

RESEARCH ARTICLE

Three Unrecorded *Penicillium* Species from Marine Environments in South Korea

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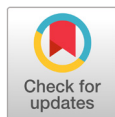
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ABSTRACT

Penicillium is the most common fungal genus in various terrestrial and marine environments. The number of new and unrecorded *Penicillium* species from various substrates and habitats are continuously increasing. As part of a project to discover indigenous fungi in South Korea, three unrecorded *Penicillium* species were isolated from mudflats and seaweeds. Based on morphological characteristics and phylogenetic analyses of β -tubulin and calmodulin loci, they were identified as *P. amaliae*, *P. infrabuccalum*, and *P. manginii*. Here, we provide a detailed morphological description and sequence information of these previously unrecorded species.

Keywords: Marine, Phylogenetic analysis, Series *Sclerotiorum*, Series *Simplicissima*, Series *Westlingiorum*



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INTRODUCTION

Penicillium is one of the most common fungal genera isolated from terrestrial environments, such as soil, indoors, plants, and food products. They play important ecological roles as decomposers, pathogens, and producers of a variety of molecules, such as bioactive compounds, enzymes, and mycotoxins [1,2]. Recently, *Penicillium* research has been expanded to various habitats, including marine (mudflats, sea sand, sea water, and algae) [3,4] and extreme environments (Antarctica, hot springs, saline soils, and deep seas) [5]. Many new species have been reported in ecological and biodiversity studies of specific substrates and habitats [6-8] and have increased significantly over the past decade [9].

Penicillium species are readily identified at the genus level according to their conidiophores and conidia, whereas they are difficult to identify at the species level because of their morphological plasticity under growth conditions and similar characteristics among closely related species [10]. Recently, the taxonomy of *Penicillium* has changed dramatically based on phylogenetic analysis. The description method of the species has been standardized, including morphology and multigene phylogenetic analysis using the nuclear rDNA internal transcribed spacer (ITS), β -tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*rpb2*). In special, ITS and *BenA* have been recommended as molecular markers for

Penicillium identification [10]. To date, *Penicillium* has been subdivided in two subgenera, 32 sections, 89 series, and 483 species worldwide [9]. Although the number of new and unrecorded species of *Penicillium* is continuously increasing [11-14], only about 24% of the described species have been found in South Korea [15]. Moreover, we have recently reported many potential new species and recorded species from mudflats and sea sand in the intertidal zones in South Korea using isolation and next generation sequencing (NGS) [4]. Therefore, it is necessary to expand our research toward new substrates and habitats using various methods, including NGS, in order to discover new species and unrecorded species.

As part of the projects organized by the Marine Fungal Resource Bank (MFRB) and the National Research Foundation of Korea (NRF), we discovered three unrecorded species from different series during the investigation of *Penicillium* diversity in marine and terrestrial environments. The main objective of this study was to describe these three unrecorded *Penicillium* species based on morphological characteristics and phylogenetic analysis of the *BenA* and *CaM* loci. They were identified as *P. amaliae*, *P. infrabuccalum*, and *P. manginii*.

MATERIALS AND METHODS

Sample collections and isolation

Using previously described methods [16], three strains were isolated from mudflats and one from seaweeds. Briefly, 5 g of mudflats was serially diluted (1/10 and 1/100) with sterilized seawater, and 100 µL of each dilution was plated on dichloran rose bengal chloramphenicol (DRBC, Difco-Becton Dickinson, MD, USA) agar. For fungal isolation from seaweed, samples were washed with seawater to remove surface debris, cut into discs of approximately 5-mm in diameter, and placed on DRBC agar. All plates were incubated at 25°C for 7 days. Isolated fungal strains were transferred to potato dextrose agar (PDA; Difco-Becton Dickinson, MD, USA) with seawater. Each strain was stored in 20% glycerol at -80°C at the Seoul National University Fungus Collection (SFC) (Table 1).

Table 1. Summary and GenBank accession numbers for *Penicillium* strains isolated from marine environments.

