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New Records of *Aspergillus allahabadii* and *Penicillium sizovae* from Crop Field Soil in Korea

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ABSTRACT

Two new records of *Trichocomaceae*, namely *Aspergillus allahabadii* and *Penicillium sizovae*, were isolated in 2016 during a survey of fungal diversity in different crop fields locations in Gyeongnam, Korea. These species were identified based on morphological characters and phylogenetic analysis using internal transcribed spacer region and β -tubulin-encoding gene sequence data. *A. allahabadii* and *P. sizovae* have not yet been reported in Korea. Thus, this is the first report of these species in Korea, and their descriptions as well as details of their morphological characters are presented.

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1. Introduction

Fungi belonging to the genera *Aspergillus* and *Penicillium* in the family *Trichocomaceae* are among the most economically important filamentous fungi [1]. The family members comprise a diverse group of species that are known for their production of various beneficial enzymes, organic acids, and pharmaceutically important compounds [2]. *Aspergillus allahabadii* was first isolated from garden soil in Allahabad, India [3]. The antimicrobial and other benefits of *A. allahabadii* have been reported by various authors [4–6]. *Penicillium sizovae* is an anamorph species of the genus *Penicillium* that produces fructose oligomers used in industrial production as a food ingredient because of their low caloric intake, low glycemic index, and ability to lower blood lipid levels, prevent urogenital infections, and reduce cancer risk [7].

A fungal diversity survey was carried out in 2016 in Gangwon-do and Gyeongsangnam-do, Korea, and subsequently, fungal species with different morphologies were isolated from field soil. Two *Trichocomaceae*, namely *A. allahabadii* and *P. sizovae*, which have not been yet reported in Korea, were isolated among those morphologically different isolates. Therefore, in this report, the detailed morphological and molecular characteristics of these two new records are presented.

2. Materials and methods

2.1. Soil sampling and isolation of fungi

Soil sample collection was carried out in 2016 at different crop field locations in Gyeongnam (N 35.2640.46°, E 128.322497° and N 35.092802°, E 128.084769°), Korea. Soil samples were collected at a depth of up to 15 cm by removing crop debris. Each soil sample was air dried and stored in a sterile polythene bag at 4 °C. A conventional soil dilution technique [8] was employed to isolate the fungi. Potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) amended with 100 $\mu\text{g L}^{-1}$ chloramphenicol was used for isolation. The Petri plates, in which diluted soil suspensions were streaked, were incubated at 25 °C for 5 days. The developing colonies were then transferred to fresh PDA medium to obtain pure cultures. The pure isolates were finally transferred to PDA slants and kept at 4 °C until further use.

2.2. Morphological examination

The fungal macro-morphological characteristics of the study isolates (KNU16-275 and KNU16-243) were evaluated on four different agar media, namely PDA, malt extract agar (MEA), Czapek yeast extract (CYA) agar, and yeast extract sucrose (YES) agar. Furthermore, the ability of KNU16-275 and KNU16-243 to grow and produce acid was tested on creatine

sucrose agar medium (CREA-creatine 3.0 g, sucrose 30 g, KCl 0.5 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.5 g, K₂HPO₄·3H₂O 1.3 g, bromocresol purple 0.05 g, agar 15.0 g, distilled water 1 L) [9]. The study isolates were inoculated at three points on a Petri plate containing each medium and incubated at 25 and 37 °C for 7 days in the dark. Colony characteristics were recorded, and a light microscope (Olympus BX50F-3; Olympus Co., Tokyo, Japan) was used to examine fungal structures. For micro-morphological examinations, mounts of all isolates, grown on PDA, were made in lactic acid, and a drop of alcohol was added to remove air bubbles. Photomicrographs were taken using a HK 3.1 CMOS digital camera (KOPTIC Korea Optics, Seoul, Korea) attached to an Olympus BX50F-3 microscope. The microscopic structures were also examined using a scanning electron microscope (LEO Model 1450VP Variable Pressure Scanning Electron Microscope; Carl Zeiss, Cambridge, MA, USA).

