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New record and enzyme activity of four species in *Penicillium* section *Citrina* from marine environments in Korea

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Several strains of *Penicillium* section *Citrina* were isolated during a survey of fungi from marine environments along the southern coast of Korea. Based on multigene phylogenetic analyses (β-tubulin and calmodulin) and morphological characteristics, the 11 strains were identified as *P. citrinum*, *P. hetheringtonii*, *P. paxilli*, *P. sumatrense*, *P. terrigenum*, and *P. westlingii*. To understand the ecological role of these species, we tested all strains for extracellular enzyme activity; six strains representing four species showed β-glucosidase activity. Four of the identified species – *P. hetheringtonii*, *P. paxilli*, *P. terrigenum*, and *P. westlingii* – are new records for Korea. For these new species records, we describe morphological characteristics of the strains and compare results to published data of type strains.

Keywords: *BenA*, *CaM*, morphology, *Penicillium* section *Citrina*, phylogeny

Introduction

The genus *Penicillium*, is one of the most common fungi found in the environment (Pitt, 1979; Samson *et al.*, 2010). This genus is important because it causes pre- and post-harvest disease on crop (Frisvad and Samson, 2004) and produces a variety of bioactive compounds that are harmful (e.g., mycotoxins) or useful (e.g., antibiotics compounds and enzymes) to humans (Visagie *et al.*, 2014). In particular, marine-derived *Penicillium* species are known to produce secondary metabolites (e.g., anti-cancer and anti-fungal compounds; Edrada *et al.*, 2002) and extracellular enzymes (Dubrovskaya *et al.*, 2012; Park *et al.*, 2014b).

Traditionally, morphological characters of the colonies, conidia, conidiophores, and stipes were used to differentiate *Penicillium* species (Pitt, 1979). However, morphological identification of species can be difficult due to the lack, independent evolution, and plasticity of distinguishing features (Pitt, 1973; LoBuglio *et al.*, 1993; Visagie *et al.*, 2014). The addition of DNA sequence data has alleviated many of the problems associated with morphological analysis. Several genes have been used to study *Penicillium*: nuc rDNA internal transcribed spacer (ITS; Peterson, 2000), nuc rDNA large subunit (LSU; Peterson, 2000), β-tubulin (*BenA*; Seifert and Louis-Seize, 2000; Samson *et al.*, 2004), calmodulin (*CaM*; Peterson *et al.*, 2005), translation elongation factor 1-α DNA (*TEF1-α*; Peterson *et al.*, 2005), RNA polymerase II largest subunit (*RPB1*; Houbraken and Samson, 2011), RNA polymerase II second largest subunit (*RPB2*; Houbraken and Samson, 2011), putative ribosome biogenesis protein (*Tsr1*; Houbraken and Samson, 2011), and putative chaperonin complex component TCP-1(*Cct8*; Houbraken and Samson, 2011). Current recommendations are to follow the polyphasic species concept, combining morphology, molecular phylogenetic analyses, and extrolite profiling for identification (Christensen *et al.*, 1999; Frisvad and Samson, 2004). Based on a polyphasic analysis (including a multigene phylogenetic analysis of ITS, *BenA*, *CaM*, and *RPB2*), there are currently 354 accepted *Penicillium* species, which have been divided into two subgenera (*Aspergilloides* and *Penicillium*) and 25 sections (Visagie *et al.*, 2014).

In Korea, over 100 *Penicillium* species have been recorded, with most originating from terrestrial environments (Lee *et al.*, 2003; Yu, 2006; Kim *et al.*, 2009). As studies using molecular methods and surveying marine environments have uncovered new species and distributional records of *Penicillium* to Korea (Park *et al.*, 2014a, 2014b, 2014c), the true *Penicillium* diversity in Korea is waiting to be discovered.

