identify the *Penicillium* isolates using morphological and molecular characteristics, (ii) use a precise molecular identification tool to compare between the species of *Penicillium*, and (iii) compare and contrast between the previously reported species and our newly identified species.

MATERIALS AND METHODS

Soil sampling and isolation of fungi

Soil samples were collected from agricultural fields at various locations in Gyeongsangbuk-do (35°33'03.95"N, 128°28'27.91"E), Korea, in 2017. Soil samples were collected from a depth of 0-15 cm, air dried, and stored in plastic bags at 4°C until further use. Fungi were isolated using a conventional dilution plating technique [12] and cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) supplemented with 100 μg/L chloramphenicol (a bacteriostatic agent) for 5-7 days at 25°C until fungal colony growth was observed. The pure cultures were preserved on PDA slants at 4°C for future use.

Morphological characterization

The morphological characteristics of the isolate KNU17-56 was observed on PDA (MB Cell, Los Angeles, CA, USA), oatmeal agar (OMA; MB Cell), malt extract agar (MEA), Czapek yeast extract agar (CYEA), and yeast extract sucrose agar (YESA). All media were prepared as previously described [12]. The strains were inoculated at three points on 9-cm petri dishes and incubated at 25°C in the dark for 7 days. After incubation, the diameter of the colonies on various agar media was measured, and the degree of sporulation was determined. Colony color (obverse and reverse sides) was described based on the guidelines provided by Kornerup and Wanscher [13]. Photomicrographs were taken using an HK 3.1 CMOS digital camera (KOPTIC Korea Optics, Seoul, Korea) attached to an Olympus BX50F-3 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a scanning electron microscope (LEO Model 1450VP Variable Pressure Scanning Electron Microscope, Carl Zeiss, Cambridge, MA, USA).

Genomic DNA extraction, PCR amplification, sequencing, and data analysis

Fungal DNA was extracted from KNU17-56 using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The ITS, *BenA*, and C*aM* gene sequences were amplified using the following primers: ITS1 (5′- TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [14]; Bt2a (5′-GGTAACCAAATCGGTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTT-3′) [15]; and CMD5 (5′-CCGAGTACAAGGCCTTC-3′) and CMD6 (5′-CCGATAGAGGTCATAACGTGG-3′) [16]. The amplicons were sequenced and then analyzed using an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Details of the PCR used for amplification and sequencing are listed in Table 1. The sequences were compared with reference ITS, *BenA*, and C*aM* gene sequences from GenBank at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool [17]. Nucleotide sequences of the isolate was deposited in the culture collection of the National Institute of Biological Resources (NIBR, Incheon, Korea). The NIBR numbers was NIBRFG0000501884. The nucleotide sequences were also deposited in GenBank and assigned the accession number MH231760 for KNU17-56. ITS, β-tubulin (*BenA*) and Calmodulin (*CaM*) gene sequences of *Penicillium* species and their strains were used to construct a phylogenetic tree for KNU17-56. GenBank accession numbers are given in Table 2. Phylogenetic relationships were analyzed using the molecular evolutionary genetic analysis (MEGA 6) software [18]. A neighbor-joining tree was constructed using the Kimura 2-parameter substitution model [19]. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

Table 1. Thermal cycle programs used for amplification.

Gene	Profile type	Initial denaturing	Cycles	Denaturing	Annealing	Elongation	Final elongation	Rest period
ITS	Standard	95°C, 5min	35	94°C, 30 s	55°C, 30 s	72°C, 1 min	72°C, 8min	4°C
BenA	Standard	95°C, 5min	35	94°C, 45 s	55°C, 45 s	72°C, 1 min	72°C, 7min	4°C
CaM	Standard	95°C, 1min	42	95°C, 1 min	55°C, 30 s	72°C, 10min	72°C, 10min	4°C

ITS: Internal transcribed spacer; *BenA*: Beta–tubulin; *CaM*: Calmodulin.