Species	Section	Strain No.	Site	Habitat	Accession No.	
					<i>BenA</i>	<i>CaM</i>
<i>P. amaliae</i>	<i>Sclerotiorum</i>	SFCP11106	Jangheung-ri, Gilsang-myeon, Ganghwa-gun, Incheon	Mudflat	MZ890585	MZ890589
<i>P. infrabuccalum</i>	<i>Lanata-Divaricata</i>	SFCP13625	Deokchon-ri, Samsan-myeon, Yeosu-si, Jeollanam-do	Seaweed	MZ890586	MZ890590
		SFCP14760	Haksan-ri, Byeollyang-myeon, Suncheon-si, Jeollanam-do	Mudflat	MZ890587	MZ890591
<i>P. manginii</i>	<i>Citrina</i>	SFCP14845	Pyeong-san-ri, Hyeongyeong-myeon, Muan-gun, Jeollanam-do	Mudflat	MZ890588	MZ890592

BenA: β-tubulin; *CaM*: Calmodulin

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon,

Korea) according to the manufacturer's instructions. *BenA* and *CaM* were amplified using previously described methods [17]. The polymerase chain reaction (PCR) products were purified using an Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. DNA sequencing was performed bidirectionally at Macrogen (Seoul, South Korea) on an ABI Prism 3730XL analyzer (Applied Biosystems, Foster City, CA, USA) using the PCR primers.

Phylogenetic analysis

The sequences were manually assembled, proofread using MEGA5 [18], and deposited in GenBank (accession Nos. are listed in Table 1). Molecular identification was performed in two steps. First, the section of the strains were determined by comparing the *BenA* sequence of each isolated strain and those from several type strains. Next, each strain was identified to the species level by analyzing the combined dataset of the two loci (*BenA* and *CaM*) with type species from each section. *P. roqueforti* CBS 221.30, *P. glabrum* CBS 125543, and *P. corylophilum* CBS 312.48 were used as the outgroups for series *Sclerotiorum*, *Simplicissima*, and *Westlingiorum*, respectively [9]. Multiple alignments were performed with MAFFT v.7 [19] using the default settings. Maximum likelihood analyses were conducted using RAxML [20] implemented on the CIPRES web portal [21], using the general time reversible (GTR)+G model with 1000 bootstrap replicates.

Morphological analysis

The morphology of the three unrecorded species was observed using previously described methods on five different culture media: creatine sucrose agar (CREA), Czapek yeast autolysate agar (CYA; Difco, Sparks, MD, USA), dichloran 18% glycerol agar (DG18), malt extract agar (MEA; Oxoid, Hampshire, UK), and yeast extract sucrose agar (YES; Difco, Sparks, MD, USA) [22]. The Methuen handbook of color was used for determining the color names and alphanumeric codes for the description of each culture [23]. Microscopic features were observed under a Nikon 80i compound light microscope (Nikon, Tokyo, Japan) using colonies grown on MEA.

RESULTS

Isolated strains were identified as *P. amaliae* in the series *Sclerotiorum* (1 strain), *P. infrabuccalum* in *Simplicissima* (2), and *P. manginii* in *Westlingiorum* (1) based on the combined dataset from *BenA* and *CaM* (Fig. 1).

SFCP11106, classified in series *Sclerotiorum* sect. *Sclerotiorum*, formed a monophyletic group with *P. amaliae* CV1875 (type strain) (sequence similarity for *BenA*=99.1% and *CaM*=100%; bootstrap support=98%). Whereas SFCP13625 and SFCP14760, classified in series *Simplicissima* sect. *Lanata-Divariata*, formed a monophyletic group with *P. infrabuccalum* KAS 2181 (type strain) (sequence similarity for *BenA*=99.8-100% and *CaM*=99.6-100%; bootstrap support=99%), and SFCP14845,

