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Research Article

A New Record of *Penicillium antarcticum* from Marine Environments in Korea

Myung Soo Park[1], Eun Ji Lee[1], Jonathan J. Fong[1], Jae Hak Sohn[2] and Young Woon Lim[1,*]

[1]*School of Biological Sciences, Seoul National University, Seoul 151-747, Korea*
[2]*Department of Biofood Materials, Silla University, Busan 617-736, Korea*

Abstract During a survey of marine fungi from the waters surrounding Jeju Island, Korea, several *Penicillium* strains were isolated from seawater and marine sponges. Based on morphological characteristics and phylogenetic analyses of the internal transcribed spacer and RNA polymerase subunit II, four strains were identified as *Penicillium antarcticum*, a fungus that, to the best of our knowledge, had not been previously reported in Korea. Here, we provide detailed descriptions of the morphological characteristics and extracellular enzyme activities of the four strains.

Keywords Extracellular enzyme activity, Internal transcribed spacer, *Penicillium antarcticum*, RNA polymerase subunit II

Penicillium species are among the most common fungi isolated from various outdoor and indoor environments [1-5]. This genus is of interest, as it produces a variety of compounds that are harmful or useful to humans, including mycotoxins, antibiotics, herbicides, antioxidants, insecticides, and anticancer compounds [4]. In particular, marine-derived *Penicillium* species are potential sources of unique bioactive compounds that are produced because of the physiochemical conditions of marine environments, such as extreme pressure, salinity, and temperature [6-8].

In Korea, over 90 *Penicillium* species have been recorded, with most isolated from terrestrial environments [9-11]. Marine-derived *Penicillium* species in Korea are poorly studied, and therefore, their diversity is not well known. Recently, several bioresource banks were established by the Ministry of Oceans and Fisheries of Korea to promote the exploration of marine biodiversity and biological resources. The present study forms part of the Ministry's long-term project to collect marine fungi in Korea. During the re-identification of marine *Penicillium* isolates by using molecular methods, we found a species that was not previously recorded in Korea—*Penicillium antarcticum* (four strains)—which belongs to the subgenus *Aspergilloides*, section *Canescentia*. In this study, we identified this species by using the nucleotide sequences of the internal transcribed spacer (ITS) and partial RNA polymerase subunit II (*rpb2*), described the macro- and micro-morphological characteristics in detail, and tested the strains for extracellular enzyme activity.

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***Corresponding author**
E-mail: ywlim@snu.ac.kr

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MATERIALS AND METHODS

Materials. Seawater and marine sponges were collected from Jeju Island, Korea in 2011. Marine sponges were processed for culture by adding two volumes of sterile seawater, followed by thorough homogenization using a blender. Before culturing, all the samples were diluted ten-fold with sterile seawater. For fungal cultures, 100 µL of each dilution was plated on potato dextrose agar (PDA; 4 g/L potato infusion, 20 g/L dextrose, 18 g/L agar, and 750 mL/L seawater), yeast extract peptone glucose agar (5 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 18 g/L agar, and 750 mL/L seawater), and glucose yeast extract agar (0.1 g/L yeast extract, 5 g/L glucose, 18 g/L agar, and 750 mL/L seawater). The plates were incubated at 25°C for 7~15 days, until the morphology of the cultured fungus could be distinguished, and then each *Penicillium* strain was picked and transferred to a new PDA plate. The strains isolated in this study were stored in 20% glycerol at −80°C at two separate institutions: the Seoul National University Fungus Collection (SFC) and Korea Institute of Ocean Science and Technology (Table 1).

