The Korean Journal of Mycology www.kjmycology.or.kr

RESEARCH ARTICLE

Two Unrecoreded Species Belonging to *Penicillium* Section *Exilicaulis* in South Korea

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ABSTRACT

Penicillium in section *Exilicaulis* is characterized by non-vesiculate monoverticillate and biverticillate stipes. Species in sect. *Exilicaulis* are commonly found in soil and plants in terrestrial environments; however, only a few species have been reported in Korea. To investigate the diversity of *Penicillium* sect. *Exilicaulis*, *Penicillium* species were isolated from terrestrial and marine environments. Based on sequence analyses of β-tubulin, calmodulin, and the second largest subunit of RNA polymerase II loci, 19 strains of *Penicillium* in sect. *Exilicaulis* were identified as *P. citreonigrum*, *P. citreosulfuratum*, *P. corylophilum*, *P. menonorum, P. rubefaciens*, *P. velutinum*, *Penicillium* sp. 1, and *Penicillium* sp. 2. Two of them, *P. citreonigrum* and *P. citreosulfuratum*, were confirmed to be new to Korea. Molecular phylogenies and detailed descriptions of the two unrecorded species are provided.

Keywords: *BenA*, *CaM*, Newly recorded species, *RPB2*

OPEN ACCESS

pISSN : 0253-651X
eISSN : 2383-5249

Kor. J. Mycol. 2020 September, 48(3): 175-185
https://doi.org/10.4489/KJM.20200019

Received: September 01, 2020
Revised: September 13, 2020
Accepted: September 16, 2020

INTRODUCTION

The genus *Penicillium* is one of the most common fungi found in various environments [1-3]. They play important ecological roles as decomposers [4]. Some *Penicillium* species are known as producer of solubilized phosphorus, siderophore, and phytohormones, which are important for plant health [5]. To date, approximately 460 species of *Penicillium* in 26 sections have been reported worldwide [6-10]. *Penicillium* in sect. *Exilicaulis* is commonly isolated from air, soil, plants, and insects [8,9]. Some species can produce metabolites that cause allergies in humans [11,12].

Section *Exilicaulis* is characterized by non-vesiculate monoverticillate stipes [1]. Recently, based on multigene phylogenetic analysis, sect. *Exilicaulis* was redefined to include species with biverticillate conidiophores, and was separated into six clades [9,13]. β-tubulin (*BenA*), calmodulin (*CaM*), and the second largest subunit of RNA polymerase II (*RPB2*) were used for accurate identification of the species in this section [9]. So far, 55 species have been reported in this section worldwide [8,9]. In Korea, a total of nine species have been reported: *P. corylophilum, P. decumbens*, *P. erubescens*, *P. melinii, P. menonorum, P. restrictum, P. rubefaciens*, *P. rubidurum*, and *P. velutinum*. They were isolated from air, mushroom

media materials, and soil. Most species were identified by morphological characteristics and/or internal transcribed spacer (ITS) sequence [14-17]. However, *Penicillium* are difficult to identify at the species level using morphology and/or ITS sequence because of their morphological plasticity and similarity and low resolution of ITS region [7]. Therefore, the accurate diversity at species level of this section in Korea is unclear.

As part of the projects organized by Basic Science Research Program through the National Research Foundation of Korea (NRF) and the Marine Biotechnology Program of the Korea Institute of Marine Science and Technology Promotion (KIMST) to excavate Korean *Penicillium* and marine fungi, we have been studying a number of *Penicillium* species from various environments in Korea. To investigate the accurate diversity of *Penicillium* sect. *Exilicaulis* in various environments, we re-identified previously isolated species in sect. *Exilicaulis* using sequence analysis of *BenA, CaM,* and *RPB2.* In this study, a total of eight species were detected in this section and two species–*P. citreonigrum,* and *P. citreosulfuratum* – were confirmed as unrecorded species in Korea. The detailed descriptions for the two unrecorded species have been provided in this study.

