RESEARCH ARTICLE

Identification and Characterization of Unreported *Penicillium* Species in Korea

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ABSTRACT

Fungal species belonging to the genus *Penicillium* are indigenous to a wide range of natural environments. Since the first published reports detailing the discovery of *Penicillium*, new species have been identified in various countries, including Korea. We present here a full characterization of five species of *Penicillium* that were previously unreported in Korea. Based on the morphologic characteristics and sequences of genes encoding fungal *β*-tubulin and calmodulin, we identified five *Penicillium* species, including *P. hetheringtonii*, *P. sublectaticum*, and *P. jacksonii*, which have been unrecorded in Korea, and confirming *P. maximae* and *P. cremeogriseum*, as the endophytic fungi isolated in Gyeongsang province, Korea. In this article, we provide detailed morphological descriptions of these fungal species.

Keywords: Endophytic fungi, Genus *Penicillium*, Identification, Morphological description

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INTRODUCTION

Ascomycota currently represent the largest phylum of fungi; at current count, this phylum includes over 64,000 fungal species [1]. In this phylum, the *Penicillium* is the most famous genus among the others. Three *Penicillium* species, *P. candidum, P. expansum* and *P. glaucum,* were first described in 1809 by Johann Heinrich Friedrich Link [2]. The mycelia of *Penicillium* consist of branched networks of polynuclear cells that share a common cytoplasm. In each branch, there are conidiospores with phialides that play a significant role in fungal reproduction. Similar to *Aspergillus*, species of the genus *Penicillium* can reproduce via an asexual pathway via its conidiospores and also via sexual reproduction pathway via ascospores. *Penicillium* species are also aerobic and prefer cool and moderate climates and oxygen–rich environments [3,4]. These fungi are normally useful to humans as sources of beneficial compounds, including antibiotics and as a means to ferment food and alcohol. Others, the other hand, are animal and plant pathogens. An exmaple of this phenomenon is the fungus *Penicillium marneffei* which has been identified as etiologic agents of human skin infections. The plant

pathogens of this phylum include those that generate apple scab, rice blast and powdery mildews [5,6]. Most species included among the ascomycetes have morphologic characteristics that include filamentous hyphae; interconnections within the hyphae result in a thallus, a structure also known as mycelium or mold. Ascomycetes can be found in various forms and shapes, including cup–shaped, potato–like, seed–like, and spongy, among others, although the shapes themselves may vary depending on environmental conditions such as growth medium and temperature [7,8]. As such, mycologists have proposed various identification schemes based on a more detailed molecular analysis. As the current time, molecular analyses contributing to fungal taxonomy have been based on sequences of internal transcribed spacer (ITS) region and DNA markers including the sequences of β–tubulin (*BenA*), calmodulin (*CaM*) and the second largest subunit of RNA polymerase II (*RPB2*) [2,9,10]. In this study, *BenA* and *CaM* were chosen for molecular analysis based on the research in 2019 [3]. The Korean peninsula has a unique and characteristic diversity of microorganisms, including fungi. Fungi promote decomposition of biomaterials and thereby produce an array of secondary metabolites; this activity contributes to the ecological biosystems at plant roots and elsewhere in the environment. Endophytic fungi have a profound influence on plant growth as they can regulate nutrients and contribute to the carbon cycle. As such, the ongoing study of these of microorganisms will be critical in order to promote further understanding of our natural environment and to maintain homeostasis with critical plant life. In 2019, several research groups presented an analysis of fungal diversity in Gyeongsang province based on both morphological characteristics and molecular analyses [11-13]. While the survey in 2018, over 200 different endophytic fungi were collected from roots of the plants in Gyeongsang province, including samples from Pohang, Jinju, Gumi, Gyeongsan and Ulleungdo Island. As part of the ongoing effort to characterize biodiversity, three fungal species, specifically, *P. hetheringtonii, P. sublectaticum, and P. jacksonii* were newly–recorded in Korea. We present the results of this investigation here.

MATERIALS AND METHODS

Isolation of endophytic fungi

In 2018, fungal diversity survey in Gyeongsang province, Korea, has been done. Among roughly two hundreds of samples of fungal isolates, five species of *Penicillium* were identified. The five fungi were isolated from plant roots of *Lespedeza cuneate*, *Chrysanthemum lavandulifolium*, *Sedum takevimense* and *Lilium lancifolium*. Roots were stored at 25℃ for 7-14 days; each root was washed with distilled water and 1% perchloric acid. Then the washed samples were cut in pieces and incubated in Hagem minimal medium with 80 parts per million (ppm) of streptomycin at 25℃. Isolated strains were transferred to potato dextrose agar (PDA) and cultivated at 25℃ for 7 days [14,15]. The isolated strains stored in 20% glycerol at -80℃ were deposited to the National Institute of Biological Resources (NIBR), Incheon, Korea. The strains have been deposited as NIBRFG0000505327– NIBRFG0000505331 at the NIBR.