The *Penicillium* section *Citrina* is of interest because it has a global distribution and is known to produce a variety of extrolites (e.g., citrinin and citreoviridin; Houbraken *et al.*, 2010, 2011). *Penicillium* species of the section *Citrina* are commonly isolated from soil and are characterized by relatively small conidia, ampulliform phialides, and symmetrically biverticillate conidiophores (Houbraken *et al.*, 2011). Currently, there are 39 recognized species in this section (Houbraken *et al.*, 2011), with seven species known from Korea (Lee *et al.*, 2003; Kim *et al.*, 2007, 2013; Paul *et al.*, 2014). For this study, we focus on species of *Penicillium* section *Citrina* from marine environments in Korea.

During recent surveys along the southern coast of Korea, we isolated 11 strains belonging to six species in the section *Citrina*. We identified these species using multigene phylogenetic analyses (*BenA* and *CaM*) and test for extracellular enzyme activity to understand their ecological role. Four of the identified species – *P. hetheringtonii*, *P. paxilli*, *P. terrigenum*, and *P. westlingii* – were previously unrecorded in Korea. For these new records, we describe their macro- and micro-morphological characteristics and compare them to published data of corresponding type strains.

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Materials and Methods

Materials

In 2014, marine substrates (sand, mud, seaweed) were collected from sites along the southern coast of Korea (Table 1). Sterile latex gloves were worn to transfer samples directly to Zip-lock bags. The samples were transported to the laboratory at 4°C and processed immediately for fungal isolation. For sand and mud, two serial dilutions (1/10, 1/100) were made using 5 g of material and artificial sea water (ASW) (Huang *et al.*, 2011); 0.1 ml of each serial dilution was transferred to the surface of three different culture media plates prepared with ASW: potato dextrose agar (PDA; Difco-Becton), glucose yeast extract agar (1 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L peptone, 15 g/L agar), and dichloran rose-bengal chloramphenicol agar (DRBC; Difco-Becton). For seaweed, each sample was gently washed with seawater to remove surface debris and soil. Discs of 5-mm in diameter were cut from each sample and placed on the three different culture media plates. All plates were incubated at 25°C until the morphology of the cultured fungi could be distinguished (7–15 days), then each *Penicillium* strain was transferred to a new PDA plate. The isolated strains are stored in 20% glycerol at -80°C at the Seoul National University Fungus Collection (SFC) (Table 1).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Rogers and Bendich, 1994). *BenA* and *CaM* have appropriate resolution for species identification in the *Penicillium* section *Citrina* (Houbraken and Samson, 2011), and were sequenced for all strains. PCR reactions were performed using primers Bt2a and Bt2b for *BenA* (Glass and Donaldson, 1995) and CF1 and CF4 for *CaM* (Peterson *et al.*, 2005). Each PCR reaction was performed in a C1000 thermal cycler (Bio-Rad) using AccuPower® PCR PreMix (Bioneer) in a final volume of 20 μl, containing 10 pmol of each primer and 10 ng of DNA. The PCR products were electrophoresed through a 1% agarose gel stained with loadingSTAR (Dyne Bio) and purified using the Expin™ PCR SV Kit (GeneAll Biotechnology) according to the manufacturer's instructions. Sequencing was performed in both forward and reverse directions using the corresponding PCR primers at Macrogen, using an ABI Prism 3700 genetic analyzer (Life Technologies).

Phylogenetic analyses

Sequences were assembled, proofread, and aligned using MEGA5 (Tamura *et al.*, 2011). The resulting consensus sequences were deposited in GenBank (accession Nos. in Table 1). Phylogenetic analyses were performed in two steps. First, we identified strains belonging to section *Citrina* by analyzing *BenA* sequences with 52 type strains spanning the diversity of *Penicillium*, using the type strain of *Aspergillus niger* CBS513.88 as the outgroup. Next, strains were identified to species by analyzing the combined dataset (*BenA* + *CaM*) with 37 GenBank sequences (33 type strains) belonging to section *Citrina*, with *P. corylophilum* CBS 330.79 (section *Exilicaulis*) used as the outgroup (Houbraken and Samson, 2011). The sequence similarities were calculated from the combined dataset (*BenA* + *CaM*) for each species using MEGA5 (Tamura *et al.*, 2011).