2.3. Genomic DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

For molecular identification, total genomic DNA of the study isolates was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The internal transcribed spacer (ITS) region of rDNA genes was amplified using the primers ITS1 (5TSTCCGTAGGTGAACCTGCG-3C) and ITS4 (5TSTCCTCCGCTTATTGATATGC-3C) [10]. PCR amplifications of *BenA* were performed using the primers Bt2a (5t2GGTAACCAATC GGTGCTGCTTTC-3T) and Bt2b (5t2ACCCTCAGTGTAGTGACCCTTGGC-3C) [11]. The PCR amplification mixture (total volume, 20 µL) for each gene was prepared in a 50 µL reaction comprising fungal DNA template, primers for each gene, and Taq DNA polymerase, dNTPs, buffer, and a tracking dye. The amplification program included the following conditions: an initial denaturation step of 2 min at 95 °C followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 1 min at 50 °C (ITS), 55–60 °C (*BenA*), extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min [12]. PCR amplification products were purified with MicroconTM filters (Millipore, Burlington, MA, USA). The amplified PCR products were sequenced using an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). All sequence information was analyzed using the BLAST program (NCBI, Bethesda, MD, USA). The phylogenetic trees were constructed by the neighbor-joining method using Kimura's two-parameter model [13] implemented in MEGA 6 software [14]. The statistical confidence of tree topology was evaluated based on bootstrap analysis of 1000 replicates.

The study isolates KNU16-275 and KNU16-243 have been deposited in the culture collection site of the National Institute of Biological Resources (NIBR, Incheon, Korea), under NIBRFG000 0499477 and NIBRFG0000499410, respectively. The nucleotide sequences of the ITS gene of isolate KNU16-275 and that of KNU16-243 have been deposited in GenBank under the accession numbers KY906226 and KY906222, respectively. In addition, the nucleotide sequences of the β -tubulin gene of isolates KNU16-275 and KNU16-243 have been deposited in GenBank under the accession numbers MG786929 and MG786928, respectively.

3. Results

3.1. Morphological characterization

In the present study, morphologically distinct fungal species isolates, KNU16-275 and KNU16-243, were found in different crop field sites, 35°26'40.46''N, 128°32'24.97''E and 35°09'28.02''N, 128°08'47.69''E, respectively. The macro-morphological characteristics of these isolates were examined on five different agar media, PDA, MEA, CREA, CYA, and YES. In addition, the microscopic structures of the isolates were also observed under compound and scanning electron microscopes. The macro- and micro-morphological descriptions of the isolates are presented below.

3.1.1. Morphological characteristics of isolate KNU16-275

3.1.1.1. Colony characters. The colony characters of the fungal isolate KNU16-275 on different media at 25 and 37 °C are shown in Figure 1. Colonies on CYA attained a diameter of 22 mm and 31–35 mm after 7 days of incubation at 25 and 37 °C, respectively. They were radially furrowed, raised at center margins entire; white mycelium; velvety texture; reverse yellow-brown. Colonies on MEA reached 23 mm and 30–35 mm in diameter after 7 days of incubation at 25 and 37 °C, respectively. They were white, plane; reverse yellow-brown. Colonies on YES attained a diameter of 20–22 mm and 28–32 mm after 7 days of incubation at 25 and 37 °C, respectively. They were radially furrowed; mycelium white; velvety texture; raised; reverse pale yellow. Colonies on PDA attained a diameter of 15–22 mm and 28–30 mm after 7 days of incubation at 25 and 37 °C, respectively. They were white; wrinkled; radially furrowed; margins undulating; velvety texture; raised; reverse orange. In addition, although isolate KNU16-275 was able to grow on CREA media, it did not form a yellow halo around its colony indicating no acid production (Figure 1). In general, colony growth of isolate KNU16-275 was

