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RESEARCH ARTICLE

Identification and Characterization of Unrecorded *Aspergillus* spp. in Korea

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ABSTRACT

A survey of fungal diversity in Gyeongsang Province, Korea, revealed two previously unreported *Aspergillus* isolates, named KMG411 and KMG412. The phylogeny of the isolates was analyzed based on β-tubulin (*BenA*) and calmodulin (*CaM*) sequencing. Morphological analyses further identified the KMG411 and KMG412 as *A. insuetus* and *A. nomius*, respectively. Here we provide detailed morphological descriptions of the previously unrecorded *Aspergillus* species.

Keywords: *Aspergillus*, Fungal diversity, Korea, Phylogeny

INTRODUCTION

The genus *Aspergillus*, a member of the Aspergillacea family, is the best-known fungi in the phylum Ascomycota, along with the genus *Penicillium* and *Talaromyces* [1]. As the largest phylum in the fungal kingdom, Ascomycota includes more than 64,000 species.

Since Pier Antonio Micheli introduced the first *Aspergillus* species in 1729 [2], over 900 species of *Aspergillus* have been reported [3]. In Korea alone, 69 *Aspergillus* species have been discovered, reflecting its diversity [4].

Aspergillus species are commonly used in agriculture, industry, human health, and plant physiology. Other *Aspergillus* species, such as *A. niger,* are pathogenic [2,5]. The name *Aspergillus* stems from the observation that its conidiophores form shapes that are reminiscent of the aspergillums used in Christian church rituals. Macroscopic and microscopic features are primarily used to characterize and analyze this fungal group [6]. Most of the members of this genus are exist in asexual states, forming asexual haploid conidia. Some species have been identified in a sexual state and form non-motile spores known as ascospores. *Aspergillus* species are aerobic and found in almost all oxygen-rich environments [2,7].

With the discovery of more species, it is no longer sufficient to rely on morphological features alone for identification [8], molecular analysis has been added to the regimen of conventional studies. Molecular identification of *Aspergillus* species depends on characterizing the internal transcribed spacer (ITS) region and DNA sequences of β-tubulin (*BenA*), calmodulin (*CaM*), and the second-largest subunit of RNA

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polymerase II (*RPB2*) [8,9]. This study used previously published sequences of *BenA* and *CaM* for molecular analysis [10,11].

In 2018, over 200 endophytic fungi were collected from plant roots in Gyeongsang Province, including Pohang, Jinju, Gumi, Gyeongsan, and Ulleungdo Island [12]. Among the isolates were two previously unreported *Aspergillus* species, named *A. insuetus* and *A. nomius*. This article describes the morphological and molecular features of these fungi found in Korea.

MATERIALS AND METHODS

Isolation of endophytic fungi

During an extensive investigation of fungal diversity in Gyeongsang Province, Korea, in 2018, two previously unreported species of *Aspergillus,* named KMG411 and KMG412, were identified (Table 1 and Table 2) [12]. KMG411 was isolated from *Lespedeza cuneate*, and KMG412 from *Chrysanthemum lavandulifolium*. The collected roots were washed with distilled water to eliminate dust. After treatment with Tween-80 solution for 5 min, the root's surface was sterilized with 1% perchloric acid. Washed roots were cut into pieces and incubated at 25℃ in Hagem minimal medium with 80 parts per million (ppm) of streptomycin [13,14]. Pure fungal strains were transferred and cultured on potato dextrose agar (PDA) from the root pieces [15]. The isolated strains were stored in 20% glycerol at -80℃ and deposited at the National Institute of Biological Resources (NIBR), Incheon, Korea, with the accession numbers NIBRFG0000505325 for KMG411 and NIBRFG0000505326 for KMG412.

Morphological analysis

Morphological characteristics of the two isolates were analyzed after growth on malt extract agar (MEA; Samson, 2010), Czapek yeast autolysate agar (CYA; Pitt, 1979), and yeast extract sucrose agar (YES; Frisvad, 1981) [10]. All plates were incubated at 25℃ in the dark for 7 days; the CYA plates underwent additional incubation at 30℃ and 37℃. Morphological characteristics, including the diameter and visible features of the fungal colonies, were observed. Fungal structures were measured and characterized by light microscopy (Eclipse 80i, Nikon, Tokyo, Japan) [16,17]. The specimens were fixed on glass slides with 85% and 99% lactic acid.