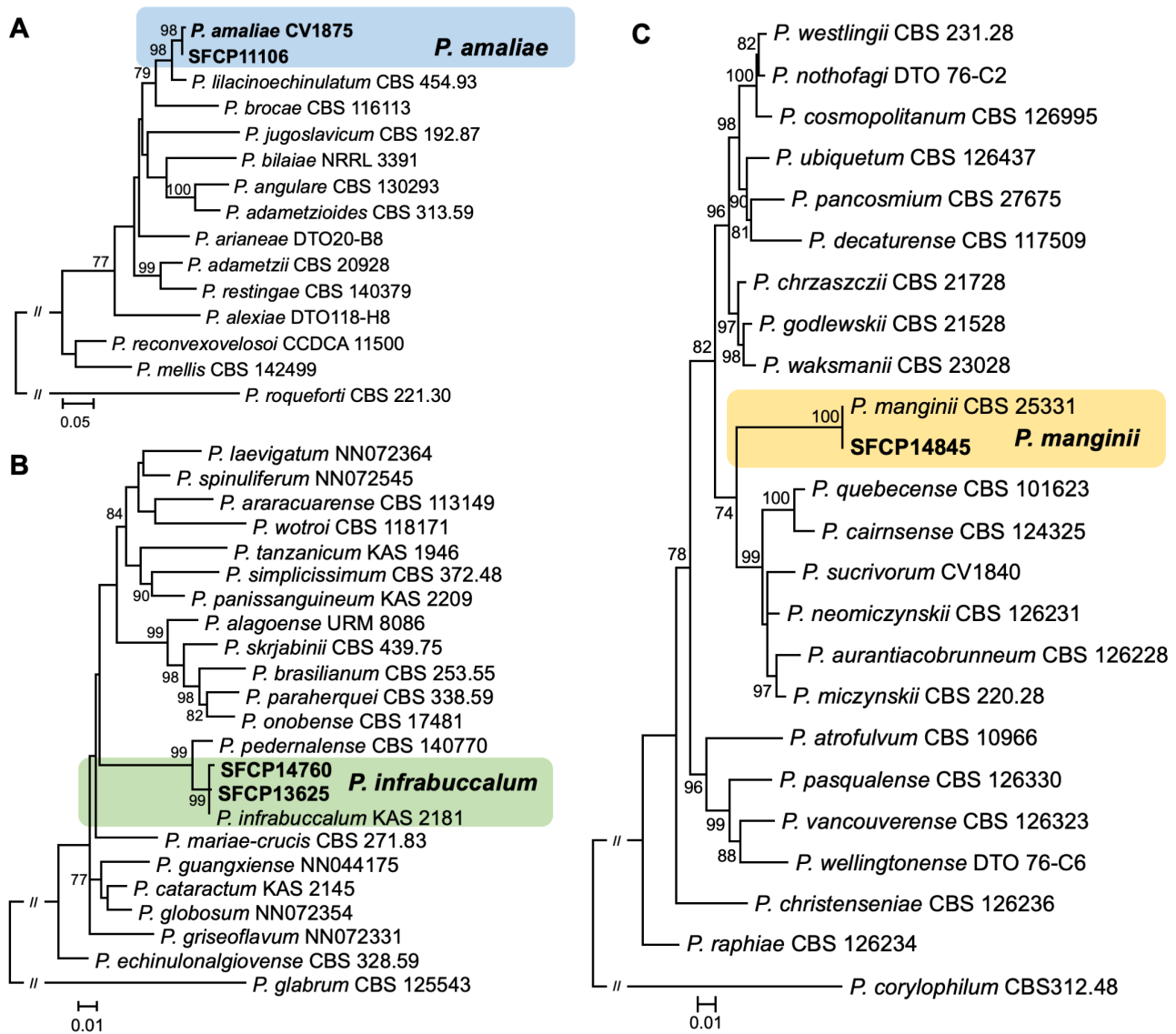


Fig. 1. Maximum likelihood (ML) phylogenetic trees of series (A) *Sclerotiorum*, (B) *Simplicissima*, and (C) *Westlingiorum* within *Penicillium* based on the combined data set of *BenA* and *CaM*. Bootstrap scores >70 are presented at the nodes. The scale bar represents the number of nucleotide substitutions per site. The unrecorded *Penicillium* species are accented in color boxes.

classified in series *Westlingiorum* sect. *Citrina*, formed a monophyletic group with *P. manginii* CBS 253.31 (type strain) (sequence similarity for *BenA*=100% and *CaM*=100%; bootstrap support=100%).

Taxonomy

Penicillium amaliae Houbraken & K. Jacobs (2013) (Fig. 2)

Description: Colony diam, 7 d, in mm: CYA 25°C 27-30; CYA 15°C 9-10; CYA 30°C 23-25; CYA 37°C no growth; MEA 25°C 25-29; YES 25°C 28-30; DG18 25°C 19-20; CREA 25°C 14-16 (Fig. 2).