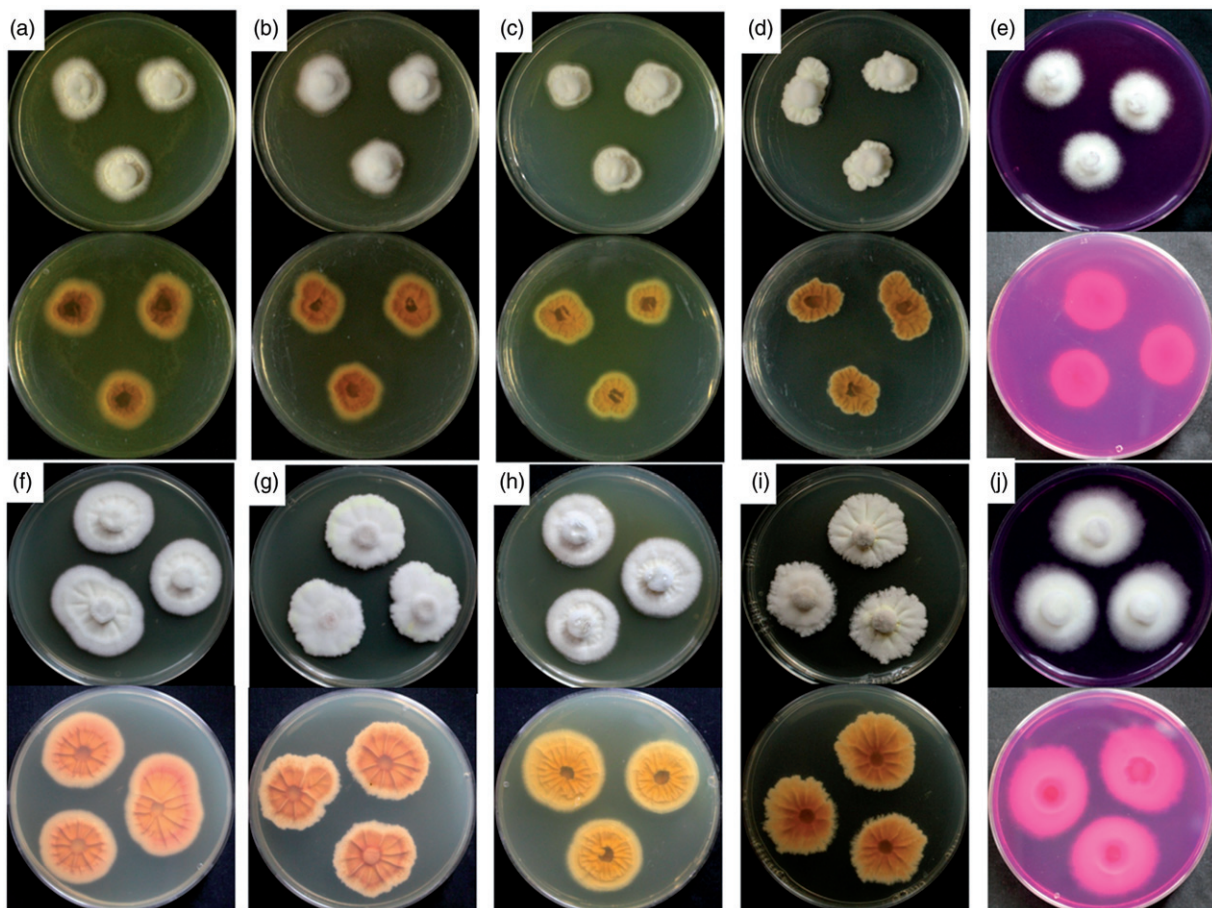


Figure 1. *Aspergillus allahabadii* KNU16-275 grown on different media at (a–e) 25 °C and (f–j) 37 °C for 7 days. (a–e) Colonies grown on CYA, MEA, YES, PDA, and CREA (top = obverse, bottom = reverse).

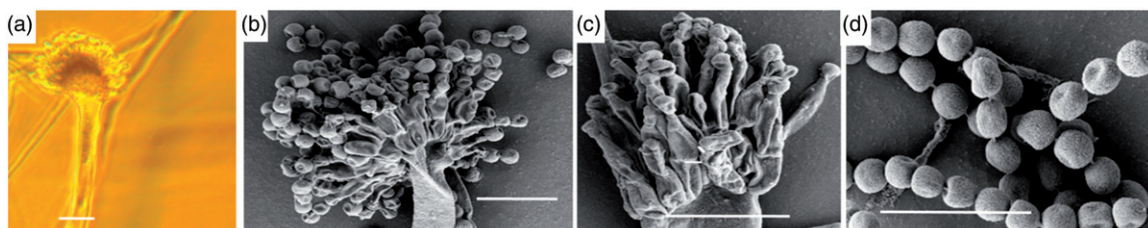


Figure 2. Micromorphology of *Aspergillus allahabadii* KNU16-275, showing (a, b, and c) conidiophores and (d) conidia (scale bars: 10 µm).

comparatively higher (35–37 mm) at 37 °C than that at 25 °C (22–25 mm) (Figure 1).

3.1.1.2. Micromorphology. Conidiophores smooth, pale, 1.6–2.0 µm width; conidial heads globose to sub-globose when young, splitting at maturity, columnar, 19–26 µm wide; metulae were wider but shorter than phialide, 4.0–4.5 × 1.9–2.5 µm; phialide were a bit longer and tapered, 3.0–4.0 × 4.8–6.0 µm; conidia globose 2–3 µm, joined into chains; smooth walled and colorless (Figure 2).