MATERIALS AND METHODS

Materials

A total of 19 *Penicillium* strains were identified in this study. Twelve strains were isolated from egg masses of *Arctoscopus japonicas* (2 strains), mudflats (4), sea sand (5), and seaweed (1) using previously described methods [18-20]. Two strains were isolated from rhizosphere soil. Five grams of soil was diluted tenfold with sterile water. Next, 100 μL of each dilution was plated on dichloran rose bengal chloramphenicol agar (DRBC, Difco, Becton Dickinson, MD, USA). Rotten pine bark (1 strain), rotten pine sapwood (3) and sponge (1) were cut to approximately 5 mm in length and were placed on DRBC agar. All plates were incubated at 25℃ for 7 days and were transferred to a PDA plate. Each strain is stored in 20% glycerol at -80℃ at the Seoul National University Fungus Collection (SFC) (Table 1).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from strain grown on malt extract agar (MEA; Oxoid, Hampshire, UK) using a cetyltrimethylammonium bromide extraction protocol [21]. *BenA, CaM* and *RPB2* were amplified using previously described methods [22]. The PCR products were purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea), according to the manufacturer's instructions. DNA sequencing was performed on an ABI Prism 3700 genetic analyzer (Life Technologies, Gaithersburg, MD, USA) at Macrogen (Seoul, Korea) using the PCR primers.

Table 1. Summary and GenBank accession numbers for *Penicillium* in section *Exilicaulis*.

Species	Strain No.	Site	Habitate	*BenA*	*CaM*	*RPB2*
P. citreonigrum[a]	SFC20200821-M01	Ocheon-dong, Yeosu-si, Jeollanam-do	Sponge	MT945077	MT945096	MT945115
	SFCP0024	Daebang-ri, Subuk-myeon, Damyang-gun, Jeollanam-do	Rotten Pine bark	MT945078	MT945097	MT945116
	SFCP0026	Sillim-dong, Gwanak-gu, Seoul	Rotten Pine sapwood	MT945079	MT945098	MT945117
	SFCP0027	Sodo-dong, Taebaek-si, Gangwon-do	Rotten Pine sapwood	MT945080	MT945099	MT945118
	SFCP0028	Sodo-dong, Taebaek-si, Gangwon-do	Rotten Pine sapwood	MT945081	MT945100	MT945119
P. citreosulfuratum	SFC20170821-M07	Oryu-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Sea sand	MT945082	MT945101	MT945120
	SFC20200821-M02	Oryu-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Sea sand	MT945083	MT945102	MT945121
	SFC20200821-M03	Oryu-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Sea sand	MT945084	MT945103	MT945122
P. corylophilum	SFC20170718-M14	Chodo-ri, Hyeonnae-myeon, Goseong-gun, Gangwon-do	Sea sand	MT945085	MT945104	MT945123
	SFC20141123-M44	Gwakji-ri, Aewol-eup, Jeju-si, Jeju-do	Seaweed	MT945086	MT945105	MT945124
P. menonorum	SFC20200821-M04	Pyeongsan-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Mud flat	MT945087	MT945106	MT945125
	SFC20200821-M05	Pyeongsan-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Mud flat	MT945088	MT945107	MT945126
P. rubefaciens	SFC20140101-M799	Wando-gun, Jeollanam-do	Mud flat	MT945089	MT945108	MT945127
P. velutinum	SFC20150915-M11	Dongmak-ri, Hwado-myeon, Ganghwa-gun, Incheon	Sea sand	MT945090	MT945109	MT945128
	SFC20200506-M51	Dongdeok-ri, Yeongok-myeon, Gangneung-si, Gangwon-do	Sailfin sandfish egg masses	MT945091	MT945110	MT945129
	SFC101366	Jeong-am-ri, Ganghyeon-myeon, Yangyang-gun, Gangwon-do	Sailfin sandfish egg masses	MT945092	MT945111	MT945130
Penicillium sp. 1	SFC20200821-M06	Jangheung-ri, Gilsang-myeon, Ganghwa-gun, Incheon	Mud flat	MT945093	MT945112	MT945131
Penicillium sp. 2	SFCP0509	Ui-dong, Gangbuk-gu, Seoul	Rhizosphere soil	MT945094	MT945113	MT945132
	SFCP0523	Ui-dong, Gangbuk-gu, Seoul	Rhizosphere soil	MT945095	MT945114	MT945133

[a] The unrecorded Penicillium species in Korea are represented in bold.