Table 1. Strain information and GenBank accession numbers for *Penicillium antarcticum*

Strain No.	Substrate	Locality	Date	Accession No.	
				ITS	*rpb2*
SFC20140101-M745	Sea water	Jeju, Korea	Feb 2011	KJ636505	KJ636508
SFC20140101-M746	Sponge	Jeju, Korea	Feb 2011	KJ527436	KJ527366
SFC20140101-M749	Sponge	Jeju, Korea	Feb 2011	KJ636506	KJ636509
SFC20140101-M838	Sponge	Jeju, Korea	Feb 2011	KJ636507	KJ636510

ITS, internal transcribed spacer; *rpb2*, RNA polymerase subunit II.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted using the modified cetyltrimethylammonium bromide extraction protocol described by Rogers and Bendich [12]. Two genomic regions for each of the four isolates, ITS and regions 5~7 of *rpb2*, were amplified and sequenced. PCR reactions were performed using ITS1F and ITS4 [13] and RPB2-5F_Eur and RPB2-7CR_Eur [14] primer sets, according to previously described methods [15]. PCR amplification was performed in a C1000 thermal cycler (Bio-Rad, Richmond, CA, USA) using Maxime PCR PreMix with StarTaq (Intron Biotechnology Inc., Seoul, Korea). Each reaction had a final volume of 20 μL and contained 10 pmol of each primer and 10 ng of DNA. The PCR products were electrophoresed on a 1% agarose gel stained with loading STAR (Dyne Bio, Seoul, Korea), and purified using the Expin PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea), according to the manufacturer's instructions. Sequencing was performed in both forward and reverse directions by using the corresponding PCR primers and an ABI Prism 3700 genetic analyzer (Life Technologies, Gaithersburg, MD, USA) at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea).

Sequence analysis. Sequences were assembled, proofread, edited, and aligned using the MEGA5 software [16]. The resulting consensus sequences were deposited in GenBank (accession Nos. in Table 1). Multiple sequence alignments were performed using the default settings of MAFFT v7 [17], and were checked and optimized by eye, with ambiguously aligned positions adjusted manually. Maximum likelihood phylogenetic analyses were performed separately for each gene, using RAxML v8.0.2 [18] under the GTRGAMMA model of evolution for tree inference and 1,000 bootstrap replicates.

Morphological analysis. To observe macroscopic culture characteristics, the strains were inoculated at three points on Czapek yeast autolysate (CYA) agar, yeast extract sucrose agar, malt extract agar (MEA; Oxoid, Basingstoke, UK), and 25% glycerol nitrate (G25N) agar and incubated at 25°C for 7 days. In addition, CYA plates were inoculated and incubated for 7 days at 4°C, 30°C, and 37°C. After incubation, the culture characteristics were recorded using the models described by Pitt [1] and Frisvad *et al.* [4]. All the culture color names and codes were based on the 'Methuen Handbook of Colour' [19]. To observe microscopic characteristics, isolate mounts were prepared in lactic acid from colonies grown on MEA, and conidiophores were washed with a drop of ethanol to remove the excess spores. Microscopy was performed using a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan).

Enzyme activity assays. Extracellular alginase, endoglucanase, and β-glucosidase activities were assessed for each strain by using plate screening methods; enzyme activity was assessed based on the formation of clear zones surrounding the colonies [20]. Alginase activity was assayed by growing the fungi on modified peptone yeast extract salt agar supplemented with 1% alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA) as the primary carbon source [21]. After incubation for 5 days, the plates were flooded with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) for 10 min. Endoglucanase activity was assayed by growing the fungi on cellulolysis basal medium agar supplemented with 2% carboxymethylcellulose (Sigma-Aldrich) as the primary carbon source [22]. After incubation for 5 days, the plates were flooded with 0.5% Congo red (Sigma-Aldrich) for 1 min that was then replaced by 1 M NaCl. β-Glucosidase activity was assayed by growing the fungi for 5 days on cellulolysis basal medium agar supplemented with 0.5% D-cellobiose (Sigma-Aldrich) as the primary carbon source [23]. Next, the plates were flooded with 0.5% Congo red or 10 min that was then replaced by 1 M NaCl.