3.1.2. Morphological characteristics of isolate KNU16-243

3.1.2.1. Colony characters. The colony characters of the fungal isolate KNU16-275 on different media at 25 and 37 °C are shown in Figure 3. Colonies on

CYA attained a diameter of 14–16 mm and 23 mm after 7 days of incubation at 25 and 37 °C, respectively. They had irregular margins; gray-green colony, white, and sharp boundaries; velvety texture; reverse yellow (Figure 3). Colonies on MEA attained a diameter of 20–21 mm and 29–35 mm after 7 days of incubation at 25 and 37 °C, respectively. They were gray-green, velvety texture, white boundaries; reverse pale. Colonies on YES attained a diameter of 17–21 mm and 32–34 mm after 7 days of incubation at 25 and 37 °C, respectively. They had irregular margins; green-gray colonies, white, and raised boundaries, velvety texture; reverse yellow. Colonies on PDA grew faster (22–23 mm in diameter and 29–31 mm at 25 and 37 °C, respectively) than those on the remaining media tested, harbored gray colonies, white and raised boundaries, smooth texture; reverse light yellow. Furthermore, isolate KNU16-

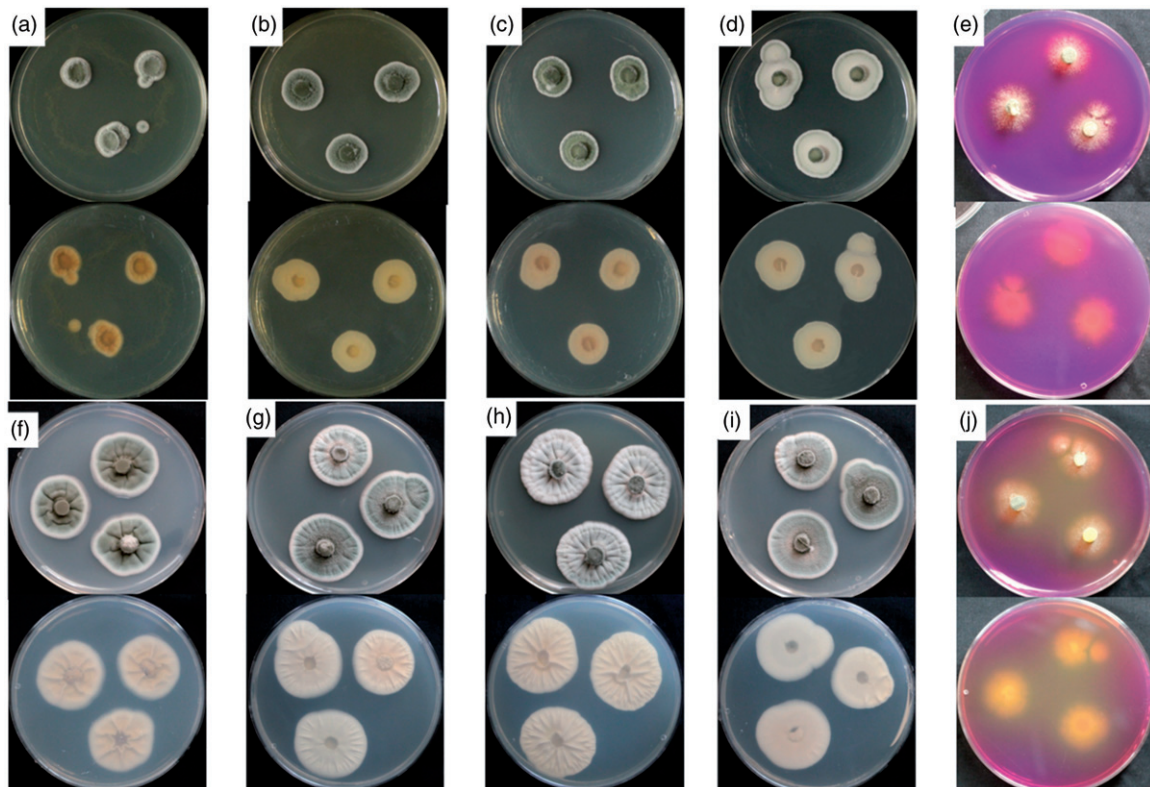


Figure 3. *Penicillium sizovae* KNU16-243 grown on different media at 25 °C for 7 days. (a–e) Colonies grown on CYA, MEA, YES, PDA, and CREA (top = obverse, bottom = reverse).