Table 2. Internal transcribed spacer (ITS), β-tubulin (*BenA*) and Calmodulin (*CaM*) gene sequences of *P. pimiteouiense* used to construct phylogenetic tree of study isolate KNU17-56 along with its strains and GenBank accession numbers

Species	Strains No.	GenBank accession no.		
		ITS	BenA	*CaM*
Penicillium pimiteouiense	NRRL 25542	NR 121258.1	-	-
Penicillium striatisporum	NRRL 26877	NR 121260.1	-	-
Penicillium erubescens	NRRL 6223	NR 121245.1	-	-
Penicillium dimorphosporum	NRRL 5207	NR 121271.1	-	-
Penicillium parvofructum	FMR 15047	NR 153309.1	-	-
Penicillium gallaicum	CBS 167.81	NR 103657.2	-	-
Penicillium laeve	CBS 136665	NR 144843.1	-	-
Penicillium katangense	NRRL 5182	NR 121240.1	-	-
Penicillium meridianum	NRRL 5814	NR 121237.1	-	-
Penicillium alutaceum	NRRL 5812	NR 121238.1	-	-
Penicillium chalabudae	CBS 219.66	NR 144845.1	-	-
Penicillium citreonigrum	NRRL 761	NR 138264.1	-	-
Penicillium citreosulfuratum	IMI 92228	NR 153252.1	-	-
Penicillium wollemiicola	CBS 137177	NR 153244.1	-	-
Penicillium wisconsinense	CBS 128279	NR 153225.1	-	-
Penicillium spinulosum	FRR 1750	NR 077158.1	-	-
Penicillium fusisporum	HMAS 244961	NR 138342.1	-	-
Penicillium thomii	FRR 2077	NR 077159.1	-	-
Penicillium alexiae	CBS 134558	NR 111869.1	-	-
Penicillium decumbens	KAS5896	-	KY469135.1	-
Penicillium waksmanii	ATHUM 5089	-	FJ004443.1	-
Penicillium rolfsii	A1S3-D87	-	KJ767045.1	-
Penicillium ochrochloron	A4S6-1	-	KJ767042.1	-
Penicillium donkii	CBS 188.72	-	JQ965072.1	-
Penicillium italicum	CBS 48984	-	AY674396.	-
Penicillium laeve	DTO270G8	-	KF667365.1	-
Penicillium ovatum	DTO270G7	-	KF667366.1	-
Penicillium erubescens	NRRL:6223	-	HQ646566.1	-
Penicillium paraherquei	-	-	KM023335.1	-
Penicillium catenatum	-	-	KJ834438.1	-
Penicillium menonorum	NRRL:50410	-	HQ646573.1	-
Penicillium pimiteouiense	a4s2 19	-	KC344994.1	-
Penicillium pimiteouiense	DTO266-B	-	-	HQ646580.1
Penicillium crustosum	KAS7461	-	-	JX141578.1
Penicillium allii	NRRL:25542	-	-	AY678584.
Penicillium solitum	-	-	-	KU896851.1
Penicillium camemberti	AS3.6669	-	-	KU896825.1
Penicillium caseifulvum	CBS 424.89	-	-	KU896826.1
Penicillium egyptiacum	CBS 299.48	-	-	EU644063.1
Penicillium kewense	CBS 101134	-	-	JX996965.1
Penicillium osmophilum	IMI40580	-	-	KU896846.1
Penicillium venetum	CBS 183.72	-	-	KU896855.1
Penicillium digitatum	CBS 462.72	-	-	AY678577.1
Penicillium italicum	CBS 201.57	-	-	DQ911135.1
Penicillium marinum	AS3.6678	-	-	KU896842.1
Penicillium expansum	CBS 339.48	-	-	AY678567.1