Phylogenetic analysis

Each sequence was assembled and proofread using MEGA5 [23]. The resulting consensus sequences were deposited in GenBank (accession Nos. are shown in Table 1). Molecular identification was performed in two steps. First, we identified strains belonging to section *Exilicaulis* by comparison to the *BenA* sequences of type strains. Next, each strain was identified to the species level by analyzing the combined dataset of the three loci (*BenA*, *CaM*, and *RPB2*). *Penicillium trzebinskii* CBS 351.51 was used as the outgroup [9]. The sequence similarities were calculated from the three loci for each species using MEGA5 [23]. Multiple alignments were performed using MAFFT v7 [24]. Maximum likelihood phylogenetic analyses were performed with RAxML [25] implemented on CIPRES web portal [26], using the GTR+G model with 1,000 bootstrap replicates.

Morphological analysis

The morphological observation of the two unrecorded species was performed using previously described methods [7] on three different media: Czapek yeast autolysate agar (CYA; Difco, Sparks, MD, USA), malt extract agar (MEA; Oxoid, Hampshire, UK), and yeast extract sucrose agar (YES; Difco, Sparks, MD, USA). The Methuen Handbook of Color was used for the color names and alphanumeric codes for macromorphological characteristics [27]. The microscopic features were observed under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan) using colonies grown on MEA at 25°C for seven days.

RESULTS

A total of 19 *Penicillium* strains were isolated from terrestrial (6 strains) and marine (13 strains) environments. They were grouped into eight groups in section *Exilicaulis* based on *BenA* sequences. For accurate identification of each strain, all strains were used for the combined dataset of *BenA*, *CaM*, and *RPB2*. These were confirmed as 8 species: *P. citreonigrum*, *P. citreosulfuratum*, *P. corylophilum*, *P. menonorum, P. rubefaciens*, *P. velutinum*, *Penicillium* sp. 1, and *Penicillium* sp. 2 (Fig. 1).

Five strains formed a monophyletic group with *P. citreonigrum* NRRL 761 (type strain), CBS 321.59, and NRRL 1187 (sequence similarity for *BenA*=98.8-100%, *CaM*=98.0-100%, and *RPB2*=98.5-99.2%; bootstrap support=89%). Three strains grouped with *P. citreosulfuratum* DTO 290-I4 (type strain), CV 2015, and NRRL 31271 (sequence similarity for *BenA*=100% and *CaM*=99.8-100%, *RPB2*=99.9; bootstrap support=100%). Two strains formed a monophyletic group with the type strain (CBS 312.48) of *P. corylophilum*, CBS 231.38 and CBS127808 (sequence similarity for *BenA*=98.8-99.0%, *CaM*=99.8%, *RPB2*=99.7; bootstrap support=100%). SFC20200821-M04 and SFC20200821-M05 grouped with *P. menonorum* NRRL 50410 (type strain) (sequence similarity for *BenA*=100% and *CaM*=99.8-100%, *RPB2*=99.7; bootstrap support=100%). SFC20140101-M799 formed a monophyletic group with *P. rubefaciens* CBS 1450.83 (type strain) and CV0597 (sequence similarity for *BenA*=99.2-99.3% and *CaM*=99.4-99.5%, *RPB2*=100; bootstrap support=99%). Three strains grouped with *P. velutinum* NRRL 2069 (type strain) (sequence similarity for *BenA*=100%, *RPB2*=99.9; bootstrap support=100%). The remaining two groups formed distinct group with previously reported species. These groups were designated as *Penicillium* sp. 1 and *Penicillium* sp. 2.

Taxonomy

Penicillium citreonigrum **Dierckx (1901)**

Description: Colony diam, 7 d, in mm: CYA 25-28; CYA 30℃ 24-27; CYA 37℃ no growth; MEA 24-26; YES 30-31 (Fig. 2).

Colony characters: CYA, 25℃, 7 d: Colonies low to moderately deep, radially sulcate; margins low, wide, entire; mycelia white to grayish yellow (2B4); texture velvety; sporulation sparse; conidia greyish green (30B4); exudates clear at center; soluble pigments yellow; reverse color deep yellow (4A8). MEA, 25℃, 7 d: Colonies low to moderately deep, radially sulcate; margins low, narrow, entire; mycelia white; texture velvety, floccose at center; sporulation moderate; conidia dull green (25D3); exudates absent; soluble pigments absent; reverse color brownish yellow (5C8). YES, 25℃, 7 d: Colonies low to moderately deep, randomly furrowed; margins low, narrow, entire; mycelia white; texture velvety; sporulation sparse to moderate; conidia greyish green (30B3) at margin, light green (30A5) elsewhere; exudates absent; soluble pigments absent; reverse color deep yellow (4A8), greyish yellow (4B5) at center.