243 was able to grow on CREA media and formed a yellow halo around the colonies indicating acid production. Notably, acid production was comparatively higher at 37 °C than that at 25 °C.

3.1.2.2. Micromorphology. Conidiophores were smooth, branched, $2.8 \times 37 \mu\text{m}$; metulae were mostly four in number on a conidiophore, smooth, $3 \times 9\text{--}11 \mu\text{m}$; phialides were bottle shaped, smooth, 6–8 in number, $2 \times 8 \mu\text{m}$; conidia globose to subglobose, $2.0\text{--}2.5(2.8) \mu\text{m}$; rough walled (Figure 4).

3.2. Phylogenetic analysis of the study isolates

Molecular analysis was carried out by comparing the sequences of ITS and β -tubulin of the study isolates with those of other strains available in GenBank using the BLAST program. The results of the phylogenetic tree evaluation based on a bootstrap analysis of 1000 replicates revealed that the study isolates KNU16-275 and KNU16-243 were *A. allahabadii* and *P. sizovae*, respectively. Briefly, the phylogenetic relationship from the ITS sequence analysis revealed that the isolate KNU16-275 was most closely related to *A. allahabadii* isolate NRRL 4539 (EF669601.1) with a very high monophyletic clade (92% of PP) in our phylogenetic tree (Figure 5). The β -tubulin gene sequence analysis also showed that isolate KNU16-275 was most closely related to *A. allahabadii* isolate NRRL 4539

(EF669531.1) with 100% sequence identity (Figure 6). In addition, the phylogenetic relationship from ITS sequence analysis indicated that KNU16-243 and the type strain *P. sizovae* NR 111487.1 were grouped with a very high monophyletic clade (100% of PP) in our phylogenetic tree (Figure 7). Similarly, the β -tubulin gene sequence analysis also showed that isolate KNU16-243 was most closely related to *P. sizovae* GU944535.1 with 100% sequence identity (Figure 8).

4. Discussion

In the present study, morphologically different isolates, KNU16-275 and KNU-243, were isolated in 2016 in different crop field locations in Gyeongnam, South Korea. Isolate KNU16-275 showed morphological similarity (Table 1) with *Aspergillus allahabadii* according to the descriptions in previous reports [3,5,15]. Briefly, conidia of the study isolate were globose, joined into chains, smooth walled, and colorless. Similar to *Aspergillus* section *Terrei* [16], our isolate showed columnar conidial heads. More importantly, the phylogenetic relationship from the sequence analysis indicated that the isolate KNU16-275 was most closely related to *A. allahabadii* with a very high monophyletic clade in our phylogenetic tree. *A. allahabadii* has not yet been reported in Korea. To the best of our knowledge, this is the first

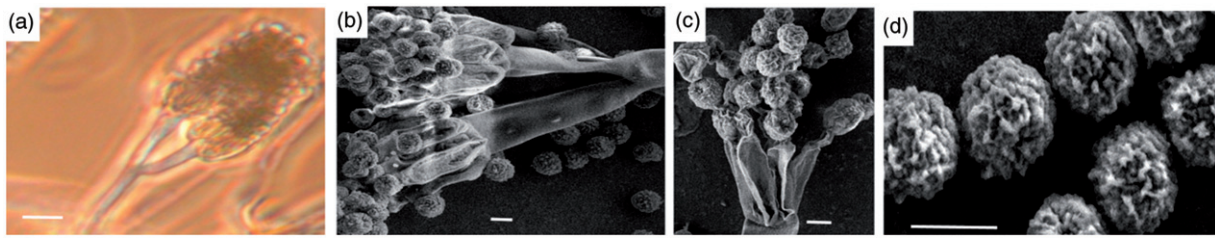


Figure 4. Micromorphology of *Penicillium sizovae* KNU16-243 showing (a, b, and c) conidiophores and (d) conidia (scale bars: 10 μ m).