Morphological analysis

Morphology of the five *Penicillium* species was investigated after growth in malt extract agar (MEA; Samson, 2010), czapek yeast autolysate agar (CYA; Pitt, 1979) and yeast extract sucrose agar (YES; Frisvad, 1981). All plates were incubated at 25℃ in the dark for 7 days; the CYA plates underwent additional incubation at 4℃ and 37℃. Characteristics measured included diameter and specific morphologic features of the fungal colonies. Fungal structures were characterized by light microscopy (Eclipse 80i, Nikon, Tokyo, Japan); 85% lactic acid and 99% ethanol were used for washing and fixing samples on glass slides [14,16,17].

DNA extraction, PCR, and sequencing

Isolated fungal strains were extracted using the Accuprep® genomic DNA extraction kit (BIONEER Corp, Daejeon, Korea). Isolated DNA preparations were stored at -20℃. In addition with a fungal barcoding maker, the internal transcribed spacer (ITS), two genes, Calmodulin (CaM) and β–tubulin (BenA) were amplified using the primer sets CMD5/CMD6 and Bt2a/Bt2b [3,9]. The resulting amplicons were isolated from a 20 uL pCR using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Amplicons were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster, CA, USA) on an ABI 310 DNA sequencer (Perkin Elmer, Foster, CA, USA).

Phylogenetic analysis

The nucleotide sequences of the amplified genes were compared to the sequences available in the National Center for Biotechnology Information (NCBI) GenBank database; we performed BLASTn search for each sequence. To construct the phylogenetic tree, the references of genus *Penicillium* were selected based on the BLASTn results. Sequences identified were aligned by BioEdit v7.2.5 and Clustal W; phylogenetic information was analyzed using Molecular Evolutionary Genetics Analysis (MEGA) 7. Maximum likelihood (ML) phylogenetic tree was constructed [18,19]. The sequence of *P. meridianum* was used as an outgroup.

RESULTS AND DISCUSSION

Sequences of *BenA* (*β*–tubulin) and *CaM* (calmodulin) amplified from the isolated fungal strains were successfully identified by BLASTn–based comparisons and phylogenetic analyses (Fig. 1). The sequences of these genes from the five *Penicillium* species were identified in the GenBank database and summarized in Table 1. Each isolate was included in monophyletic groups with the type strain for each *Penicillium* species. The isolate NIBRFG0000505327 was included in a monophyletic group with the type strain of *P. hetheringtonii* (sequence similarity for *BenA* at 99.8-100% with GU944538 and *CaM* at 94.1-99.8% with GU944642; bootstrap value, 100%). The isolate NIBRFG0000505328 was included in a monophyletic group with the type strain of *P. sublectaticum* (sequence similarity for *BenA*

Table 1. Summary and GenBank accession numbers for *Penicillium* strains isolated from environment of Gyeongsang province.

Species	Strain No.	Substrate	GPS coordinates	Collector	Collect date	Accession No.	
						BenA	*CaM*
P. maximae	NIBRFG0000505329	*Chrysanthemum lavandulifolium*	35°9'42.94"N 128°17'45.64"E	Doo-Ho Choi Jong-Guk Kim	24-Oct-18	MN850488	MN850484
P. jacksonii	NIBRFG0000505331	*Lilium lancifolium*	37º31'53.23"N 130º52'36.88"E	Doo-Ho Choi Jong-Guk Kim	24-Nov-18	MN862312	MN862316
P. sublectaticum	NIBRFG0000505328	*Phedimus takesimensis*	37°28'54.97"N 130°54'32.76"E	Doo-Ho Choi Jong-Guk Kim	27-Oct-18	MN850487	MN850485
P. hetheringtonii	NIBRFG0000505327	*Phedimus takesimensis*	37°28'54.97"N 130°54'32.76"E	Doo-Ho Choi Jong-Guk Kim	24-Oct-18	MN841277	MN850486
P. cremeogriseum	NIBRFG0000505330	*Lespedeza cuneata*	35°47'56.64"N 128°47'20.08"E	Doo-Ho Choi Jong-Guk Kim	15-Nov-18	MN862311	MN862315