Conidiophores monoverticillate, occasionally biverticillate; stipes smooth walls; phialides ampulliform, 6.0-9.0×2.0-3.0 μm; conidia smooth walls, globose to subglobose, 1.8-2.6 μm diam; sclerotia absent; asci and ascospores not observed.

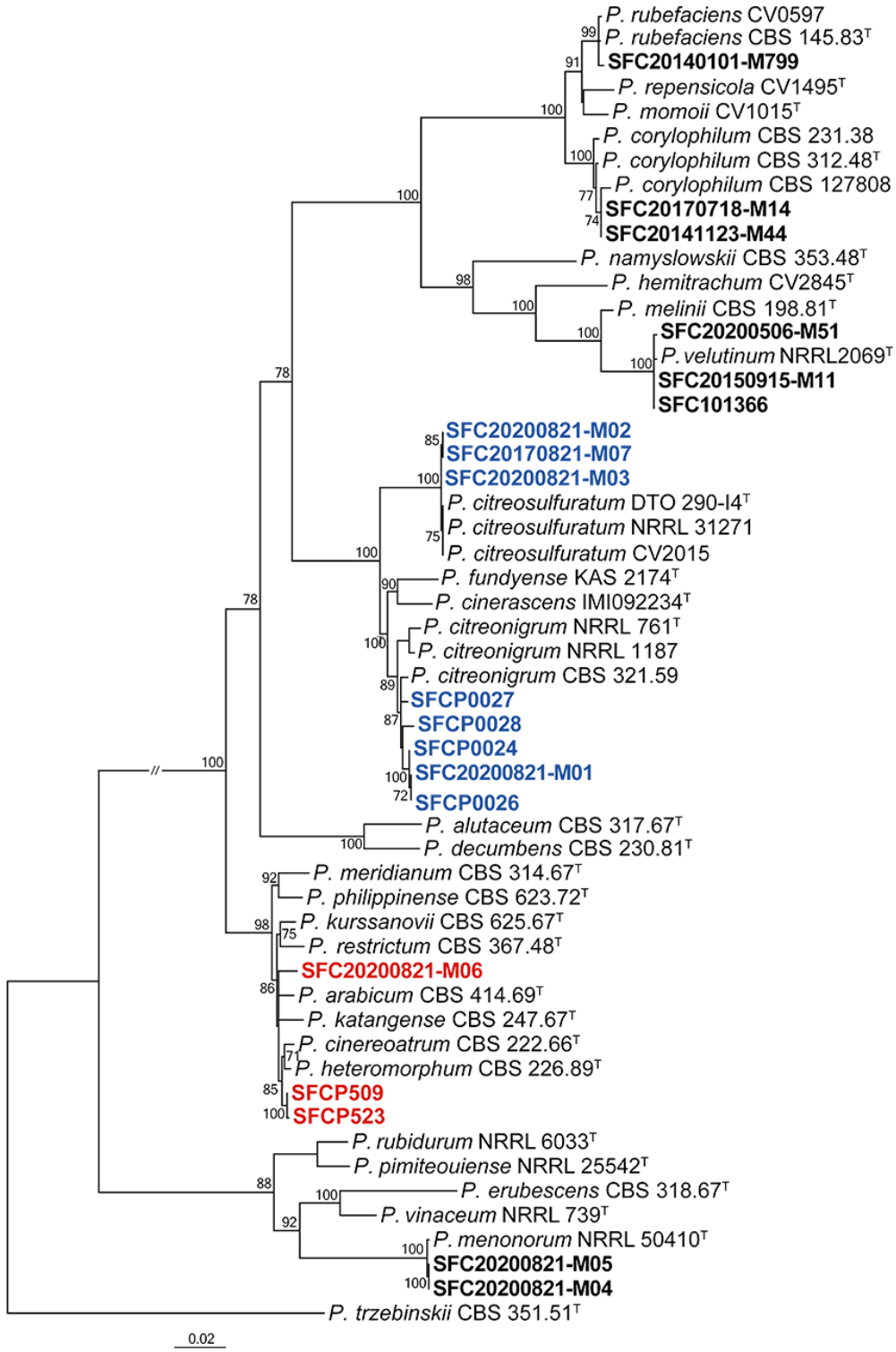

Fig. 1. Maximum likelihood phylogenetic tree of the combined data set of *BenA*, *CaM*, and *RPB2* gene sequences used to identify strains to the species level in *Penicillium* section *Exilicaulis*. Bootstrap scores of >70 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site. "T" indicates the ex-type strains. Strains reported in the current study are represented in bold. The species labeled in blue represent previously unrecorded species in South Korea. The names in red are potential new species.