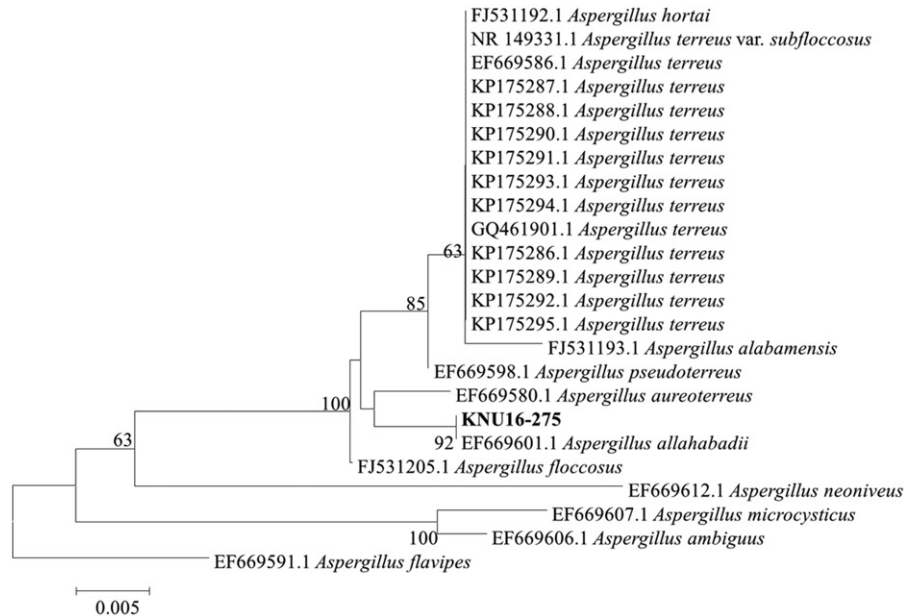


Figure 5. Neighbor-joining phylogenetic analysis of the partial 18S-ITS1-5.8S-ITS2-partial 28S rDNA sequences of *Aspergillus allahabadii* KNU16-275, obtained from field soil in Korea. Species examined in this study are in bold. Bootstrap scores of >60 are presented at the nodes. The scale bar represents the number of substitutions per site. Sequences of type specimens are indicated by the symbol (T).

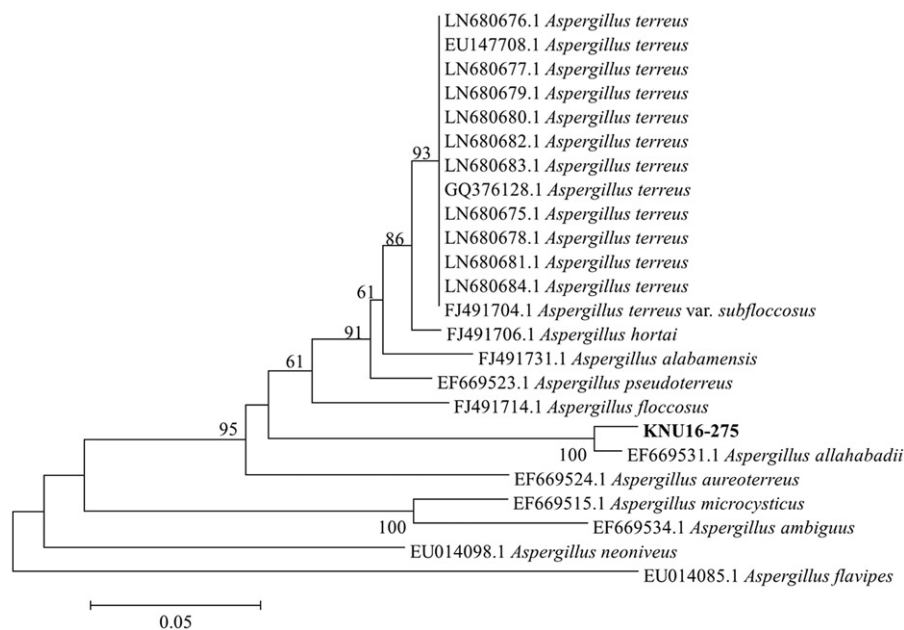


Figure 6. Neighbor-joining phylogenetic analysis of the β -tubulin gene sequences of *Aspergillus allahabadii* KNU16-275, obtained from field soil in Korea. Species examined in this study are in bold. Bootstrap scores of >60 are presented at the nodes. The scale bar represents the number of substitutions per site. Sequences of type specimens are indicated by the symbol (T).