DNA extraction, PCR, and sequencing

The Accuprep® genomic DNA extraction kit (BIONEER Corp, Daejeon, USA) was used to extract DNA from the fungal isolates. DNA was stored at -20℃ before amplification. PCR amplification primers for Calmodulin (*CaM*) and β-tubulin (*BenA*), CMD5/CMD6 and Bt2a/Bt2b, were constructed based on prior publications by doctor Lee's team and doctor Lim's team [10,11]. After amplification in a 20-μL volume, a QIAquick PCR purification kit (Qiagen) was used to purify the PCR amplicons. Sequencing was performed on an ABI 310 DNA sequencer (Perkin Elmer, Foster, CA, USA) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster, CA, USA) [1,18].

Table 1. Summary and GenBank accession numbers for *Aspergillus* strains isolated from Gyeongsang Province, Korea.

Species	Strain No.	Substrate	GPS coordinates	Collector	Collect date	Accession No.	
						BenA	CaM
Aspergillus insuetus	NIBRFG0000505325	*Lespedeza cuneata*	36°11'47.33"N 129°20'04.79"E	Doo-Ho Choi Jong-Guk Kim	2018-11-17	MN862313	MN862317
Aspergillus nomius	NIBRFG0000505326	*Chrysanthemum lavandulifolium*	35°09'42.94"N 128°17'45.64"E	Doo-Ho Choi Jong-Guk Kim	2018-11-14	MN862314	MN862318

Table 2. Accession numbers for fungal strains used for the phylogenetic analysis.

Species	Collection no.	GenBank accession no.	
		BenA	*CaM*
Aspergillus albertensis	NRRL 20602 (T)	EF661464	EF661537
A. alliaceus	CBS 536.65 (T)	EF661465	EF661534
A. avenaceus	CBS 109.46 (T)	FJ491481	FJ491496
A. baeticus	NRRL 62501 (T)	HE615092	HE615117
A. bombycis	CBS 117187 (T)	AY017547	AY017594
A. caelatus	CBS 763.97 (T)	EF661470	EF661522
A. calidoustus	CBS 121601 (T)	FJ624456	HE616559
A. carlsbadensis	CBS 123894 (T)	FJ531179	FJ531126
A. contaminans	CBS 142451 (T)	LT594443	LT594425
A. coremiiformis	CBS 553.77 (T)	EU014104	EU014112
A. deflectus	CBS 109.55 (T)	EF652261	EF652349
A. elongatus	CBS 387.75 (T)	EF652326	EF652414
A. flavus	CBS 569.65 (T)	EF661485	EF661508
A. germanicus	CBS 123887 (T)	FJ531172	FJ531141
A. granulosus	NRRL 1932 (T)	EF652254	EF652342
A. heterothallicus	CBS 488.65 (T)	EF652323	EF652411
A. insuetus	CBS 107.25 (T)	EF652281	EF652369
A. insuetus	**KMG411**	**MN862313**	**MN862317**
A. keveii	CBS 209.92 (T)	EU076376	EU076365
A. lanosus	CBS 650.74 (T)	EF661468	EF661539
A. leporis	CBS 151.66 (T)	EF661499	EF661541
A. lucknowensis	CBS 449.75 (T)	EF652283	EF652371
A. mottae	CBS 130016 (T)	HM803086	HM803015
A. nomius	CBS 260.88 (T)	AF255067	AY017588
A. nomius	**KMG412**	**MN862314**	**MN862318**
A. oryzae	CBS 102.07 (T)	EF661483	EF661506
A. parvisclerotigenus	CBS 121.62 (T)	EF203130	EF202077
A. porphyreostipitatus	CBS 138203 (T)	KJ775080	KJ775338
A. pseudocaelatus	CBS 117616 (T)	EF203128	EF202037
A. pseudodeflectus	CBS 756.74 (T)	EF652331	EF652419
A. pseudonomius	CBS 119388 (T)	EF661495	EF661529
A. pseudotamarii	CBS 766.97 (T)	EF203125	EF202030
A. pseudoustus	CBS 123904 (T)	FJ531168	FJ531129
A. puniceus	CBS 495.65 (T)	EF652322	EF652410
A. subolivaceus	CBS 501.65 (T)	EF203144	EF202064
A. tamarii	CBS 104.13 (T)	EF661474	EF661526
A. thesauricus	NRRL 62487 (T)	HE615095	HE615120
A. togoensis	CBS 205.75 (T)	FJ491477	FJ491489
A. turkensis	CBS 504.65 (T)	FJ531191	FJ531145
A. ustus	CBS 261.67 (T)	EF652279	EF652367