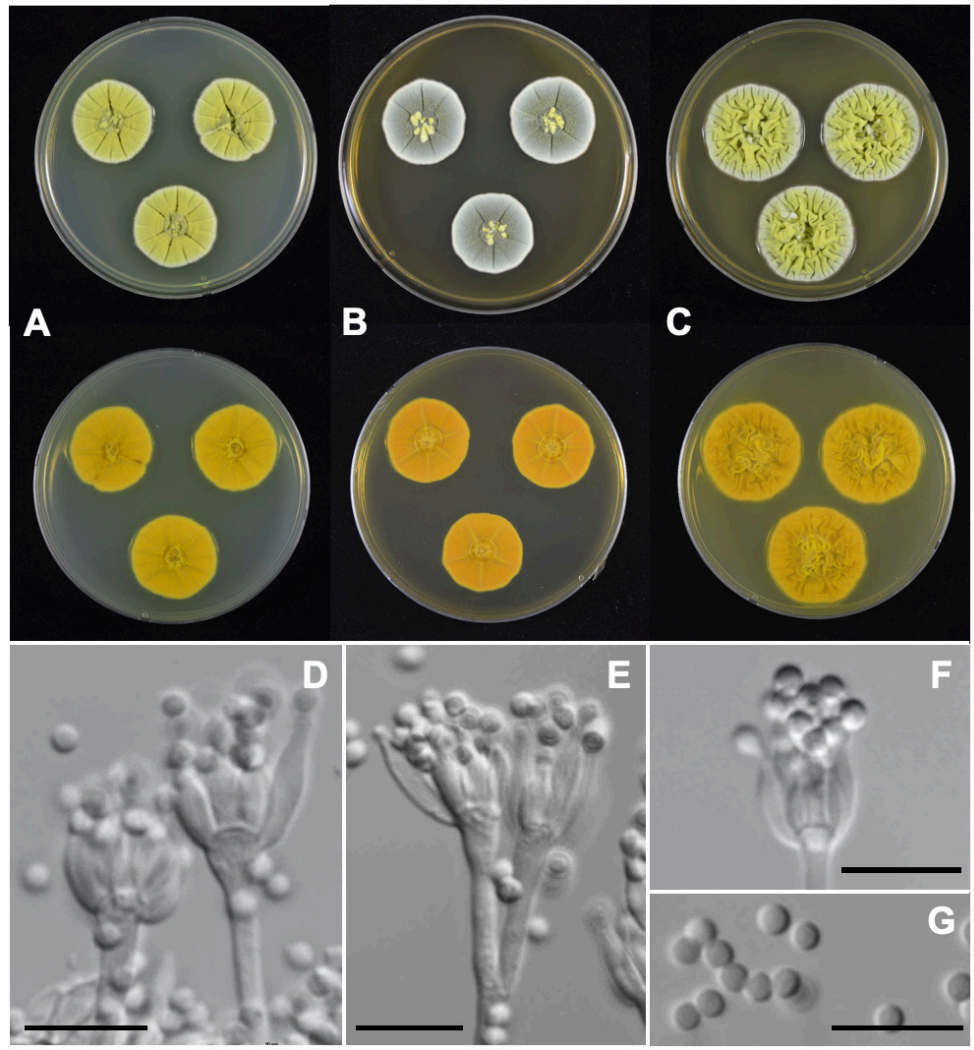

Fig. 2. *Penicillium citreonigrum* SFCP0024 in 7-day-old cultures at 25℃. (A-C) Colonies grown on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES) from left to right (top=obverse, bottom=reverse). (D-F) Conidiophores; (G) Conidia (scale bar: D-G=10 μm).

Strain examined: SFCP0024

Note: — *Penicillium citreonigrum* is morphologically similar to *P. citreosulfuratum*, *P. cinerascens*, and *P. fundyense*. *Penicillium citreonigrum* and *P. cinerascens* can be distinguished from *P. citreosulfuratum* by no growth on CYA at 37℃ [8,9]. *Penicillium citreonigrum*, *P. cinerascens*, and *P. fundyense* are difficult to identify based on morphological characteristics. They are accurately identified based on ITS or *BenA* sequences.