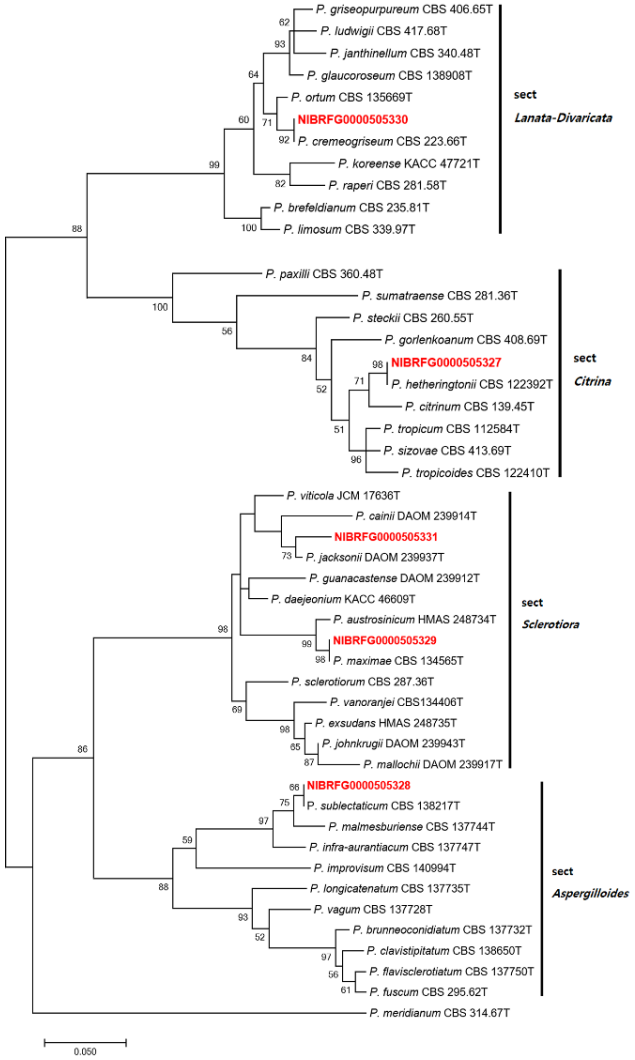

Fig. 1. Maximum–likelihood phylogenetic analysis of combined sequence data of *BenA* and *CaM* genes from known species of the genus *Penicillium*. The sequence of *P. meridianum* was used as an outgroup. Bootstrap scores >50 is presented. The number of nucleotide substitution per site was denoted by the scale bar. "T" identifies the type strains of the given fungal species.

at 94.9-100% with KM089010 and for *CaM* at 99.5-100% with KM089397; bootstrap value, 100%). The NIBRFG0000505329 was included in a monophyletic group with the type strain of *P. maximae* (sequence similarity for *BenA* at 99.6-100% with KC773795 and *CaM* at 99.5-100% with KC773821; bootstrap value, 100%). The isolate NIBRFG0000505330 was included in a monophyletic group with the type strain of *P. cremeogriseum* (sequence similarity for *BenA* at 97.1-100% with GU981624 and for *CaM* at 100% with KF296403; bootstrap value, 100%). *P. jacksonii* (sequence similarity for *BenA* at 100% with JN686368 and *CaM* at 94.9-95.1% with JN686391; bootstrap value, 100%). The previously unreported endophytic fungi featured here were identified in both marine and mountain environments in Korea. In this study, totally five *Penicillium* species were identified, of which *P. maximae* and *P. cremeogriseum* have been already reported in 2019 [3,20]. To the best of our knowledge, this is the first report of *P. hetheringtonii, P. sublectaticum,* and *P. jacksonii* identified in Korea. Additional taxonomic information is presented in the section to follow.

Taxonomy

Penicillium maximae **C.M. Visagie, J. Houbraken & R.A. Samson (2013)**

Description: Colony diam, 7 d, in mm: CYA 30-33; CYA 30℃ 29-32; CYA 37℃ no growth; MEA 15-24; YES 33-36 (Fig. 2).