RUSULTS AND DISCUSSION

Phylogenetic analysis. ITS and *rpb2* alignments were 494 and 913 bp long, respectively. Molecular analyses were performed using BLAST to compare ITS and *rpb2* sequences to those of the type strains available on GenBank. First, ITS sequence analysis was performed on broad taxonomic sampling across *Penicillium* species to determine the overall placement of the strains in the section *Aspergilloides*. *rpb2* sequences have been shown to be excellent markers for species identification in *Penicillium* section *Aspergilloides* [14]; we sequenced *rpb2* for all the strains and compared these data against those of the type strains in Genbank. The four strains had identical ITS sequences that showed 99.3%, 100%, and 99.8% sequence similarity to *Penicillium canescens* NRRL 910, *Penicillium coralligerum* CBS 123.65,

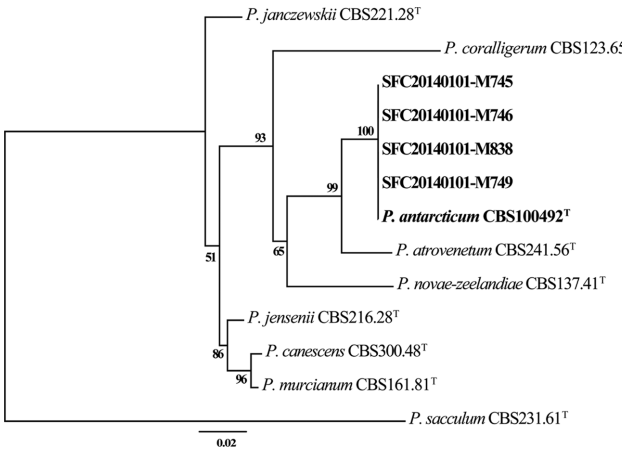

Fig. 1. Phylogenetic tree for *Penicillium antarcticum* and related species based on maximum likelihood analysis of RNA polymerase subunit II (*rpb2*). Bootstrap scores of > 50 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site, and the letter T indicates the ex-type strains.

and *Penicillium novae-zeelandiae* NRRL 2128, respectively (ITS tree is not shown). The four strains also had identical *rpb2* sequences. All the strains formed a monophyletic group with *P. antarcticum* CBS 100492 (100% bootstrap value), with 100% sequence similarity. This clade was clearly distinct from the species identified as being highly similar based on ITS analyses (*P. canescens* NRRL 910, *P. coralligerum* CBS 123.65, and *P. novae-zeelandiae* NRRL2128) (Fig. 1).

Morphological characterization. Morphologies are shown in Fig. 2. In comparison to the original description for *P. antarcticum* [2], all the morphological characteristics are similar, excluding the growth rate on CYA and G25N media, where our isolates exhibited slightly faster rates. We believe that these slight differences are due to morphological variation between the strains.

***Penicillium antarcticum* A. D. Hocking & C. F. McRae 1999 (Fig. 2).**

Colonies grown on CYA for 7 days at 25°C (Fig. 2A): 33~45 mm in diameter, velvety, strong sporulation at the center, but light at the margins. Dull green (25E3) at the center, 1~2 mm white mycelia at the margins, exudates absent (except SFC20140101-M749, producing small droplets of clear exudate), soluble pigment not produced, reverse grayish yellow (4B4).

Colonies grown on CYA for 7 days at 4°C: No growth.

Colonies grown on CYA for 7 days at 30°C: 19~25 mm in diameter.

Colonies grown on CYA for 7 days at 37°C: No growth.

Colonies grown on MEA for 7 days at 25°C (Fig. 2B): 34~37 mm in diameter, velvety, and weak sporulation. Dull green with 1-mm white mycelia at the margins, exudates absent, soluble pigment not produced, margin entire, reverse light brown.