Multiple sequence alignments were performed using the default settings of MAFFT v7 (Katoh and Standley, 2013), and ambiguously aligned positions adjusted manually. We performed maximum likelihood phylogenetic analyses on datasets with RAxML (Stamatakis, 2006), using the GTR+G model of evolution and 1,000 bootstrap replicates.

Enzyme assay

Enzyme assays were done for all 11 strains belonging to *Penicillium* section *Citrina* identified in this study. We screened for extracellular alginase, endoglucanase, and β-glucosidase activity using the modified plate screening methods of Park *et al.* (2014b).

Morphological analysis

Detailed observation of morphology was done for the species with new distributional records in Korea. To observe macroscopic culture characteristics, strains were inoculated at three points on five different culture media – Czapek yeast autolysate agar (CYA, yeast extract, Difco), CYA supplemented 5% NaCl (CYAS), yeast extract sucrose agar (YES,

Table 1. Collection information and GenBank accession numbers for *Penicillium* strains used in this study

Species	Strain No.	Substrate	Locality	Date	Accession No.	
					BenA	*CaM*
P. citrinum	SFC20140423-M87	Seaweed	Yeosu, Korea	April 2014	KP235300	KP235289
	SFC20140723-M14	Sand	Buan, Korea	July 2014	KP235301	KP235290
P. hetheringtonii	SFC20141120-M06	Seaweed	Jeju, Korea	November 2014	KP235302	KP235291
P. paxilli	SFC20141120-M03	Seaweed	Jeju, Korea	November 2014	KP235303	KP235296
P. sumatrense	SFC20140423-M88	Seaweed	Yeosu, Korea	April 2014	KP235304	KP235292
	SFC20141120-M01	Seaweed	Jeju, Korea	November 2014	KP235305	KP235293
	SFC20141120-M04	Seaweed	Jeju, Korea	November 2014	KP235306	KP235294
	SFC20140723-M15	Mud	Taean, Korea	July 2014	KP235307	KP235295
P. terrigenum	SFC20141120-M02	Seaweed	Jeju, Korea	November 2014	KP235308	KP235297
	SFC20141120-M05	Seaweed	Jeju, Korea	November 2014	KP235309	KP235298
P. westlingii	SFC20140423-M69	Seaweed	Yeosu, Korea	April 2014	KP235310	KP235299

BenA, β-tubulin; *CaM*, calmodulin

yeast extract Difco), malt extract agar (MEA, Oxoid), and creatine sucrose agar (CREA) – and incubated at 25°C for seven days. In addition, CYA plates were inoculated and incubated for 7 days at 15°C, 30°C, and 37°C. All media were prepared as described in Visagie *et al.* (2014). After incubation, the culture characteristics were recorded using the models described by Pitt (1979) and Frisvad and Samson (2004). All the culture color names and codes were based on the 'Methuen Handbook of Colour' (Kornerup and Wanscher, 1963).

To observe microscopic characters, mounts of strains were made in lactic acid from colonies grown on MEA, and conidiophores were washed with a drop of ethanol to remove excess spores. Microscopy was performed using a light microscope (Nikon Eclipse 80i).

Results and Discussion

The phylogenetic analysis based on the *benA* dataset identified 11 strains in the section *Citrina* (bootstrap support= 97%; data not shown). Phylogenetic analysis of the combined dataset (*BenA* + *CaM*) identified these 11 strains as six species; strains were highly similar to and formed strongly supported monophyletic groups with type strains (Fig. 1). Strain SFC20140423-M87 and SFC20140723-M14 were identified as *P. citrinum* (sequence similarity=99.8–100%; bootstrap support=83%), strain SFC20141120-M06 as *P. hether-*