Bold letters indicate the strain and accession numbers of the isolate identified in this study.

Phylogenetic analysis

Nucleotide sequences were aligned using BioEdit v7.2.5 (Clustal W) [19], then transferred to Molecular Evolutionary Genetics Analysis (MEGA) 7 software [20]. A phylogenetic tree was constructed based on the sequence similarities, a maximum likelihood (ML) using a Tamura 3-parameter model. The ML heuristic method was set to level 3 for subtree pruning regrafting (SPR), and bootstrap replicates were set to 1,000. The species similarity was calculated as a percentage based on a BLASTn search at the National Center for Biotechnology Information (NCBI) [16]. Two different groups were selected to construct the phylogenetic tree; *A. versicolor* for NIBRFG0000505325 [21] and *A. muricatus* for NIBRFG0000505326 [22].

RESULTS AND DISCUSSION

Sequences of *BenA* (β-tubulin) and *CaM* (calmodulin) amplified from the isolated fungal strains were successfully identified through BLASTn-based comparisons and phylogenetic analyses. The gene sequences from the two *Aspergillus* species were identified in the GenBank database. Two isolates were included in monophyletic groups with the type strain for each *Aspergillus* species. The NIBRFG0000505325 isolate was homologous to the *A. insuetus* type strain (sequence similarities of 95.7-100% with EF652281 for *BenA* and 94.1-99.8% with EF652369 for *CaM*; bootstrap value of 99%). The NIBRFG0000505326 isolate was included in a monophyletic group with type strain *A. nomius* (sequence similarities of 98-99.6% with AF255067 for *BenA* and 98.9-99.4% with AY017588 for *CaM*; bootstrap value of 63%).

Currently, 72 species of *Aspergillus* have been revealed in Korea [4,10]. In this study, two *Aspergillus* species, named *A. insuetus* and *A. nomius*, were identified. To the best of our knowledge, these species have not been reported in Korea. Additional taxonomic information is presented in the following section.

Taxonomy

Aspergillus insuetus Thom & Church (1929), Mycobank No. 267997

Description: Colony diam, 7 d, in mm: CYA 28-31; CYA 30℃ 43-47; CYA 37℃ 5-9; MEA 20-24; YES 30-33 (Fig. 1 and Fig. 2).

CYA, 25℃: Colonies moderately deep, angular shape, radially sulcate with a yellowish-gray color; margins low, narrow, entire; mycelia white; texture floccose; sporulation sparse, soluble pigment present, reverse pigmentation reddish-brown at center fading to pale.

MEA, 25℃: Colonies moderately deep, lightly sulcate with a dull greyish-green color; margins low, narrow, entire; mycelia white; texture floccose; sporulation dense at the center, soluble pigment absent, reverse pigmentation brown at center fading to pale.

YES, 25℃: Colonies moderately deep, lightly radially sulcate with a dull reddish-brown color; margins high, moderate, entire; mycelia white; texture floccose and velutinous; sporulation dense at margins, soluble pigment absent, reverse pigmentation deep reddish-orange at center fading to reddish-yellow.