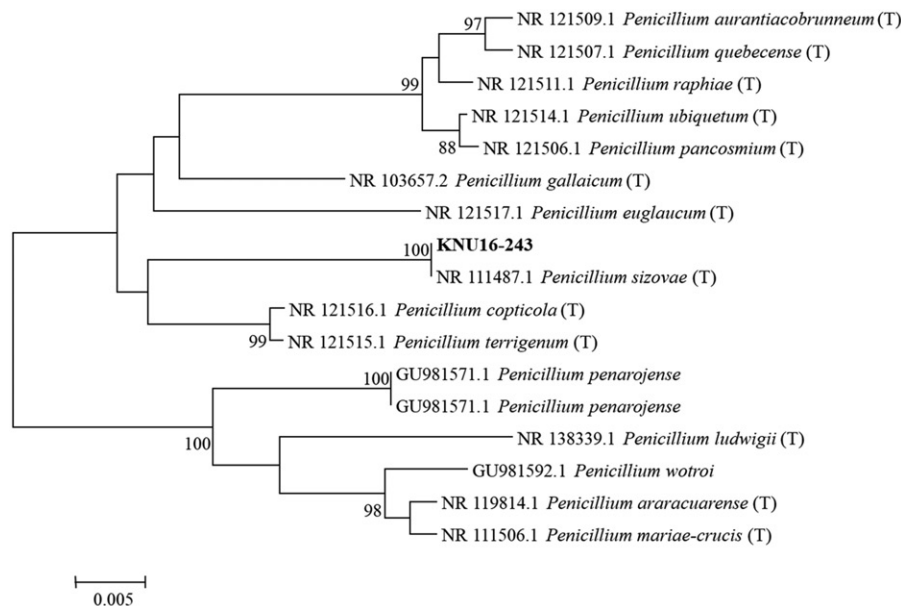


Figure 7. Neighbor-joining phylogenetic analysis of the partial 18S-ITS1-5.8S-ITS2-partial 28S rDNA sequences of *Penicillium sizovae* KNU16-243, obtained from field soil in Korea. Species examined in this study are in bold. Bootstrap scores of >60 are presented at the nodes. The scale bar represents the number of substitutions per site. Sequences of type specimens are indicated by the symbol (T).

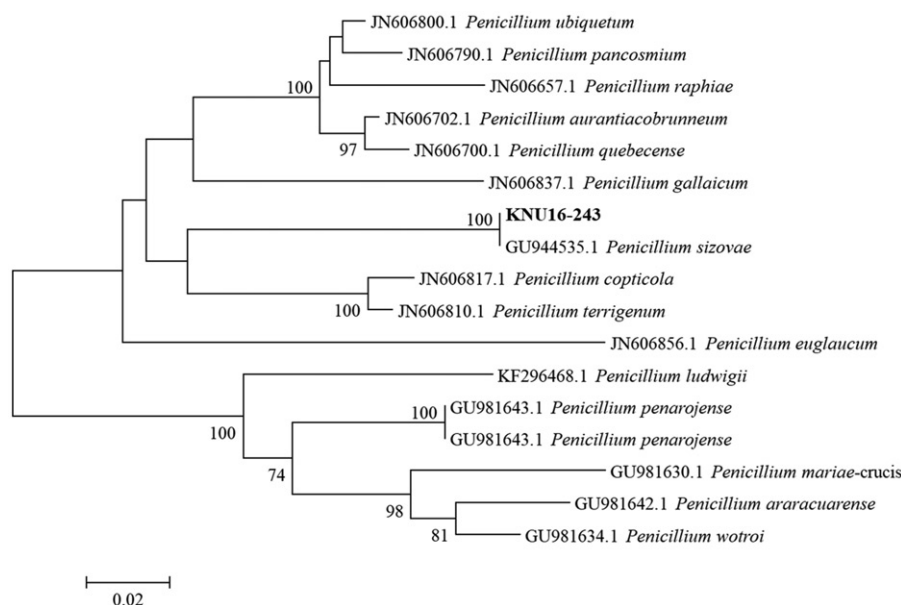


Figure 8. Neighbor-joining phylogenetic analysis of the β -tubulin gene sequences of *Penicillium sizovae* KNU16-243, obtained from field soil in Korea. Species examined in this study are in bold. Bootstrap scores of >60 are presented at the nodes. The scale bar represents the number of substitutions per site. Sequences of type specimens are indicated by the symbol (T).

Table 1. Morphological characteristics of KNU16-275 and the reference species *Aspergillus allahabadii* on malt extract agar medium at 25 °C.