RESULTS

Morphology of fungal isolate KNU17-56

Macromorphology of KNU17-56

Photomicrographs of the morphological structures of KNU17-56 are shown in Fig. 1. On PDA, the colonies grew moderately and reached a diameter of 40-45 mm after 7 days at 25°C. The front side of the mycelium had a white margin (6 mm) and a creamy white center, while the back side of the colony had a white margin (5 mm) at the edge and red center (Figs. 1A and 1F). The colony sporulation was dense, conidia were in mass, surface was smooth, texture was floccose, form was circular, elevation and entire margin was flat, and exudate was absent. On CYEA, the colonies grew slowly and reached a diameter of 20-25 mm after 7 days at 25°C. The front side of the colony was white and back side was light yellow (Figs. 1B and 1G). The colony sporulation was moderate to dense, conidia were in mass, surface was smooth, texture was floccose, form was circular, elevation and entire margin was raised, and exudate was absent (Figs. 1B and 1G). A woolly appearance was observed on the front side of the colony (Figs. 1B and 1G). On MEA, the colonies grew moderately and reached a diameter of 25-30 mm after 7 days at 25°C. Front side of the colony was white and back side was light yellow (Figs. 1C and 1H). The colony texture was floccose, form was circular, elevation and entire margin was flat (Figs. 1C and 1H). Sporulation was moderate to dense, surface was smooth, exudate was absent, and conidia were in mass. On YESA, the colonies grew moderately and reached a diameter of 22-27 mm after 7 days at 25°C. Front side of the colony was white and back side was pale yellow (Figs. 1D and 1I). The colony sporulation was moderate to dense, exudates were absent, form was circular form, elevation and entire margin was flat (Figs. 1D and 1I), surface was smooth, conidia were in mass. On OMA, the colonies grew rapidly and reached a diameter of 35-40 mm after 7 days at 25°C. Front side of the colony was powdery white and back side was pale yellow (Figs. 1E and 1J). The colony sporulation was dense, texture was floccose, surface was smooth, exudate was absent, form was circular, elevation and entire margin was flat, and conidia were in mass.

Micromorphology of KNU17-56

The conidiophores were monoverticillate and approximately 7-32 μm in length. Penicilli was monoverticillate. The stipes was short (8-12×1.8-2.1 μm) (Fig. 1K). Phialides were ampuliform (4.6-5.7×1.4-3.1 μm; (Table 3). The conidia were present in short chains, globose to subglobose, finely roughened, and 2.0-3.1 μm in size (Fig. 1M and 1N).

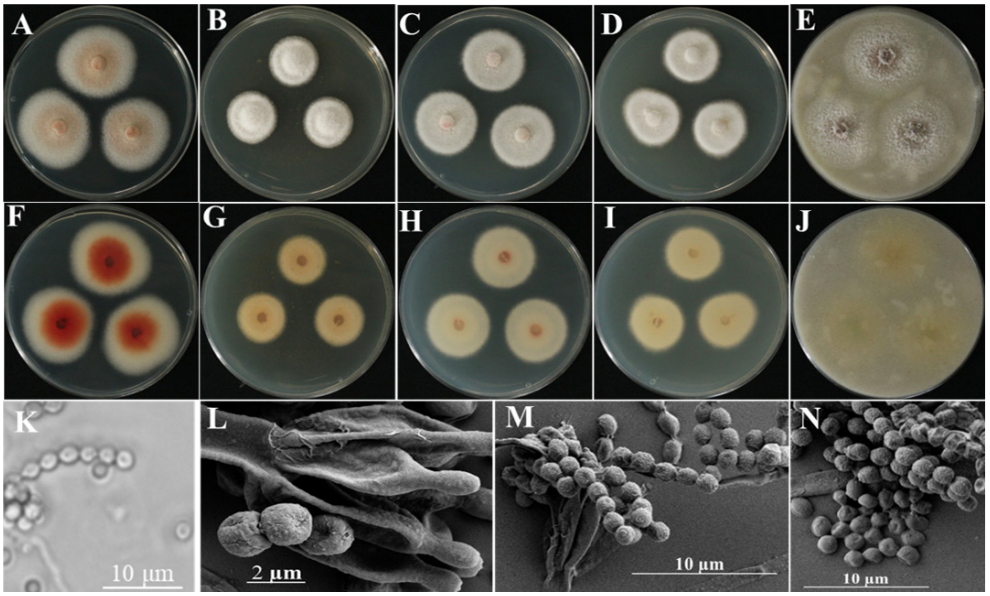

Fig. 1. Morphological characteristics of *Penicillium pimiteouiense* (KNU17-56) grown for 7 days on potato dextrose agar (PDA), czapek yeast extract agar (CYEA), malt extract agar (MEA), yeast extract sucrose agar (YESA), and oatmeal agar (OMA), at 25℃. Front colony from left to right (A-E) and back colony from left to right (F-J) grown on PDA, CYEA, MEA, YESA, and OMA. Conidiophores and conidia images taken by simple microscope (K, L). Scanning electron microscope images of conidiophore (M) and conidia (N) (scale bars: K, M, N=10 μm, L=2 μm).

Table 3. Morphological comparison of the study isolate KNU17-56 with the previously reported *Penicillium pimiteouiense*.