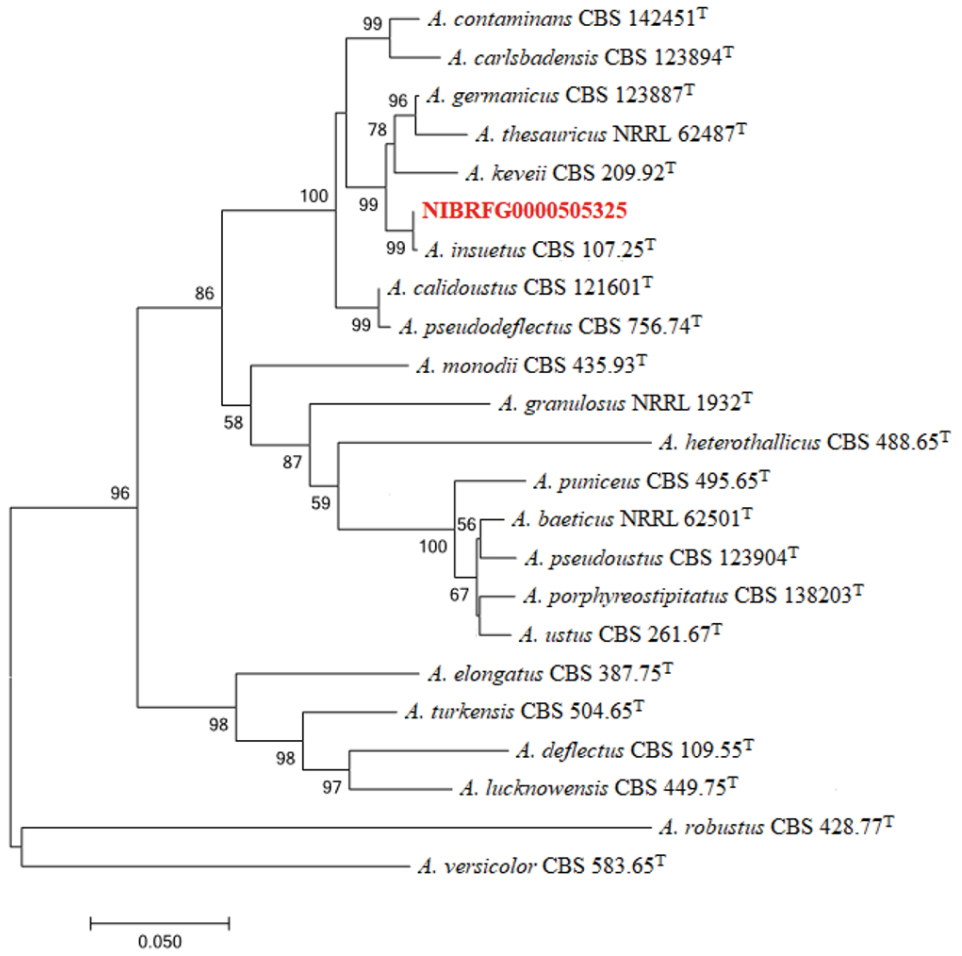

Fig. 1. Maximum-likelihood phylogenetic analysis of combined sequence data of *BenA* and *CaM* genes from NIBRFG0000505325. The sequence of *A. versicolor* was used as an outgroup. Bootstrap scores >50 are presented. The number of nucleotide substitutions per site was denoted by the scale bar. "T" identifies the type strains of the given fungal species. The isolate NIBRFG0000505325 is marked in red.

Micromorphology: Conidiophores had a floccose conidial head, distinct rough-walled stipes, 6-8.4×280 μm; vesicle, roughened, globose to subglobose, 9-15 μm; phialides were ampulliform type, navicular shape, smooth; conidium globose to subglobose, color with echinulations, 3-3.8 μm; distinct roughened and inner and outer-walled.

Strain examined: NIBRFG0000505325, isolated from *Lespedeza cuneata* in Pohang (36°11'47.33"N, 129°20'04.79"E).

Note: When compared with the type strain of *Aspergillus insuetus*, *A. insuetus* from Korea can be easily distinguished by colony growth on CYA at 37℃. Colony growth on other mediums showed similar growth in both species. Like type strain of *A. insuetus*, the isolated strain had a similar shape of conidial head, vesicle, and conidium, but was slightly smaller. Type A. *keveii,* close to type *A. insuetus* in the phylogenetic tree, had lower growth activity in medium and rough-walled stipes not smooth-walled [22,23].