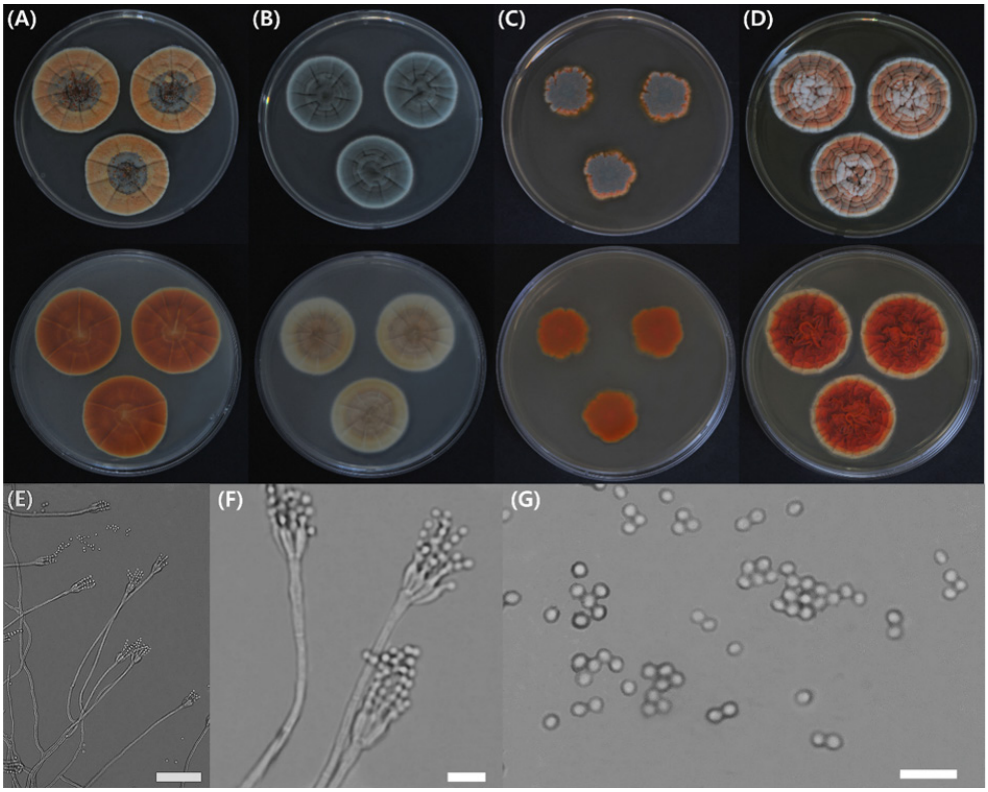

Fig. 2. *Penicillium maximae* (isolate NIBRFG0000505329) after 7 days in culture. Colonies grown on (A) czapek yeast autolysate (CYA) agar at 25°C and (B) 30°C, (C) on malt extract agar (MEA) at 25°C and (D) yeast extract sucrose (YES) agar at 25°C; images include the top and undersides of the plates. (E–F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

CYA, 25°C: Colonies moderately deep, angular shape, radially sulcate with a grayish–green color; margins low, narrow, entire; mycelia white and pinkish orange; texture floccose; sporulation sparse, soluble pigment present, reverse pigmentation pinkish–brown at center and fading to white.

MEA, 25°C: Colonies moderately deep, lightly sulcate, with a grayish–green color; margins low, narrow, entire; mycelia white and pinkish orange; texture floccose and velutinous; sporulation dense at center, soluble pigment absent, reverse pigmentation pinkish–brown at center and fading to orange.

YES, 25°C: Colonies moderately deep, randomly sulcate, with a white and reddish–orange color; margins low, narrow, entire; mycelia white and reddish–orange; texture floccose; sporulation sparse, soluble pigment absent, reverse pigmentation bright reddish–brown at center fading to orange.

Micromorphology : Conidiophores were strictly monoverticillate, smooth–walled stipes, 1.6-4.1×104.5-152.2 μm; single metula on a conidiophore, smooth, 1.6-2.5×20.3-27.7 μm; phialides were of ampulliform type, navicular shape, smooth, 3-6 in number, 2.8-3.6×7.9-9.6 μm; conidia ellipsoidal, 2.5-3×3.1-3.4 μm; smooth–walled.

Strain examined: NIBRFG0000505329, isolated from *Chrysanthemum lavandulifolium* in Jinju (35°9'42.94"N, 128°17'45.64"E).

Note: When compared with the type strain of *Penicillium maximae*, the Korean isolate showed less vigorous growth on MEA and YES at 25°C [3,20].

***Penicillium jacksonii* K.G. Rivera, Houbraken & Seifert (2011)**

Description: Colony diam, 7 d, in mm: CYA 25-29; CYA 30°C 30-33; CYA 37°C no growth; MEA 12-15; YES 26-31 (Fig. 3).