***Penicillium citreosulfuratum* Biourge (1923)**

Description: Colony diam, 7 d, in mm: CYA 27-28; CYA 30℃ 28-30; CYA 37℃ 7-10; MEA 23-25; YES 28-30 (Fig. 3).

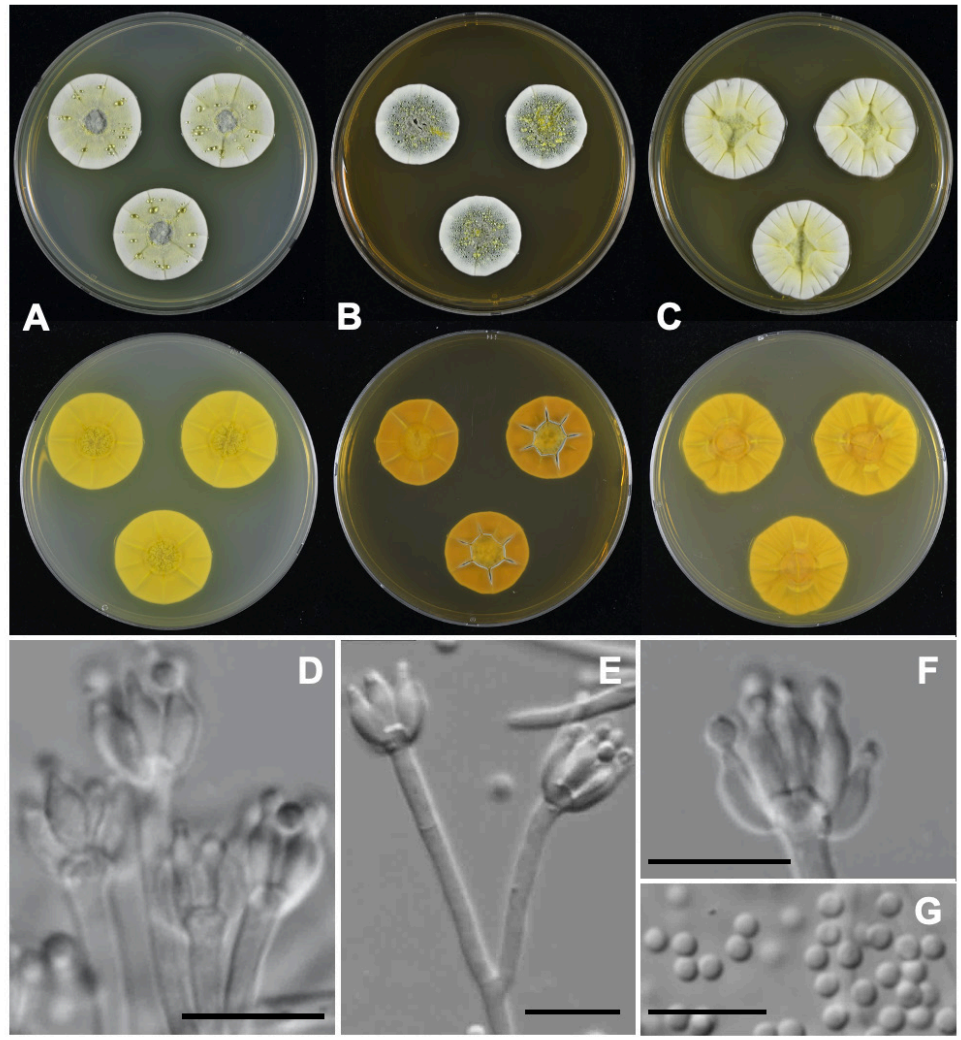

Fig. 3. *Penicillium citreosulfuratum* SFC20170821-M07 in 7-day-old cultures at 25℃. (A-C) Colonies grown on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES) from left to right (top=obverse, bottom=reverse). (D-F) Conidiophores; (G) Conidia (scale bar: D-G=10 μm).