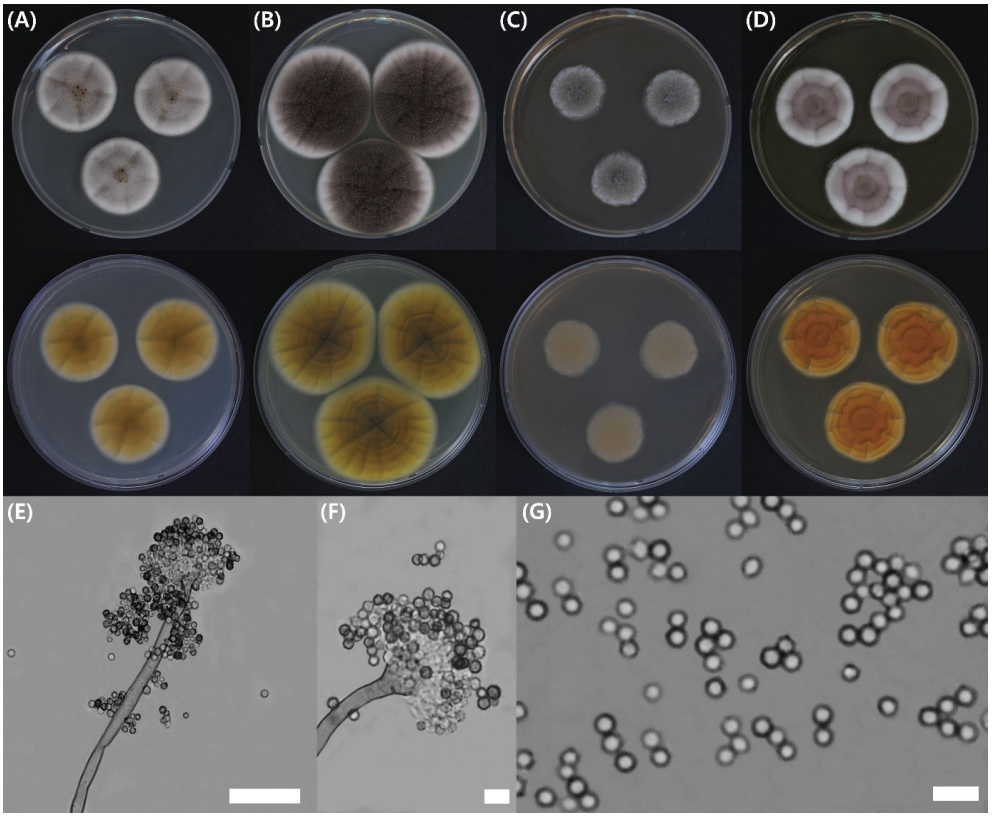

Fig. 2. *Aspergillus insuetus* KMG411 cultures after 7 days in culture. Colonies were grown on (A) Czapek yeast autolysate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E-F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

Aspergillus nomius Kurtzman, B.W. Horn & Hesselt (1987), Mycobank No. 133392

Description: Colony diam, 7 d, in mm: CYA 52-55; CYA 30℃ 61-67; CYA 37℃ 53-57; MEA 56-62; YES 66-69 (Fig. 3 and Fig. 4).

CYA, 25℃: Colonies highly deep, lightly radially sulcate, with a yellowish-white color; margins low, narrow, entire; mycelia white; texture floccose and velutinous; plentiful sporulation distributed evenly, soluble pigment absent, reverse pigmentation yellowish-brown at center fading to cream to brown.

MEA, 25℃: Colonies moderately deep, lightly sulcate with a dull greyish-green color; margins low, moderate, entire; mycelia pale greyish-yellow; texture floccose and velutinous; sporulation dense at the center, soluble pigment absent, reverse pigmentation pale greyish-green at center fading to translucent.

YES, 25℃: Colonies high and deep, randomly sulcate, have a dull reddish-white color; margins low, narrow, entire; mycelia yellowish-white; texture floccose and velutinous; sporulation dense at margins, soluble pigment absent, reverse pigmentation deep reddish-orange at center fading to reddish-yellow.