CYA, 25°C: Colonies moderately deep, lightly radially sulcate with a deep green color; margins low, narrow, entire; mycelia white to yellowish white; texture floccose; sporulation sparse, soluble pigment absent, reverse pigmentation vivid yellow at center fading to white.

MEA, 25°C: Colonies moderately deep, lightly sulcate, with a deep green color; margins low, narrow, entire; mycelia grayish–white; texture floccose and velutinous; sporulation dense at center, soluble pigment absent, reverse pigmentation deep yellowish–brown at center and fading to pale.

YES, 25°C: Colonies fairly deep, randomly sulcate with a grayish–green color; margins entire; mycelia pale grayish green and white; texture floccose; sporulation sparse, soluble pigment absent, reverse pigmentation reddish–yellow at center fading to yellowish–white.

Micromorphology : Conidiophores were biverticillate or monoverticillate, smooth and rough–walled stipes, 3-4.1×28.4-164.8 μm; single metula was on a conidiophore, smooth, 2.2-3.1×8.7-14.8 μm; phialides were ampulliform type, navicular shape, smooth, 5-11 in number, 2-2.9×6-9.6 μm; conidia globose, 2.2-2.8 μm; smooth–walled.

Strain examined: NIBRFG0000505331, isolated from *Lilium lancifolium* in Ulleungdo island (37°31'53.23"N, 130°52'36.88"E).

Note: When compared with the type strain of *Penicillium jacksonii*, the isolate from Korea can be easily

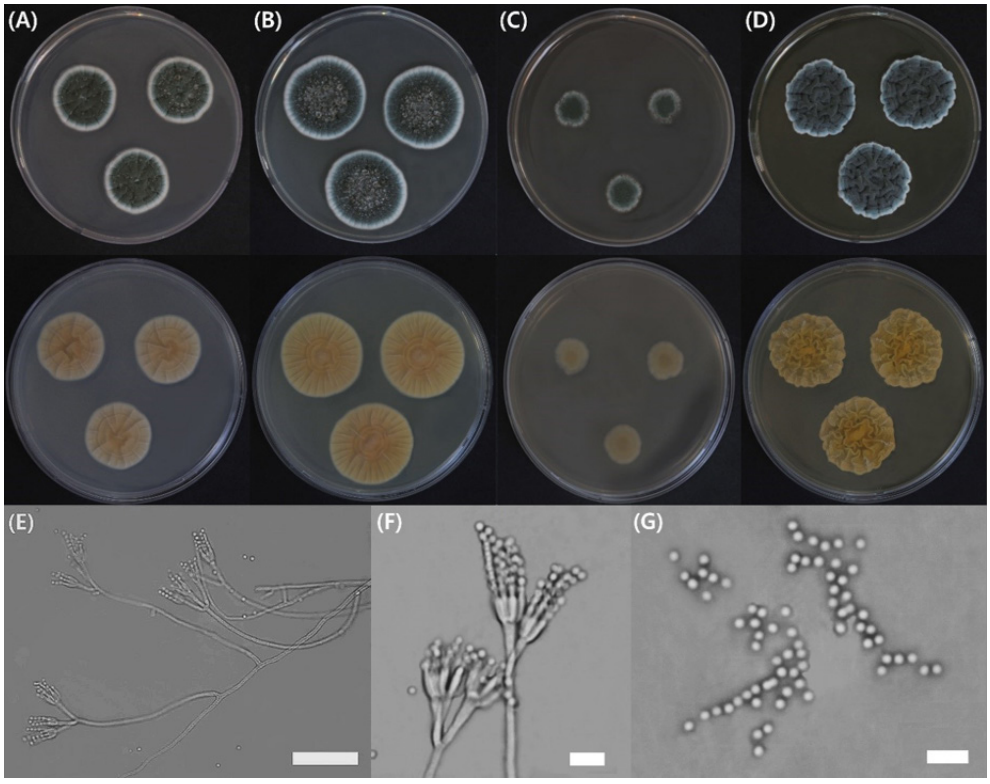

Fig. 3. *Penicillium jacksonii* NIBRFG0000505331 after 7 days in culture. Colonies grown on (A) czapek yeast autolysate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E–F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

distinguished by the conidiophore with monoverticillate and biverticillate. Except the MEA, this species showed the similar growth with type strain [18].

Penicillium sublectaticum **Houbraken, Frisvad, Samson & Seifert (2014)**

Description: Colony diam, 7 d, in mm: CYA 26-30; CYA 30℃ 6-10; CYA 37℃ no growth; MEA 21-24; YES 30-33 (Fig. 4).