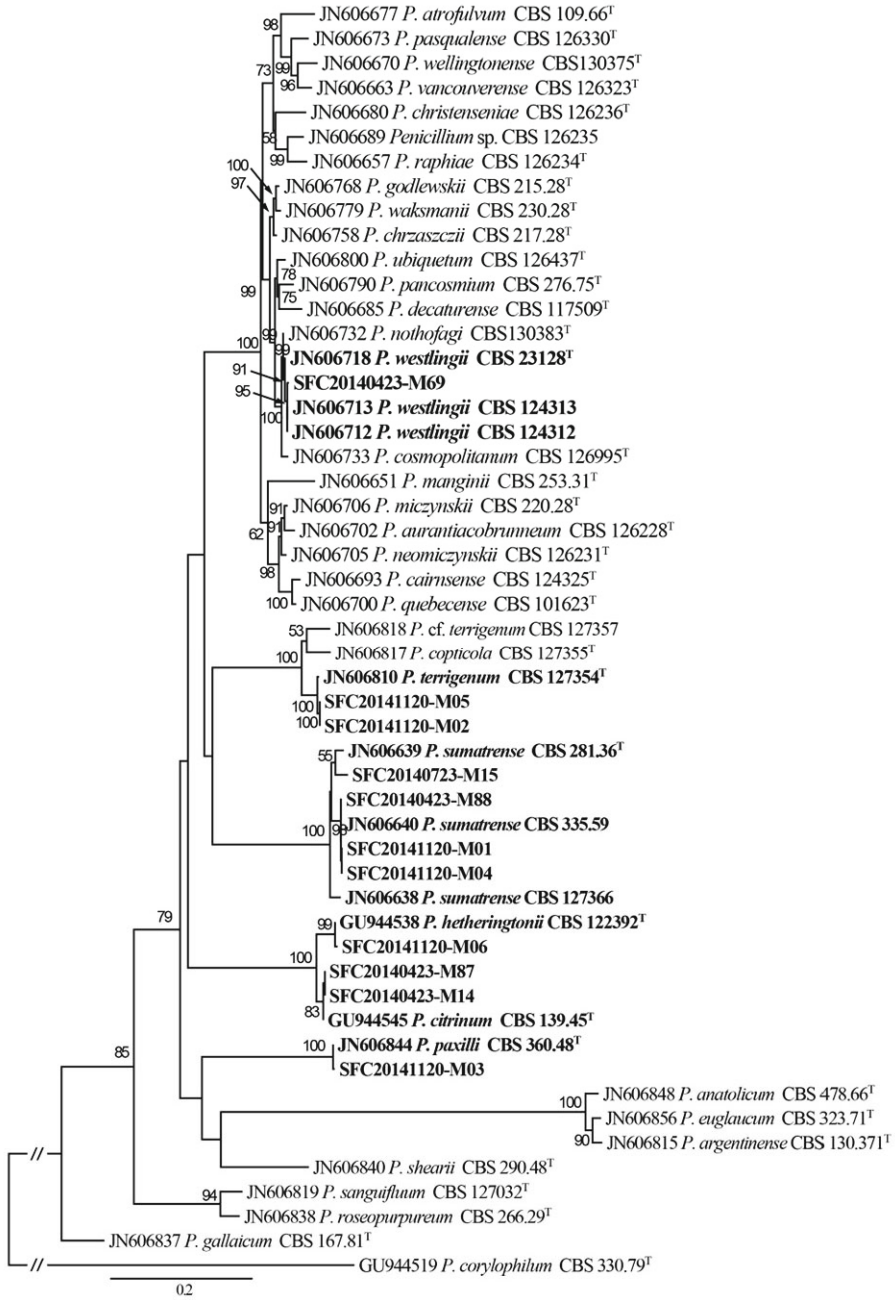

Fig. 1. Maximum likelihood phylogenetic analysis of the combined data set of β-tubulin (*BenA*) and calmodulin (*CaM*) used to identify strains to species in the *Penicillium* section *Citrina*. Bootstrap scores of >50 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site, and "T" indicates the ex-type strains.