Characteristic		Study isolate KNU17-56	*P. pimiteouiense*[a]
Colony	Diameter (mm)	PDA=40-45, CYEA=20-25, MEA=25-30, YESA=22-27, OMA=35-40	PDA=NA, CYEA=16-18, YESA=NA, OMA=NA, MEA=20-22
Conidiophore	Structure	Moderate, born on aerial hyphae	Moderate, born on aerial hyphae
Penicilli	Structure	Monoverticillate	Strictly monoverticillate and nonvesiculate.
Stipe	Size	8~12×1.8~2.1 μm	5~10 (~16)×2~3 μm
	Structure	Short	Short
Phialide	Size	4.6~5.7×1.4~3.1μm	5~6 (~8)×1.5~2.2 (~3) μm
	Structure	Ampuliform	Ampuliform
Conidia	Size	2.0~3.1 μm	1.5~3 μm
	Structure	Born in short chains, globose to subglobose, finely roughened	Born in short chains, globose to subglobose, finely roughened

PDA: Potato Dextrose Agar; CYEA: Czapek Yeast Extract Agar; MEA: Malt Extract Agar; YESA: Yeast Extract Sucrose Agar; OMA: Oat Meal Agar.

Molecular phylogeny of the fungal isolates

Molecular phylogeny of KNU17-56

The ITS, *BenA*, and C*aM* gene sequences were used to study and compare the phylogenetic relationships between KNU17-56 and previously described *Penicillium* species. 18S-ITS1-5.8S-ITS2-28S rDNA sequences of KNU17-56 most closely related to *P. pimiteouiense* (NR121258.1), forming a monophyletic clade group with a bootstrap value of 99% (Fig. 2). In addition, tub2/BenA gene sequences of KNU17-56 were compared with the sequences deposited in the NCBI database, and KNU17-56 showed a close resemblance to *P. pimiteouiense* (KC344994.1) (Fig. 3), with a bootstrap value of 99%. Moreover, for further confirmation, the C*aM* gene sequence of KNU17-56 was compared with the C*aM* gene sequences of previously reported *P. pimiteouiense* in the NCBI database. The results obtained from the neighbor-joining phylogenetic tree suggested that KNU17-56 closely resembled *P. pimiteouiense* (HQ646580.1), forming a monophyletic clade with a bootstrap value of 100%. (Fig. 4).

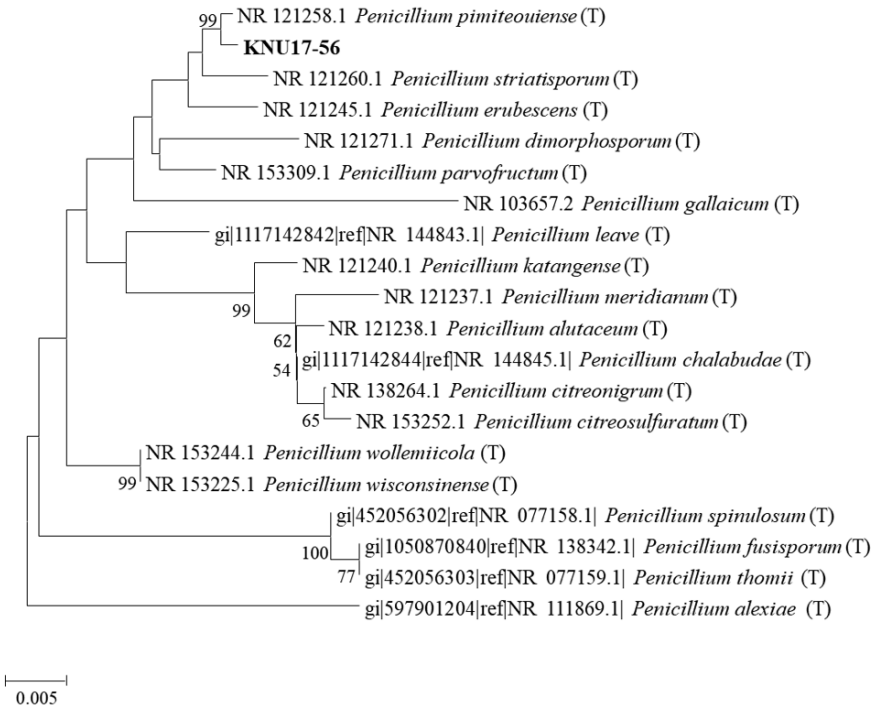

Fig. 2. Neighbor-joining phylogenetic analysis of the partial 18S-ITS1-5.8S-ITS2-28S rDNA sequences of *Penicillium pimiteouiense* (KNU17-56) obtained from field soil samples in South Korea. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in boldface. The mark (T) indicates type strain. Numerical values (>50) on branches are the bootstrap values as percentage of bootstrap replication from a 1,000-replicate analysis. Scale bar represents the number of substitutions per site.