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

MEA, 25℃: Colonies moderately deep, lightly sulcate with a grayish–green color; margins low, narrow, entire; mycelia pale grayish green; texture floccose; moderate sporulation evenly, soluble pigment absent, reverse pigmentation brown at center fading to pale.

YES, 25℃: Colonies fairly deep, lightly randomly sulcate with a grayish–green color; margins entire; mycelia yellowish–white; texture floccose and velutinous; sporulation sparse, soluble pigment absent, reverse pigmentation reddish–brown at center and fading to cream to brown.

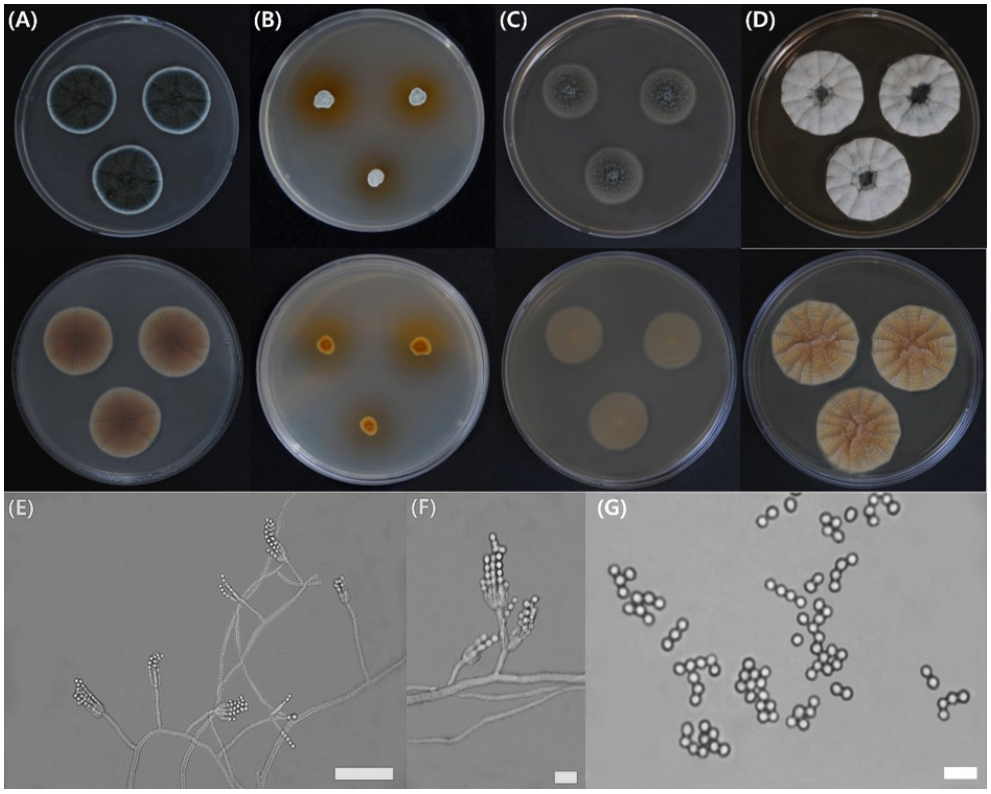

Fig. 4. *Penicillium sublectaticum* NIBRFG0000505328 after 7 days in culture. Colonies grown on (A) czapek yeast autolysate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E–F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

Micromorphology: Conidiophores were monoverticillate with an additional monoverticillate branch, smooth–walled stipes, 3.3-4.4×25.7-62.9 μm; single metula was on a conidiophore, smooth, 2.6-3.1×10.8-15.4 μm; phialides were ampulliform type, cylindrical shape, smooth, 3-7 in number, 2.3-2.9×6-8.2 μm; conidia globose to subglobose, 3.1-3.4 μm; smooth–walled.

Strain examined: NIBRFG0000505328, isolated from *Sedum takevimense* in Ulleungdo island (37°28'54.97"N, 130°54'32.76"E).

Note: When compared with the type strain of *Penicillium sublectaticum***,** the present isolate showed similar growth on all media. This species can be distinguished by the smaller size of conidiophores [12].

***Penicillium hetheringtonii* Houbraken, Frisvad & Samson (2010)**

Description: Colony diam, 7 d, in mm: CYA 30-35; CYA 30℃ 21-24; CYA 37℃ almost no growth; MEA 15-21; YES 33-36 (Fig. 5).