ingtonii (sequence similarity=99.7%; bootstrap support=99%), strain SFC20141120-M03 as *P. paxilli* (sequence similarity=99.8%; bootstrap support=100%), strains SFC20140423-M88, SFC20141120-M01, SFC20141120-M04, and SFC20140723-M15 as *P. sumatrense* (sequence similarity=97.8–99.8%; bootstrap support=100%), strains SFC20141120-M02 and SFC20141120-M05 as *P. terrigenum* (sequence similarity=99.4–100%; bootstrap support=100%), and strain SFC20140426-M69 as *P. westlingii* (sequence similarity=99.3–99.9%; bootstrap support=91%).

The section *Citrina* comprises 39 species worldwide (Houbraken *et al.*, 2011). In Korea, seven species in the section *Citrina* have been reported from terrestrial soil: *P. citrinum*, *P. copticola*, *P. miczynskii*, *P. raphiae*, *P. steckii*, *P. sumatrense*, and *P. waksmanii* (Lee *et al.*, 2003; Kim *et al.*, 2007, 2013; Paul *et al.*, 2014). Two species identified in our study were previously reported (*P. citrinum* and *P. sumatrense*), while, to the best of our knowledge, four are new species records to Korea (*P. hetheringtonii*, *P. paxilli*, *P. terrigenum*, and *P. westlingii*). The four new species records found in our study increases the species diversity of *Penicillium* section *Citrina* in Korea to 11.

Macroalgae (i.e. seaweed) are common in marine environments, and their cell walls often contain alginate and/or cellulose for structural strength (Wei *et al.*, 2013). Many organisms play an important ecological role in decomposing macroalgae organic material (Barnes and Mann, 1980), including marine fungi (Hyde *et al.*, 1998). Marine fungi produce several extracellular enzymes, such as alginase to degrade alginate (Schaumann and Weide, 1990), and β-glucosidase and endoglucanase to degrade cellulose (Pointing 1999). We tested for these three extracellular enzymes in the isolated strains. Neither alginase nor endoglucanase activity were identified, although other studies have found *Penicillium* species that produce these extracellular enzymes (Wood *et al.*, 1980; Jorgensen *et al.* 2003; Dutta *et al.*, 2008; Burtseva *et al.*, 2010). However, six strains representing four species (*P. citrinum*, *P. hetheringtonii*, *P. sumatrense*, and *P. terrigenum*) showed β-glucosidase activity (Table 2). β-glucosidase contributes to cellulose degradation by breaking the β 1-4 bond between two glucose molecules (Pei *et al.*, 2012). These results not only demonstrate the ability of these strains to degrade cellulose, but also their potential industrial application to biomass degradation.

Comparing morphological characters of these four species, Korean strains were similar to their corresponding type strains (Houbraken *et al.*, 2011). We describe the morphology and taxonomic information of these four species in detail.

Taxonomy

Penicillium hetheringtonii Houbraken, Frisvad & Samson 2010 (Fig. 2)

Description: Colony diameters, 7 d, 25°C (unless otherwise stated), in mm: CYA 27–31; CYA 15°C 12–13; CYA 30°C 24–26; CYA 37°C 1–3; MEA 25–27; YES 34–35; CREA 20–21. Ratio CYAS:CYA 0.9–1.0.

Colonies on CYA; conidia dull green (27E3); colony texture velvety; sporulation moderate to strong, absent towards the margins; non-sporulating margins 4–5 mm; small hyaline exudates droplets; reverse color pale yellow (3A3). Colonies on MEA; conidia dull green (26D3); colony texture velvety; sporulation strong, absent towards the margins; non-spor-