Micromorphology: Conidiophores with typically radiate conidial head, hyaline, distinct rough-walled stipes, over 1,000 μm; vesicle, roughened, globose to subglobose, 20-33 μm; phialides were ampulliform type, navicular shape, smooth; conidium globose to subglobose, color with pale green, 3-3.4 μm; distinct roughened and inner and outer-walled.

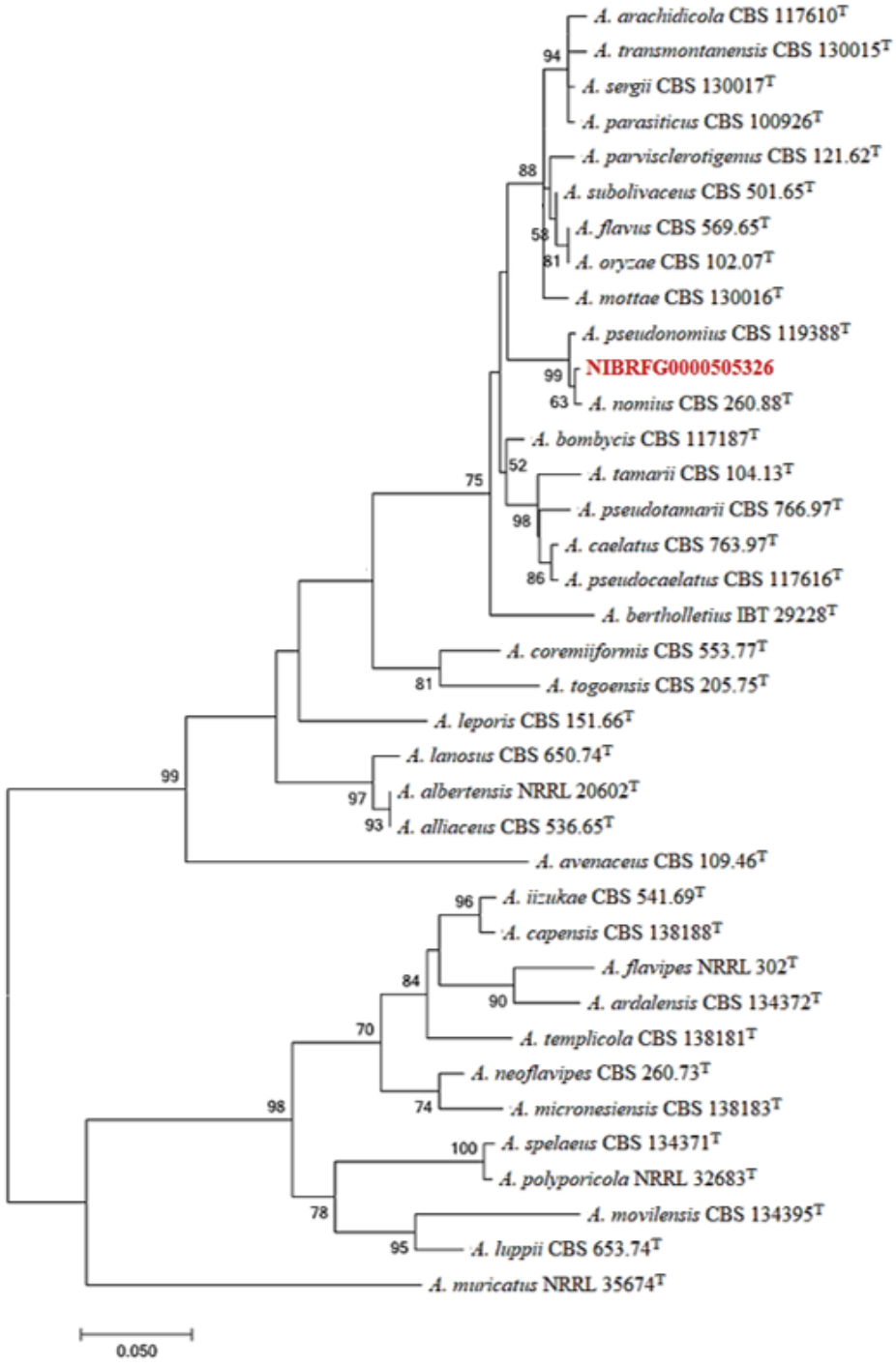

Fig. 3. Maximum-likelihood phylogenetic analysis of combined sequence data of *BenA* and *CaM* genes from NIBRFG0000505326. The sequence of *A. muricatus* was used as an outgroup. Bootstrap scores >50 are presented. The number of nucleotide substitutions per site was denoted by the scale bar. "T" identifies the type strains of the given fungal species. The isolate NIBRFG0000505326 is marked in red.