Colony characters: CYA, 25℃, 7 d: Colonies low to moderately deep, radially sulcate; margins moderately deep, entire; mycelia white at margins, and light green (30A4) elsewhere; texture velvety, floccose at center; sporulation sparse; conidia greenish grey (28D3); exudates pale yellow (1A3), scattered overall; soluble pigments yellow; reverse color grayish yellow (3B5). MEA, 25℃, 7 d: Colonies low to moderately deep, radially sulcate; margins white, entire; mycelia white; texture velvety, floccose at center; sporulation moderate; conidia greyish green (26C3); exudates abundant, pale yellow (1A3); soluble pigments absent; reverse color brownish yellow (5C7). YES, 25℃, 7 d: Colonies low to deep, sulcate; margins low, narrow, entire; mycelia white; texture velvety, floccose at center; sporulation sparse; conidia greyish green (28B4); exudates absent; soluble pigments absent; reverse color reddish yellow (4A7), light orange (5A5) at center.

Conidiophores monoverticillate, occasionally biverticillate, stipes smooth walls; phialides ampulliform, 6.0-8.0×2.0-3.0 μm; conidia smooth walls, globose to subglobose, 2.3-3.2 μm diam; sclerotia absent; asci and ascospores not observed.

Strain examined: SFC20170821-M07

Note: *Penicillium citreosulfuratum* is morphologically similar to *P. cinerascens*, *P. citreonigrum*, and *P. fundyense*. *P. citreosulfuratum* can be distinguished from them by growth on CYA at 37℃ [8,9].

DISCUSSION

We confirmed that eight species in sect. *Exilicaulis* exist in Korea. Four species of them were previously reported in Korea: *P. corylophilum, P. menonorum, P. rubefaciens*, and *P. velutinum*. *Penicillium citreonigrum* and *P. citreosulfuratum* have been recorded for the first time in Korea. Although other two species were clearly separated from the previously described species based on phylogenies of *BenA*, *CaM*, and *RPB*2, they were designated as *Penicillium* sp. due to the minor morphological differences. Additional examination for a more detailed morphological comparison with phylogenetically similar species will be required to identify these species.

Sect. *Exilicaulis* is divided into six clades based on phylogenies of ITS, *BenA*, *CaM*, and *RPB*2. *Penicillium citreonigrum* clade consists of four species: *P. cinerascens*, *P. citreonigrum*, *P. citreosulfuratum*, and *P. fundyense*. Although *P. citreosulfuratum* can be distinguished from the rest by its ability to grow at 37℃ [8,9], these species are difficult to identify based on morphological characteristics due to only a few significant or consistent morphological differences [9]. Phylogeny based on ITS or *BenA* sequences have been proposed for species identification in *P. citreonigrum* clade [9]. The morphological and phylogenetic characteristics of two unrecorded species were consistent with those of the respective type species. *Penicillium citreonigrum* and *P. citreosulfuratum* were isolated from plant and soil in terrestrial environments [9] and produced citreoviridin [28] correlated with yellow rice disease [29,30]. These two species are reported for the first time in Korea as well as from marine environment.

Nine species in section *Exilicaulis* have been previously reported in Korea [31]. Five species of them were not found in this study: *P. decumbens*, *P. erubescens*, *P. melinii, P. restrictum* and *P. rubidurum*. Two species (*P. erubescens* and *P. melinii*) were recently reported as unrecorded species in Korea based on morphological characteristics and *CaM* sequence [32]. *Penicillium rubidurum* KNU14-12 was reported from soil based on the ITS sequence (accession no. KP055596) [14]. However, *P. rubidurum* KNU14-12 formed a phylogenetically distinct group with the type strain (CBS 609.73) of *P. rubidurum* and showed the highest similarity of ITS sequence with *P. parvum* CBS 570.73 (sequence similarity for ITS=99.8%). Based on these results, *P. rubidurum* KNU14-12 might be a new species rather than *P. rubidurum*. *Penicillium decumbens and P. restrictum* were identified by morphological characteristics [17]. There are no stored strains of *P. decumbens* and *P. restrictum*, so the identity cannot be verified.

In conclusion, we found eight species including two new species candidates and two unrecorded species in sect. *Exilicaulis* in this study. Three of nine species previously reported in Korea were confirmed by sequence analysis. As a result, there are 11 *Penicillium* species in section *Exilicaulis* in Korea.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2019R1I1A1A01061954) and the Marine Biotechnology Program of the Korea Institute of Marine Science and Technology Promotion (KIMST) funded by the Ministry of Oceans and Fisheries (MOF) (No. 20170431).

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