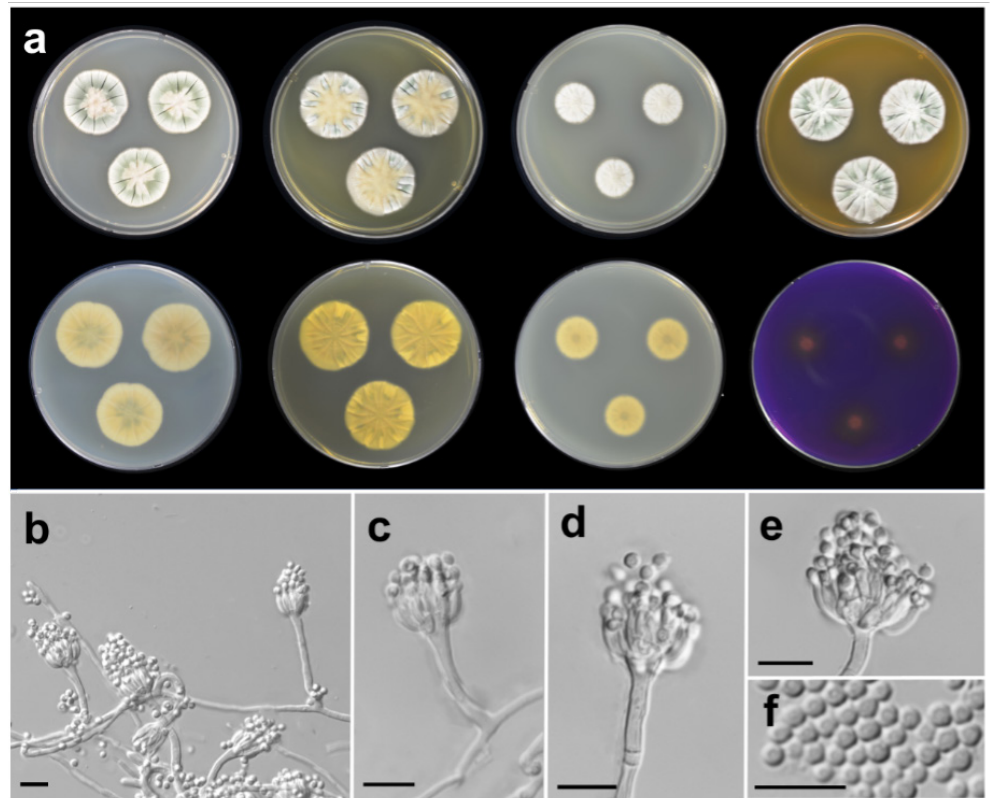


Fig. 2. Morphological characteristics of *Penicillium amaliae* SFCP11106. (a) Colonies after 7 days at 25°C, from left to right (top row) Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), dichloran 18% glycerol agar (DG18), malt extract agar (MEA); (bottom row) CYA reverse, YES reverse, DG18 reverse, creatine sucrose agar (CREA) reverse; (b-e) Conidiophores; (f) Conidia (scale bar: b-f=10 μ m).

Colony characteristics: CYA, 25°C, 7 d: Colonies moderately deep, radially sulcate; margins low, narrow, entire; mycelia white; texture velvety; sporulation moderately dense; conidia dull green (27E3) to grayish green (27B3); exudates clear to almost yellowish brown; soluble pigments absent; reverse color light orange (5A5) to pale yellow (2A3). MEA, 25°C, 7 d: Colonies low, radially sulcate; margins low, narrow, entire; mycelia white; texture velvety; sporulation moderate to dense; conidia grayish green (26B3); exudates absent; soluble pigments absent; reverse color deep orange (6A8). YES, 25°C, 7 d: Colonies moderately deep, radially sulcate; margins low, narrow, entire; mycelia white; texture velvety; sporulation sparse to moderate; conidia grayish green (26B4); exudates absent; soluble pigments absent; reverse color light grayish yellow (3B5). DG18, 25°C, 7 d: Colonies low, radially sulcate; margins low, wide, entire; mycelia white; texture floccose; sporulation absent to sparse; conidia grayish turquoise (24B4); exudates absent; soluble pigments absent; reverse color grayish yellow (3B3). CREA, 25°C, 7 d: Growth poor; acid production weak.