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

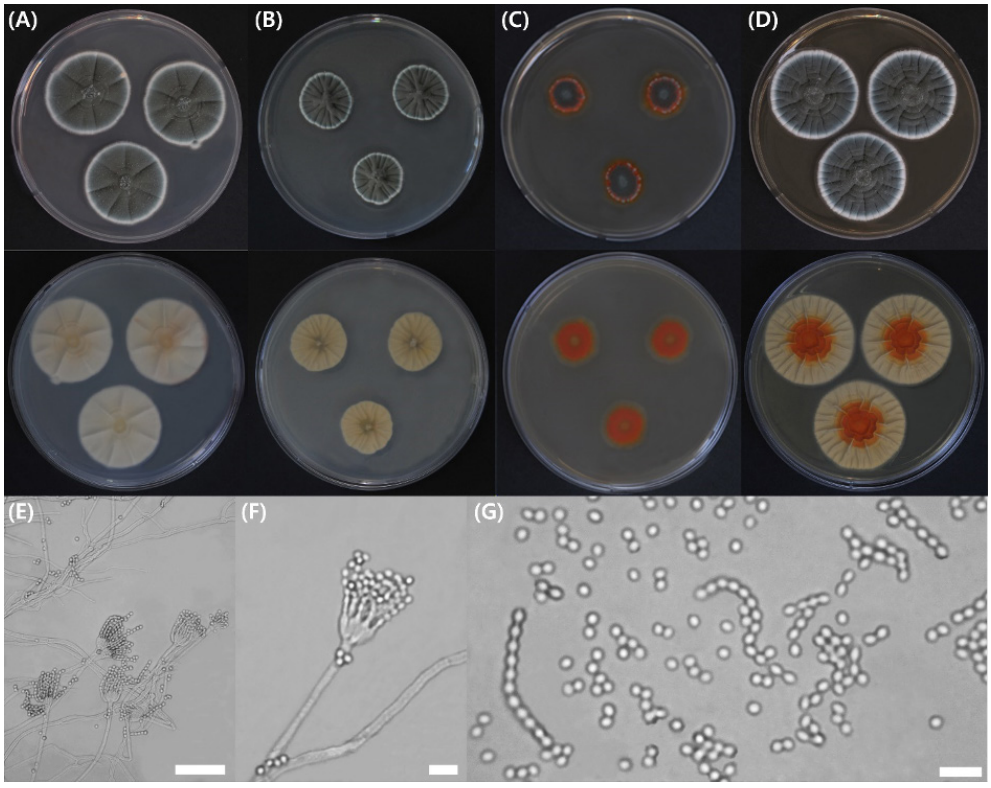

Fig. 5. *Penicillium hetheringtonii* NIBRFG0000505327 after 7 days in culture. Colonies grown on (A) czapek yeast autolyate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E–F) Conidiophores; (G) Conidia (scale bars; E, 50 μm; F and G, 10 μm).

MEA, 25℃: Colonies moderately deep, lightly sulcate with a grayish–green color; margins low, moderate, entire; mycelia orange and translucent; texture floccose and velutinous; moderate sporulation dense at center, soluble pigment absent, reverse pigmentation velvety or orange color at center fading to translucent.

YES, 25℃: Colonies moderately deep, lightly angular shape, lightly randomly sulcate with a grayish–green color; margins low, narrow, entire; mycelia white; texture floccose; sporulation sparse, soluble pigment present, reverse pigmentation orange at center and brownish–yellow at margins.

Micromorphology: Conidiophores were strictly monoverticillate, smooth–walled stipes, 2.8-3.9×54-198 μm; single metula was on a conidiophore, smooth, 3.2-3.8×22.1-27.5 μm; phialides were ampulliform type, navicular shape, smooth, 5-7 in number, 2.3-4.7×7.6-11.2 μm; conidia ellipsoidal, 2-2.4 μm; smooth–walled.

Strain examined: NIBRFG0000505327, isolated from *Sedum takevimense* in Ulleungdo island (37°28'54.97"N, 130°54'32.76"E).

Note: When compared with the type strain of *Penicillium hetheringtonii,* the present Korean isolate *showed* similar growth on all media. Otherwise, the form of conidiophores showed quit difference;

biverticillate form in type strain and monoverticillate form in this species [14].

Penicillium cremeogriseum **Chalab., Bot. Mater. Otd. Sporov. Rast (1950)**

Description: Colony diam, 7 d, in mm: CYA 33-37; CYA 30℃ 42-48; CYA 37℃ 30-34; MEA 35-41; YES 32-37 (Fig. 6).