Character	Study isolate, <i>Aspergillus allahabadii</i> KNU16-275	Previous reports, <i>Aspergillus allahabadii</i> ^a
Colony color	Radially furrowed, raised at center; white colony; reverse yellow-brown	White; reverse yellow brown
Colony diameter	23 mm in a week at 25 °C	45 mm in two weeks at 25 °C
Exudate	Absent	Absent
Conidial heads	Columnar	Columnar
Conidia	Globose to sub globose, 2–3 μ m, joined into chains; smooth walled and colorless	Globose, 2.0–2.8 μ m, joined into chains; smooth and colorless

^aFrom the description of Mehrotra and Agnihotri [5].

report of *A. allahabadii* in Korea. *A. allahabadii* has many benefits. Previous studies [5,6] reported that *A. allahabadii* has beneficial effects, including

antibacterial, antioxidant, mild antidiabetic, and antifungal properties without cytotoxicity and strong antiradical and antioxidant properties.

Table 2. Morphological characteristics of KNU16-243 and the reference species *Penicillium sizovae* on malt extract agar medium at 25 °C.

Characteristics	(KNU16-243), study isolate	<i>Penicillium sizovae</i> ^a , previous report
Colony color	On MEA gray-green, reverse, pale; On YES yellowish reverse	On MEA, gray-green, reverse, pale; On YES yellowish to crème reverse
Colony diameter	20–21 mm at 25 °C in a week	On MEA 27–35 mm at 30 °C in a week
Acid production	Weak to moderate acid production	Weak acid production
Conidiophore	Smooth; 2.8–3.5 µm	Symmetrically biverticillate, stipes smooth, 2.5–3.2 µm
Metulae	Metuli were mostly four in number on a conidiophore, smooth, 3 × 9–11 µm	In whorls of 2–5, 2.5–3.2 × 11–16 µm
Phialides	Bottle shaped, smooth, 6–8 in number, 2 × 8 µm	Ampulliform, 2.0–3.0 × 7.0–9.4 µm
Conidia	Globose to sub globose, 2–2 × 5 (2.8) µm, rough-walled	Globose to sub globose, 2–2.5 µm; finely roughened conidia

^aFrom the description of Houbraken et al. 2010 [15].

Hence, further research is warranted to confirm the beneficial activities of this isolate.

In addition, the macro-morphology of the Korean isolate *Penicillium sizovae* KNU16-243 has much similarity (Table 2) with the descriptions in a previous report by Houbraken et al. [17]. Briefly, the colony colors of the study isolate on the obverse and reverse sides were gray-green and yellowish, respectively. The isolate grew faster on PDA and MEA. In addition, the conidia shape and roughness of the study isolate were similar to those in the Houbraken et al. [17] report. Interestingly, the authors stated that fast growth on MEA, conidia roughness, and pale reverse side were the most important diagnostic features of *P. sizovae* compared with those of other related species. The faster growth of *P. sizovae* on MEA particularly at 25 °C distinguished this species from its close relatives such as *P. hetheringtonii* and *P. tropicoides* [18]. In agreement with our result, previous studies [17,19] reported that *P. sizovae* has a higher maximum growth temperature than other species, including *P. steckii* and *P. westlingii*. However, there are very few differences in conidia size. More importantly, the results of the ITS and β -tubulin gene sequence analysis revealed that isolate KNU16-243 was most closely related to *P. sizovae* with 100% sequence identity. *P. sizovae* has not yet been reported in Korea. To the best of our knowledge, this is the first report of *P. sizovae* in Korea. Previous reports [20–22] showed that *P. sizovae* has many benefits, such as antimicrobial and transfructosylating activity and the production of fructose oligomers, which can be used for industrial production as food ingredients. Hence, further research is warranted to explore these untapped resources.

Furthermore, the ability of KNU16-275 and KNU16-243 to grow and produce acid was tested on CREA medium at different temperatures (25 and 37 °C). The results indicated that both isolates were able to grow on CREA media; however, only isolate KNU16-243 was able to form a yellow halo around its colony, indicating its acid production potential.

In addition, acid production by KNU16-243 was comparatively higher at 37 °C than at 25 °C. This result agrees with that in a previous report [19] that *P. sizovae* was able to produce acid. Similar to our result, some species in *Aspergillus* section *Terrei* do not produce acid on CREA media [16].

Disclosure statement

No potential conflict of interest was reported by the authors.

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