Conidiophores monoverticillate; stipes smooth walled, 16-59 \times 2-3 μ m, phialides ampulliform, 9-19 per stipe, 6.3-16.5 \times 1.8-2.9 μ m. Conidia rough walled, globose to subglobose, 2.3-3.0 \times 1.9-2.8 μ m. Sclerotia absent; asci and ascospores not observed.

Strains examined: SFCP11106

Note: *P. amaliae* is phylogenetically similar to *P. lilacinoechinulatum*. Both species can be distinguished by their different growth rates on CYA at 30°C and acid production on CREA: *P. amaliae* grows slower than *P. lilacinoechinulatum* on CYA at 30°C after 7 d (23-25 vs. 30-33 mm) [24]. *P. amaliae* produced a weak acid on CREA, whereas *P. lilacinoechinulatum* produced strong acid on CREA. Interestingly, the strain in this study showed no growth on CYA at 37°C, whereas the type strain had a low growth rate of 3-11 mm.

***Penicillium infrabuccalum* Visagie, David Clark & Seifert (2016) (Fig. 3)**

Description: Colony diam, 7 d, in mm: CYA 25°C 53-63; CYA 15°C 18-21; CYA 30°C 52-56; CYA 37°C no growth; MEA 25°C 33-39; YES 25°C 48-51; DG18 25°C 21-23; CREA 25°C 38-40 (Fig. 3).

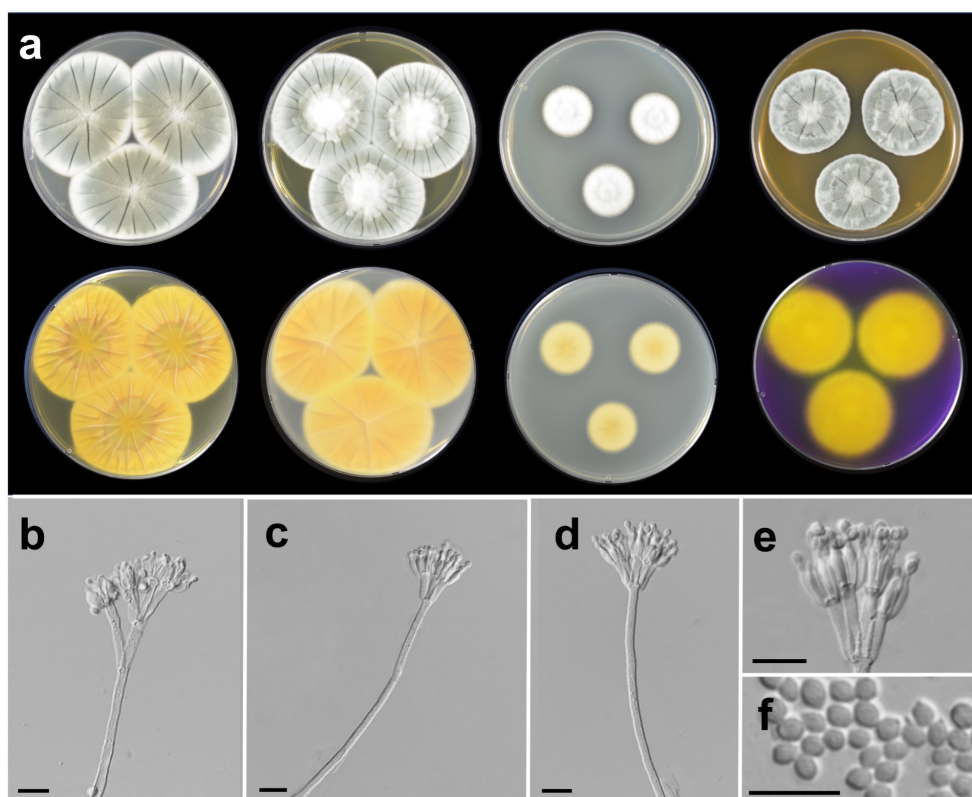


Fig. 3. Morphological characteristics of *Penicillium infrabuccalum* SFCP13625. (a) Colonies after 7 days at 25°C, from left to right (top row) Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), dichloran 18% glycerol agar (DG18), malt extract agar (MEA); (bottom row) CYA reverse, YES reverse, DG18 reverse, creatine sucrose agar (CREA) reverse; (b-e) Conidiophores; (f) Conidia (scale bar: b-f=10 µm).