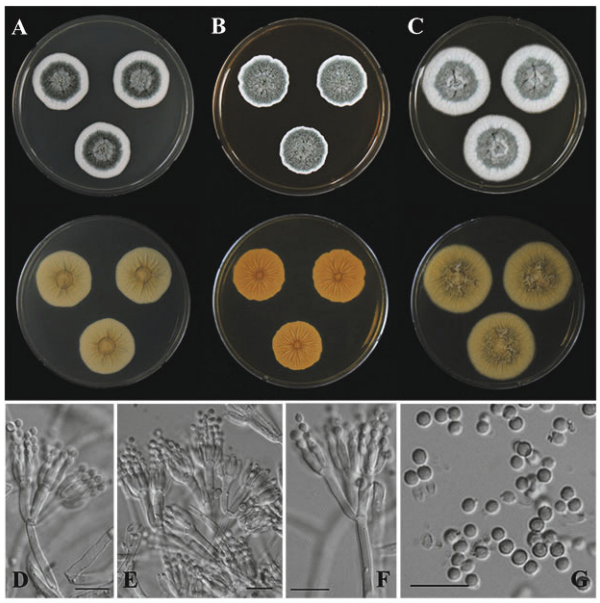

Fig. 2. Morphology of *P. hetheringtonii* SFC20141120-M06 (A–C), 7-day-old cultures, at 25°C. Left to right, first row, all obverse, (A) Czapek yeast autolysate agar (CYA), (B) malt extract agar (MEA), (C) yeast extract sucrose agar (YES); second row, CYA reverse, MEA reverse, YES reverse; (D–F) Conidiophores; (G) Conidia (scale bars: D–H = 10 μm).

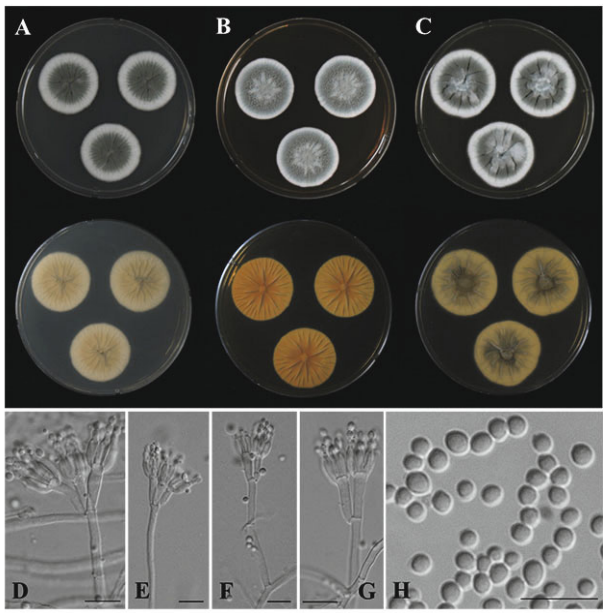

Fig. 3. Morphology of *P. paxilli* SFC20141120-M03 (A–C), 7-day-old cultures, at 25°C. Left to right, first row, all obverse, (A) Czapek yeast autolysate agar (CYA), (B) malt extract agar (MEA), (C) yeast extract sucrose agar (YES); second row, CYA reverse, MEA reverse, YES reverse; (D–G) Conidiophores; (H) Conidia (scale bars: D–H = 10 μm).

ulating edges 1–2 mm; small hyaline exudates droplets; reverse color brownish orange (5C4). Colonies on YES strongly wrinkled; conidia greenish white (25A2) and dull green (25E3); colony texture floccose; sporulation moderate; non-sporulating margins 5–7 mm; exudates absent; reverse color greyish yellow (4B3). Growth on CREA poor; no acid production.

Sclerotia absent. Asci and ascospores not observed. Conidiophores predominantly biverticillate, smooth or finely roughened walls, 2.3–3.3 μm wide, 3–6 metulae in a compact whorl, 10–14 × 2.4–3.8 μm; phialides ampulliform, 9–12 × 2.4–3.2 μm (Fig. 2D-F). Conidia globose to subglobose, 2.3–3.0 × 2.3–2.6 μm, with smooth or finely roughened walls (Fig. 2G).

Extracellular enzymatic activity: β-glucosidase (clear zone = 5.0 mm).