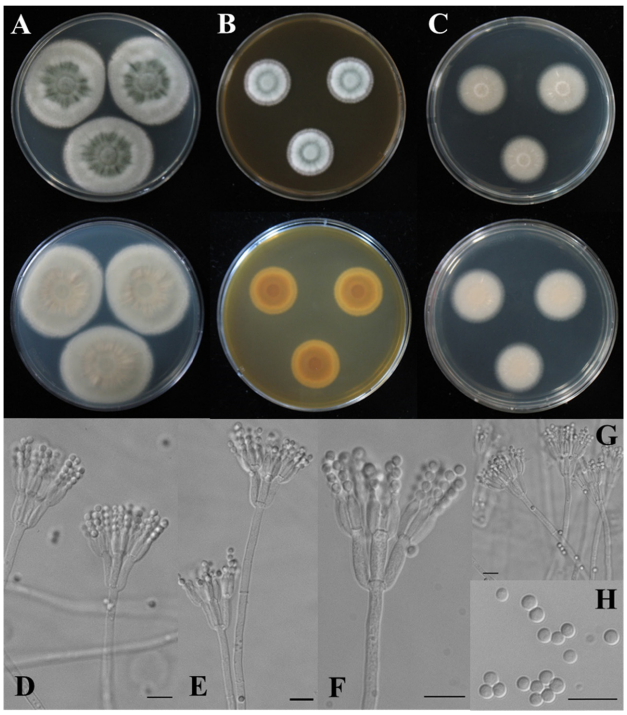

Fig. 2. Morphologies of *Penicillium antarcticum* SFC20140101-M745 (A~C), 7-day-old cultures, at 25°C. Left to right, first row, Czapek yeast autolysate (CYA) agar (A), malt extract agar (MEA) (B), 25% glycerol nitrate (G25N) agar (C); second row, all obverse, CYA reverse, MEA reverse, G25N reverse; Conidiophores (D~G); Conidia (H) (scale bars: D~H = 10 μm).

Colonies grown on G25N for 7 days at 25°C (Fig. 2C): 20~25 mm in diameter, velvety and weak sporulation. White with 1-mm white mycelia at the margins, exudates absent, soluble pigment not produced, margin entire, reverse pale yellow. Conidiophores (Fig. 2D~2G) were mostly biverticillate on MEA, arising from aerial hyphae or agar surface. Stipes were simple, smooth walled, 105~230 (~350) × 3.2~3.8 μm. Phialides were ampulliform, 8.2~10.8 × 2.3~3.3 μm. Conidia (Fig. 2H) were subglobose to globose, smooth-walled or finely roughened, 2.2~3.1 μm in diameter.

Enzyme activity. All the strains were screened for alginase, endoglucanase, and β-glucosidase extracellular enzyme activities. Only β-glucosidase activity was observed in the strains, generating clear zones approximately 6~11.5 mm in diameter (Table 2, Fig. 3). Marine-derived *Penicillium* species are known as important producers of extracellular enzymes [24, 25]. β-Glucosidase and endoglucanase are important enzymes for cellulose degradation [22]. Yoon *et al.* [23] reported that the majority of the tested species from terrestrial environments in Korea show strong β-glucosidase activity. Although several *Penicillium* species have been known to produce alginase activity [24] and endoglucanase activity [26-28], these activities were not observed in this species.

Table 2. Extracellular enzyme activity of *Penicillium antarcticum* on media supplemented with alginic acid sodium salt (alginase), carboxymethycellulose (endoglucanase), and D-cellobiose (β-glucosidase)

Strain	Clear zone (mm)		
	Alginase	Endoglucanase	β-Glucosidase
SFC20140101-M746	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 1.0
SFC20140101-M749	0.0 ± 0.0	0.0 ± 0.0	8.0 ± 0.5
SFC20140101-M838	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 0.7

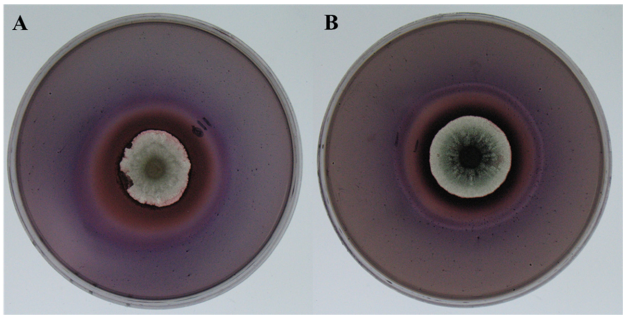

Fig. 3. β-Glucosidase activity in *Penicillium antarcticum* strain SFC20140101-M745 (A) and strain SFC20140101-M749 (B).

Penicillium antarcticum has been isolated from soil, nest scrapings, Antarctic moss [2], *Cedrus deodara* stems [29], and food [30]. Four *Penicillium* strains were isolated from seawater and sponge substrates and identified as *P. antarcticum* based on two nucleotide sequences, ITS and *rpb2*. To the best of our knowledge, this is the first record of *P. antarcticum* isolated from a marine environment in Korea. All the strains had no alginase and endoglucanase activities, but exhibited strong β-glucosidase activity.

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