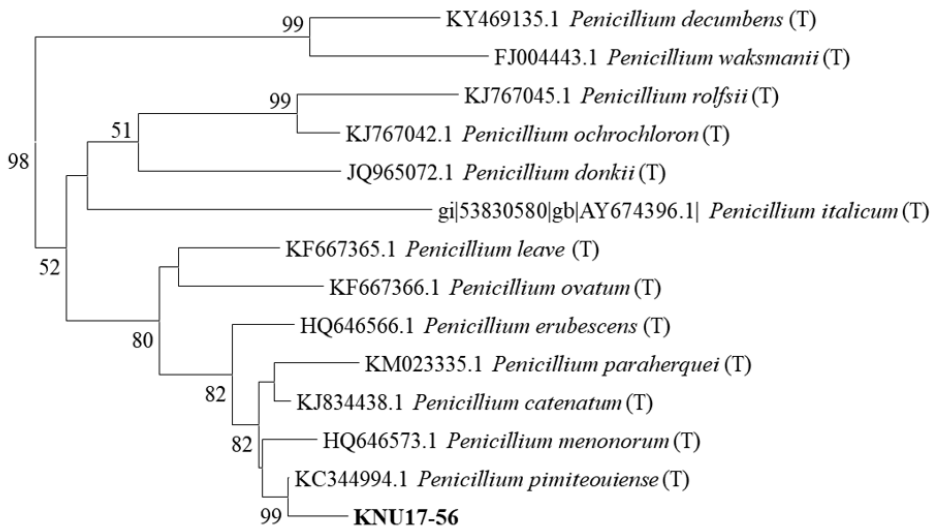

Fig. 3. Neighbor-joining phylogenetic analysis of β-tubulin (*BenA*) gene sequences of *Penicillium pimiteouiense* (KNU17-56) obtained from field soil samples in South Korea. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in boldface. The mark (T) indicates type strain. Numerical values (>50) on branches are the bootstrap values as percentage of bootstrap replication from a 1,000-replicate analysis. Scale bar represents the number of substitutions per site.