Colony characteristics: CYA, 25°C, 7 d: Colonies moderately deep, radially sulcate; margins low, wide, entire; mycelia white; texture floccose; sporulation moderately dense; conidia grayish green (25B3); exudates; soluble pigments absent; reverse color light orange (5A5), light yellow (4A4) at margin. MEA, 25°C, 7 d: Colonies low to moderately deep, radially sulcate; margins low, narrow, entire; mycelia white;

texture floccose; sporulation moderately dense; conidia dull green (25D4) at center, grayish green (25B3) elsewhere; exudates absent; soluble pigments absent; reverse color orange (5B8). YES, 25°C, 7 d: Colonies moderately deep, radially sulcate; margins low, wide, entire; mycelia white; texture floccose at center, velvety elsewhere; sporulation sparse to moderately dense; conidia grayish green (27E4) to greenish gray (26B2); exudates absent; soluble pigments absent; reverse color brownish orange (5C6) to grayish yellow (4C3) at center, deep yellow (4A8) elsewhere. DG18, 25°C, 7 d: Colonies low, radially sulcate; margins low, narrow, entire; mycelia white; texture floccose; sporulation sparse; conidia greenish white (25A2); exudates absent; soluble pigments absent; reverse color light yellow (4A5) at center, pale yellow (4A3) elsewhere. CREA, 25°C, 7 d: Growth moderate; acid production absent.

Conidiophores biverticillate, terverticillate; stipes rough walled, $100\text{--}410 \times 2\text{--}3\text{ }\mu\text{m}$; branches $24\text{--}37\text{ }\mu\text{m}$; metulae divergent, 2-5 per stipe, $9.7\text{--}13 \times 2.5\text{--}3.5\text{ }\mu\text{m}$; phialides ampulliform, 4-10 per metula, $6.1\text{--}11.2 \times 1.6\text{--}3.2\text{ }\mu\text{m}$. Conidia finely rough walled, subglobose to ellipsoidal, $2.5\text{--}3 \times 2\text{--}3\text{ }\mu\text{m}$. Sclerotia absent; asci and ascospores not observed.

Strains examined: SFCP13625 and SFCP14760.

Note: *P. infrabuccalum* is phylogenetically similar to *P. pedemalense*. *P. infrabuccalum* is characterized by no acid production on CREA, whereas *P. pedemalense* produces strong acid on CREA [25]. Korean *P. infrabuccalum* tends to grow faster than the type strain KAS 2181 of *P. infrabuccalum* on CYA and MEA at 25°C.

***Penicillium manginii* Duché & Heim (2011) (Fig. 4)**

Description: Colony diam, 7 d, in mm: CYA 25°C 29-31; CYA 15°C 14-16; CYA 30°C no growth; CYA 37°C no growth; MEA 25°C 28-30; YES 25°C 35-38; DG18 25°C 18-19; CREA 25°C 12-15 (Fig. 4).

Colony characteristics: CYA, 25°C, 7 d: Colonies moderately deep, radially sulcate; margins low, narrow, entire; mycelia light yellow (2A4); texture velvety; sporulation moderately dense; conidia grayish green (27B4); exudates absent or yellow; soluble pigments yellow; reverse color deep orange (5A8). MEA, 25°C, 7 d: Colonies low, radially sulcate; margins low, narrow, entire; mycelia white; texture velvety; sporulation poor to moderately dense; conidia grayish green (3B4); exudates clear; soluble pigments absent; reverse color deep orange (5A8). YES, 25°C, 7 d: Colonies deep, randomly furrowed; margins low, narrow, entire; mycelia white; texture velvety; sporulation moderately dense; conidia grayish green (26B4); exudates absent; soluble pigments reddish brown (9D8); reverse color reddish brown (8D8). DG18, 25°C, 7 d: Colonies low; margins low, narrow, entire; mycelia white; texture velvety; sporulation moderately dense; conidia grayish green (26B3); exudates absent; soluble pigments brownish red (10D8); reverse color violet brown (10F8). CREA, 25°C, 7 d: Growth poor; acid production weak; reverse color red (11A6).