CYA, 25℃: Colonies moderately deep, lightly angular shape, radially sulcate with a grayish–white color; margins high, moderate, entire; mycelia white; texture floccose and velutinous; sporulation sparse, soluble pigment absent, reverse pigmentation cream to brown at center fading to pale.

MEA, 25℃: Colonies moderately deep, lightly sulcate, with a grayish–green color; margins low, moderate, entire; mycelia grayish–green; texture floccose and velutinous; sporulation dense at center, soluble pigment present, reverse pigmentation velvety or yellowish–brown at center fading to pale.

YES, 25℃: Colonies moderately deep, angular shape, radially sulcate with a pale grayish– green color; margins entire; mycelia high, moderate, entire; mycelia yellowish–white; texture floccose; sporulation dense at center, soluble pigment present, reverse pigmentation deep reddish–brown at center and cream to brown at margins.

Micromorphology : Conidiophores were biverticillate or monoverticillate with an additional

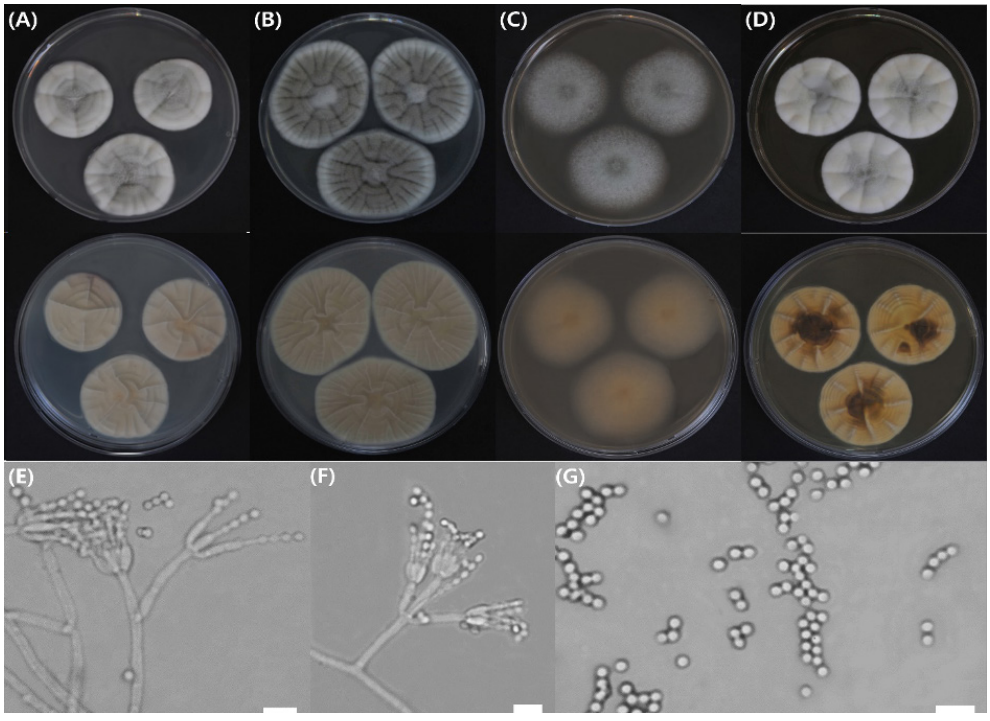

Fig. 6. *Penicillium cremeogriseum* NIBRFG0000505330 after 7 days in culture. Colonies grown on (A) czapek yeast autolyate (CYA) agar at 25℃ and (B) 30℃, (C) on malt extract agar (MEA) at 25℃ and (D) yeast extract sucrose (YES) agar at 25℃; images include the top and undersides of the plates. (E–F) Conidiophores; (G) Conidia (scale bars; E – G, 10 μm).

monoverticillate branch, smooth–walled stipes, 1.8-2.1×14.3-39.5 μm; single metula was on a conidiophore, smooth, 1.6-2.4×6.3-8.6 μm; phialides were ampulliform type, subcylindrical shape, smooth, 3-6 in number, 1.4-1.7×3.4-4.3 μm; conidia elliptical, 2.6-3 μm; smooth–walled.

Strain examined: NIBRFG0000505330, isolated from *Lespedeza cuneata* in Gyeongsan (35°47'56.64"N, 128°47'20.08"E).

Note: When compared with the type strain of *Penicillium cremeogriseum*, the isolate from Korea showed less vigorous growth on MEA and YES at 25℃. Not like the type strain, this strain showed the conidiophores with biverticillate. Both of them show no soluble pigments [3,10].

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