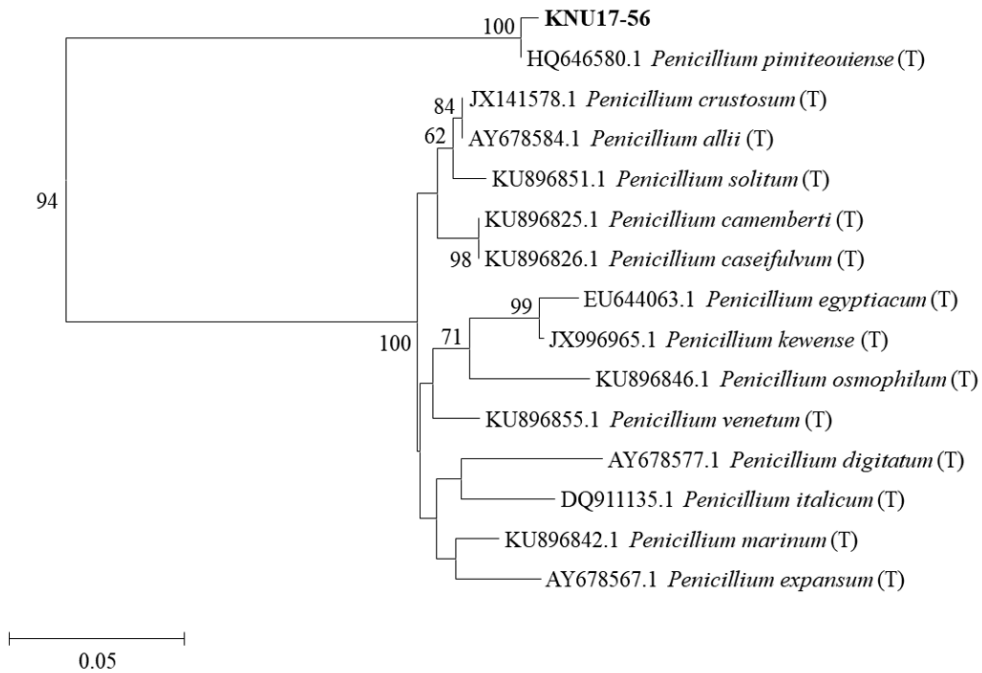

Fig. 4. Neighbor-joining phylogenetic analysis of Calmodulin (*CaM*) gene sequences of *Penicillium pimiteouiense* (KNU17-56) obtained from field soil samples in South Korea. A phylogenetic tree was constructed using the MEGA ver. 6 program. Sequences obtained in the study are shown in boldface. The mark (T) indicates type strain. Numerical values (>50) on branches are the bootstrap values as percentage of bootstrap replication from a 1,000-replicate analysis. Scale bar represents the number of substitutions per site.

DISCUSSION

Morphological and molecular characterizations are the most commonly used scientific tools for fungal species identification. Mycologists have traditionally used the morphology of fungal species, such as spore producing structures formed from sexual and asexual reproduction, to identify the species [20]. In recent years, morphological identification techniques in combination with molecular techniques are used for species identification within the mycological community [21]. In this study, we characterized our study isolate KNU17-56 as *P. pimiteouiense* based on their morphological characteristics. The use of differential media such as MEA and CYEA is a simple, easy, and reliable method for identifying *Penicillium* species [22]. We used five different media (PDA, CYEA, YESA, MEA, and OMA) for the morphological identification and characterization of our study isolate. KNU17-56, isolated from the field soil in Gyeongsangbuk-do province of Korea, was likely to be *P. pimiteouiense* based on the shape, size, and structure of conidiophores, conidia, phialides, and stipes. The macromorphology and micromorphology of the isolate KNU17-56 matched that of *P. pimiteouiense*, as previously reported [9, 10]. KNU17-56 only has slight differences from the original description made in previous reports. *P. pimiteouiense* identified in our study have not been reported in Korea before, therefore, herein is the first report isolated from soil samples in Korea.

The identification of fungal species using molecular tools is a widely used technique by mycologists and taxonomists. DNA sequencing is one of the most reliable tools as it displays the relationship within the species and also facilitates sequence-based identification [2]. rDNA ITS is the most widely used molecular marker in fungi identification [23]. In this study, we used 18S-ITS1-5.8S-ITS2-28S rDNA for the identification of KNU17-56 our results confirmed that our study isolates belong to *P. pimiteouiense* (Fig. 2). *P. pimiteouiense* lies near the clade of *P. stratisporum* when using ITS rDNA for identification [9]. This was also observed in our study (Fig. 2). Therefore, because of this limitation associated with using ITS as a species marker for *Penicillium*, tub2/BenA was considered the best option for a more precise identification [2, 24]. *BenA* gene maker analysis showed that KNU17-56 had 99% similarity with *P. pimiteouiense* (Fig. 3). Another possible secondary marker for *Penicillium* species identification is Ca*M* [2], which has proven to be a powerful tool for the molecular exploration of taxa related to *Penicillium* [25, 26]. Phylogenetic analysis using the *CaM* gene further confirmed that KNU17-56 belongs to *P. pimiteouiense* (Fig. 4). Some members of the genus *Penicillium* are responsible for the production of penicillin, which kills or inhibits the growth of some bacteria [27]. *Penicillium* has a wider application in biotechnology, in the production of enzymes, vitamins, valuable chemicals, and proteins, due to its low cost [28]. Although it also produces mycotoxins which are responsible for food spoilage, the economic importance supersedes the negative effects [28].

In conclusion, this is the first report of *P. pimiteouiense* from field soil samples in South Korea, identified based on its morphological and molecular characteristics. This fungal isolate may be of biotechnological importance in the field of mycology, therefore, further studies on their use and implications are warranted.

DISCLOSURE STATEMENT

No potential conflict of interest.

ACKNOWLEDGEMENTS

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