Conidiophores biverticillate; stipes smooth to finely rough walled, $150\text{--}450 \times 2\text{--}4\text{ }\mu\text{m}$; metulae 2-5 per branch, $8.0\text{--}11.9 \times 2.7\text{--}3.8\text{ }\mu\text{m}$; phialides ampulliform, 6-8 per metula, $5.0\text{--}8.3 \times 1.4\text{--}2.8\text{ }\mu\text{m}$. Conidia smooth walled, subglobose to ellipsoidal, $2.3\text{--}3.6 \times 1.2\text{--}2.4\text{ }\mu\text{m}$. Asci and ascospores not observed.

Strains examined: SFCP14845.

Note: *P. manginii* is morphologically similar to *P. vancouverense*. Both species can be distinguished by

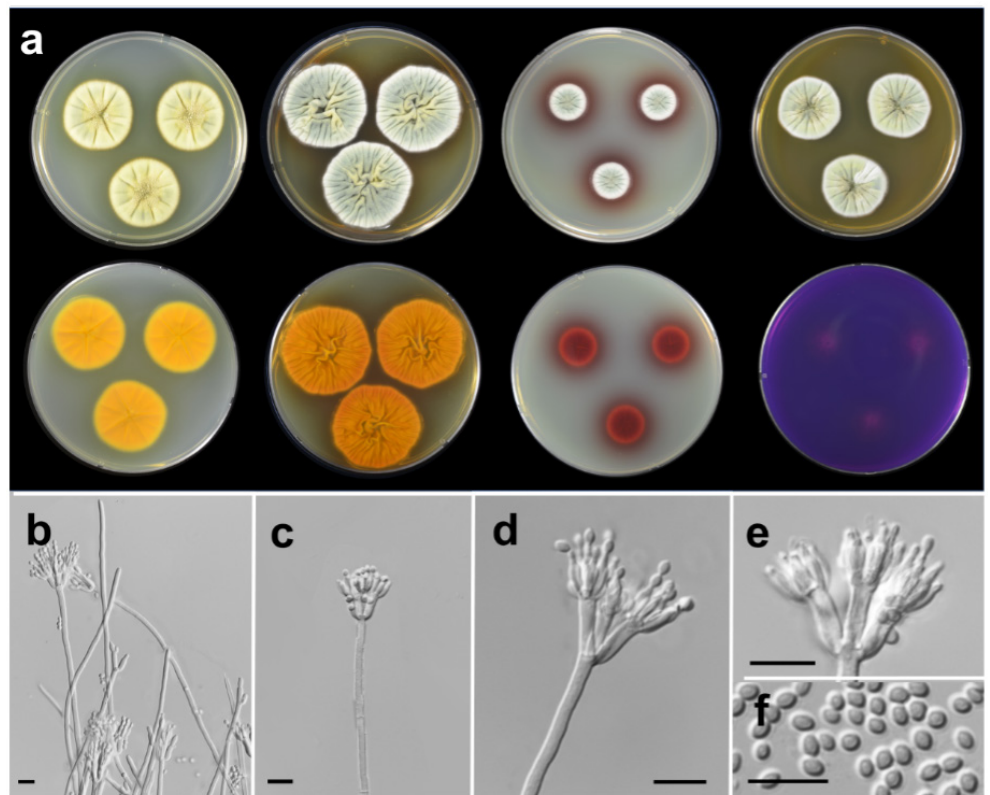


Fig. 4. Morphological characteristics of *Penicillium manginii* SFCP14845. (a) Colonies after 7 days at 25°C, from left to right (top row) Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), dichloran 18% glycerol agar (DG18), malt extract agar (MEA); (bottom row) CYA reverse, YES reverse, DG18 reverse, creatine sucrose agar (CREA) reverse; (b-e) Conidiophores; (f) Conidia (scale bar: b-f=10 µm).

their growth rate and soluble pigment [26]. *P. manginii* grows faster than *P. vancouverense* on MEA and YES at 25°C. *P. manginii* produces a reddish brown color on YES, whereas *P. vancouverense* does not produce any soluble pigment. Korean *P. manginii* tends to grow slower than the type strain CBS 253.31 on CYA at 25°C.