Penicillium paxilli Banier 1907 (Fig. 3)

Description: Colony diameters, 7 d, 25°C (unless otherwise stated), in mm: CYA 31–33; CYA 15°C 14–16; CYA 30°C 24–25; CYA 37°C no growth; MEA 31–32; YES 33–38; CREA 20–22. Ratio CYAS:CYA 1.0–1.03.

Colonies on CYA; conidia dull green (27E3); colony texture velvety; sporulation strong, absent towards the margins; non-sporulating margins 3–4 mm; exudate absent; reverse color greyish yellow (4B3). Colonies on MEA; conidia dull green (27E3); colony texture velvety; sporulation strong, absent towards the margins; non-sporulating edges 2–3 mm; exudates absent; reverse color greyish orange (5B4). Colonies on YES slightly wrinkled; conidia dull green (27E3); colony texture velvety; sporulation strong; non-sporulating margins 3–4 mm; exudates absent; reverse color pale yellow (3A3) with yellowish grey (2B2). Growth on CREA poor; no acid production.

Sclerotia absent. Asci and ascospores not observed. Conidiophores predominantly biverticillate, smooth or finely roughened walls, 2.3–3.3 μm wide, 3–6 metulae in a compact whorl, 10–14 × 2.4–3.8 μm ; phialides ampulliform, 9–12 × 2.4–3.2 μm (Fig. 3D–G). Conidia globose to subglobose, 2.3–3.0 × 2.3–2.6 μm, with smooth or finely roughened walls (Fig. 3H).

Penicillium terrigenum Seifert, Houbraken, Frisvad & Samson 2011 (Fig. 4)

Description: Colony diameters, 7 d, 25°C (unless otherwise stated), in mm: CYA 28–30; CYA 15°C 15–17; CYA 30°C 18–26; CYA 37°C no growth; MEA 26–29; YES 31–36; CREA 19–22. Ratio CYAS:CYA 1.0–1.2.

Colonies on CYA; conidia dull green (27E3); colony texture velvety and floccose in center; sporulation strong, absent towards the margins; non-sporulating margins 1–2 mm; exudate absent; reverse color orange grey (5B2). Colonies on MEA; conidia dull green (27E3); colony texture velvety; sporulation strong, absent towards the margins; non-sporulating edges 1–2 mm; exudates absent; reverse color brownish orange (5C4). Colonies on YES strongly wrinkled; conidia greenish grey (26D2); colony texture velvety; sporulation strong; non-sporulating margins 1–2 mm; exudates absent; reverse color greyish yellow (4C3). Growth on CREA poor; no acid production.

Sclerotia absent. Asci and ascospores not observed. Conidiophores predominantly biverticillate, smooth or finely roughened walls, 2.3–3.2 μm wide, 3–6 metulae in a compact whorl, 12–16 × 2.4–3.6 μm ; phialides ampulliform, 7.5–10.5