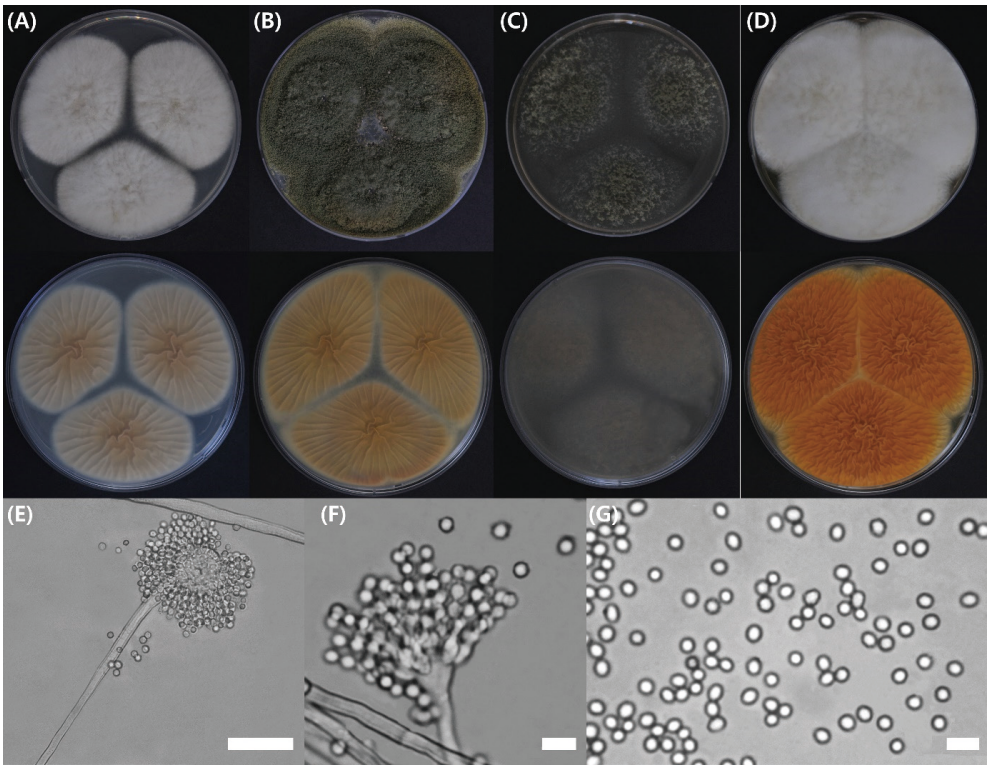

Fig. 4. *Aspergillus nomius* KMG412 after 7 days in culture. Colonies were grown on (A) Czapek yeast autolysate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E-F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

Strain examined: NIBRFG0000505326, isolated from *Chrysanthemum lavandulifolium* in Jinju (35° 09'42.94"N, 128°17'45.64"E).

Note: When compared with the type strain of *Aspergillus nominus*, *A. nominus* from Korea can be easily distinguished because the isolated strain did not show small bullet-shaped sclerotia. Otherwise, both strains had similar growth activity on all media. Comparing with *A. pseudonominus*, *A. nominus* showed similar growth activity except on CYA at 25℃ and 37℃. In both conditions, *A. nominus* had slower growth than type strain *A. pseudonominus*. *A. nominus* also had larger conidiophores than type *A. pseudonominus* [24,25].