DISCUSSION

Penicillium species are one of the most common fungi genera found in various substrates of marine environments, such as sea sand, seaweeds, and sailfin sandfish egg masses [3,27,28]. We identified three *Penicillium* species new to South Korea, that were isolated from marine environments; according to phylogenetic analyses based on the *BenA* and *CaM* loci, these species are *P. amaliae*, *P. infrabuccalum*, and *P. manginii*. Each of these *Penicillium* species was included in different sections and series: *P. amaliae* belonged to series *Sclerotiorum*, sect. *Sclerotiorum*; *P. infrabuccalum* to series *Simplicissima*, sect. *Lanata-Divaricata*; and *P. manginii* to series *Westlingiorum*, sect. *Citrina*.

The series *Sclerotiorum* included in sect. *Sclerotiorum* consists of 17 species and is characterized by

monoverticillate conidiophores, moderately fast colony growth, dull green to gray-green conidia, and globose to ellipsoidal conidia [9]. In South Korea, 11 *Penicillium* species have been reported, including *P. acidum*, *P. austrosinicum*, *P. cainii*, *P. daejeonium*, *P. exsudans*, *P. jacksonii*, *P. johnkrugii*, *P. mallochii*, *P. maximae*, *P. sclerotiorum*, and *P. ulleungdoense* [14]. They have been isolated from various substrates in marine and terrestrial environments [29-33]. *P. amaliae* was previously reported in the infructescence of *Protea repens* and insect larva [24]. Although SFCP11106 from mudflats showed a difference in mycelial growth at 37°C on CYA compared with that of the type strain of *P. amaliae*, there were no differences in other morphological characteristics [24]. SFCP11106 formed a monophyletic group with the type strain of *P. amaliae* and was clearly different from other species in the series *Sclerotiorum*, based on *BenA* and *CaM* sequence analysis. Therefore, the strain could be confidently identified as *P. amaliae*. The series *Simplicissima* included in sect. *Lanata-Divaricata* consists of 20 species. Although the species in the series *Simplicissima* are morphologically characterized by biverticillate or divaricate conidiophores, colony growth at 37°C, gray-green or dull green conidia, and conidia of various sizes and shapes, classification of the series is difficult to establish based on morphological features [9]. In South Korea, six species have been reported, including *P. brasilianum*, *P. echinulonalgiense*, *P. globosum*, *P. onobense*, *P. paraherquei*, and *P. simplicissimum* [15,34]. They were isolated from terrestrial environments, such as fresh water, indoors, and soil [4,29,34-36]. SFCP13625 and SFCP14760 were isolated from mudflats and seaweeds in marine environments and showed slight differences in sporulation and growth rate with previously reported *P. infrabuccalum* [25]. However, based on *BenA* and *CaM* sequence analysis and other morphological features, the strains were identified as *P. infrabuccalum*.

The series *Westlingiorum* included in sect. *Citrina* comprises 22 species and is morphologically characterized by biverticillate conidiophores, no growth at 37°C, and blue green to grayish green conidia [9]. A total of seven species have been reported in South Korea, including *P. caimsense*, *P. decaturense*, *P. miczynskii*, *P. pasqualense*, *P. raphiae*, *P. waksmanii*, and *P. westlingii* [12,15]. With the exception of *P. westlingii*, most species have been isolated from soils in marine and terrestrial environments [4,12,16,37,38]. SFCP14845 was isolated from mudflats, and although it grew slower than the type strain of *P. manginii* on CYA at 25°C, most of its morphological features were similar to those of the type strain [26]. SFCP14845 formed a well-supported monophyletic group with *P. manginii* CBS 253.31 (type strain). Therefore, we identified it as *P. manginii*.

In conclusion, we confirmed the presence of three unrecorded *Penicillium* species in South Korea using morphological and molecular approaches. The results of this study contribute to the update of the fungal inventory in this country. Continuous fungal surveys, along with accurate identification based on morphological and molecular characteristics, will increase the possibility of discovering more unrecorded species or even new ones.

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