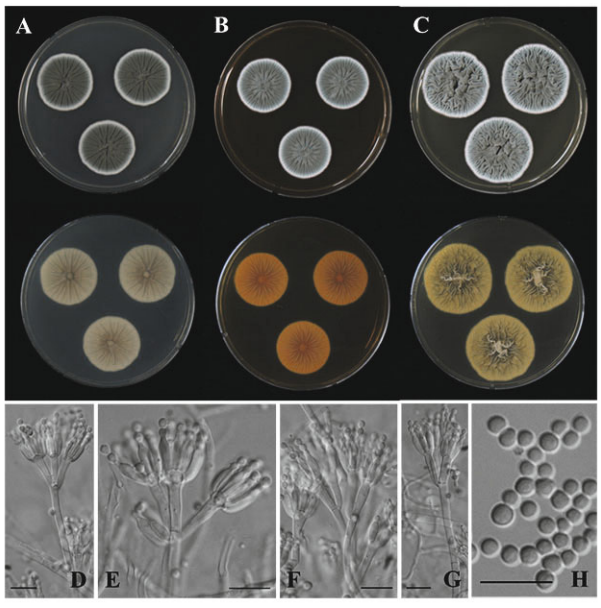

Fig. 4. Morphology of *P. terrigenum* SFC20141120-M02 (A–C), 7-day-old cultures, at 25°C. Left to right, first row, all obverse, (A) Czapek yeast autolysate agar (CYA), (B) malt extract agar (MEA), (C) yeast extract sucrose agar (YES); second row, CYA reverse, MEA reverse, YES reverse; (D–G) Conidiophores; (H) Conidia (scale bars: D–H = 10 μm).

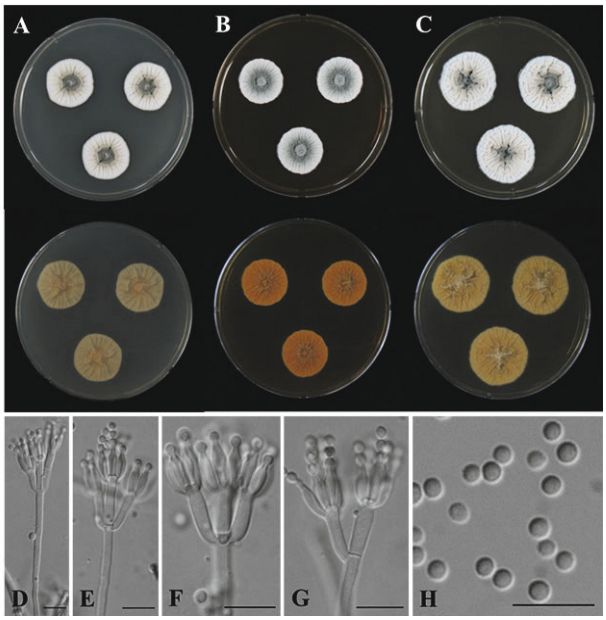

Fig. 5. Morphology of *P. westlingii* SFC20141120-M06 (A–C), 7-day-old cultures, at 25°C. Left to right, first row, all obverse, (A) Czapek yeast autolysate agar (CYA), (B) malt extract agar (MEA), (C) yeast extract sucrose agar (YES); second row, CYA reverse, MEA reverse, YES reverse; (D-G) Conidiophores; (H) Conidia (scale bars: D–H = 10 μm).

× 2.5–3.3 µm (Fig. 4D–G). Conidia globose to broadly ellipsoidal, 2.4–3.2 × 2.2–3.7 µm, with smooth or finely roughened walls (Fig. 4H).

Extracellular enzymatic activity: β-glucosidase (clear zone = 3.0–5.0 mm).

Penicillium westlingii Zaleski 1927 (Fig. 5)

Description: Colony diameters, 7 d, 25°C (unless otherwise stated), in mm: CYA 24–26; CYA 15°C 17–18; CYA 30°C 7–8; CYA 37°C No growth; MEA 22–24; YES 29–32; CREA 15–17. Ratio CYAS:CYA 0.9–1.0.

Colonies on CYA; sparse sporulation in center; conidia greenish grey (27E2); exudate absent; reverse color greyish yellow (4B3) with greyish orange (5B3) in center; margin polygonal. Colonies on MEA; conidia dull green (27E3); colony texture velvety, floccose in the center; sporulation weak; exudates absent; reverse color greyish orange (5B5). Colonies on YES; sparse sporulation in center; exudates absent; reverse color orange white (5A2). Growth on CREA poor; no acid production.

Sclerotia absent. Asci and ascospores not observed. Conidiophores predominantly biverticillate, smooth, 2.3–3.2 µm wide, 3–5 metulae in a compact whorl, 9–14(–16) × 2.0–3.5 µm; phialides ampulliform, 6.3–9.0 × 2.4–3.0 µm (Fig. 5D–G). Conidia globose, 2.2–2.7 µm, with finely roughened walls (Fig. 5H).

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