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REFERENCES

1. Kirk PM, Cannon PF, David JC, Stalpers JA. Dictionary of the fungi. 9th ed. Wallingford: CABI Publishing; 2001.
2. Berbee ML, Taylor JW. Dating the evolutionary radiations of the true fungi. Can J Bot 1993;71:1114-27.
3. Gams W, Christensen M, Onions AH, Pitt JI, Samson RA. Infrageneric taxa of *Aspergillus*. Advances in *Penicillium* and *Aspergillus* systematics. In: Samson RA, Pitt JI, editors. Boston: Springer; 1985. p. 55-61.
4. National Center for Biotechnology Information. GenBank overview [Internet]. Bethesda (MD): National Center for Biotechnology Information; 2015 [cited 2018 September 10]. Available from: https://www.ncbi.nlm.nih.gov/genbank/.
5. Samson RA, Varga J. What is a species in *Aspergillus*? Med Mycol 2009;47:S13-20.
6. Raper KB, Fennell DI. The genus *Aspergillus*. Baltimore: Williams and Wilkins; 1965.
7. Goldman GH, Osmani SA. The aspergilli: Genomics, medical aspects, biotechnology, and research methods. Boca Raton: CRC Press; 2008.
8. Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M. Identification of *Aspergillus* species using morphological characteristics. Pak J Med Sci 2007;23:867-72.
9. Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K, Meijer M, Amend AS, Seifert KA, Samson RA. *Aspergillus*, *Penicillium*, and *Talaromyces* isolated from in house dust samples collected around the world. Stud Mycol 2014;78:63-139.
10. Nguyen TTT, Pangging M, Bangash NK, Lee HB. Five new records of the family Aspergillaceae in Korea, *Aspergillus eruopaeus*, *A. pragensis*, *A. tennesseensis*, *Penicillium fluviserpens* and *P. scabrosum*. Mycobiol 2020;48:81-94.
11. Park MS, Chung D, Baek K, Lim YW. Three Unrecorded species belonging to *Penicillium* section *Sclerotiora* from marine environments in Korea. Mycobiol 2019;47:165-72.
12. Choi DH, Kwon HJ, Kim MG, Kim DH, Kim YG, Kim JG. A-11: Diversity of endophytic fungi isolated from plant's roots in part of Korean Peninsula. Kor J Mycol 2019;31:74.
13. Khan SA, Hamayun M, Kim HY, Yoon HJ, Seo JC, Choo YS, Lee IJ, Kim SD, Rhee IK, Kim JG. A new strain of *Arthrinium phaeospermum* isolated from *Carex kobomugi* Ohwi is capable of gibberellin production. Biotechnol Lett 2009;31:283-7.
14. Wang XC, Chen K, Zeng ZQ, Zhuang WY. Phylogeny and morphological analyses of *Penicillium* section *Sclerotiora* (Fungi) lead to the discovery of five new species. Sci Rep 2017;7:8233-46.
15. Kil YJ, Eo JK, Eom AH. Molecular identification and diversity of endophytic fungi isolated from *Pinus densiflora* in Boeun, Korea. Kor J Mycol 2009;37:130-3.
16. Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA. Identification and nomenclature of the genus *Penicillium*. Stud Mycol 2014;78:343-71.
17. Pitt JI. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. London: Academic Press; 1979.
18. Kim HJ, Kim JS, Cheon KH, Kim DH, Seok SJ, Hong SB. Species list of *Aspergillus*, *Penicillium*, and *Talaromyces* in Korea, based on 'One Fungus One Name' system. Kor J Mycol 2016;44:207-19.

19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82.
20. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33:1870-4.
21. Samson RA, Varga J, Meijer M, Frisvad JC. New taxa in *Aspergillus* section *Usti*. Stud Mycol 2011;69:81-97.
22. Frisvad JC, Hubka V, Ezekiel CN, Hong SB, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenář F, Mahakarnchanakul W. Houbraken J. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Stud Mycol 2019;93:1-63.
23. Ropars J, Dupont J, Fontanillas E, Rodríguez de la Vega RC, Malagnac F, Coton M, Giraud T, López-Villavicencio M. Sex in cheese: Evidence for sexuality in the fungus *Penicillium roqueforti*. PLoS ONE 2012;7:e49665.
24. Kurtzman CP, Horn BW, Hesseltine CW. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 1987;53:147-58.
25. Samson RA, Varga J, Meijer M, Frisvad JC. New taxa in *Aspergillus* section *Usti*. Stud Mycol 2011;69:81-97.