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Penicillium jejuense sp. nov., isolated from the marine environments of Jeju Island, Korea

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Abstract: Three strains of an unidentified *Penicillium* species were isolated during a fungal diversity survey of marine environments in Korea. These strains are described here as a new species following a multigene phylogenetic analyses of nuc rDNA internal transcribed spacer barcodes (ITS1–5.8S–ITS2), genes for β-tubulin, calmodulin and RNA polymerase II second largest subunit, and observation of macro- and micromorphological characters. Phylogenetic analyses revealed that the three strains formed a strongly supported monophyletic group distinct from previously reported species of section *Aspergilloides*. Morphologically this species can be distinguished from its sister species, *P. crocicola*, by the reverse color on Czapek yeast autolysate agar, abundant production of sclerotia on malt extract agar and colony characters on yeast extract sucrose agar. We name this new species *P. jejuense*, after the locality where it was discovered. At 25 C for 7 d, *P. jejuense* colonies grew to 55–60 mm on CYA, 45–48 mm on MEA, 48–52 mm on YES and 23–26 mm on CREA. Conidia (2.2–3.4 × 2.0–2.6 μm) and sclerotia (160–340 × 125–210 μm) were globose to ellipsoidal.

Key words: new species *Penicillium*, phylogenetic analyses, section *Aspergilloides*

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Introduction

Marine fungi have an estimated diversity of 1500 species and are frequently isolated from wood, algae, corals, sponges and sand (Kohlmeyer and Kohlmeyer 1979, Hyde 1996, Hyde et al. 1998). They act as pathogens, decomposers of organic material and symbionts of other marine organisms (Hyde et al. 1998). Species belonging to the genera *Penicillium*, *Aspergillus*, *Trichoderma*, *Chaetomium* and *Cladosporium* are commonly found in marine environments as facultative marine fungi that originate from a terrestrial environment and adapt to marine conditions (Steele 1967, Khudyakova et al. 2000, Cantrell et al. 2006).

Penicillium species commonly are isolated from various outdoor and indoor environments (Pitt 1979, McRae et al. 1999, Gunde-Cimerman et al. 2003, Frisvad and Samson 2004, Houbraken et al. 2010, Samson et al. 2010), including marine substrates such as sponges, algae and sand (Raghukumar 2008, Li and Wang 2009, Paz et al. 2010). *Penicillium* is especially important because numerous bioactive compounds, such as mycotoxins and anticancer agents, have been isolated (Frisvad et al. 2004). There are currently more than 200 recognized species, divided among 25 sections based on a multigene phylogenetic analysis (Houbraken and Samson 2011).

The Marine Microbe Extract Bank of Korea, managed by the Ministry of Oceans and Fisheries, includes many strains of marine *Penicillium* species from Korea. During molecular reevaluation of the collection we detected three strains of an unknown *Penicillium* species belonging to section *Aspergilloides*. Section *Aspergilloides* corresponds to series *Glabra* of Pitt (1979) and ''group two'' of Peterson (2000) and was delineated for species with fast growth on agar, velvety colonies and predominantly monoverticillate conidiophores. The most recent revision of *Penicillium* section *Aspergilloides* identified 51 species (Houbraken et al. 2014).

The aim of this study was to describe the unknown *Penicillium* species collected from Jeju Island, Korea. Because it is phylogenetically distinct from known species of section *Aspergilloides* based on multigene phylogenetic analysis, we describe it as a new species with macro- and micromorphological characters.

Materials and methods

Materials studied.—Three strains of an unknown *Penicillium* species were isolated from Jeju Island, Korea, during two

sampling trips (2009, 2011), from three different substrates (barnacle, sponge, sand). Strains were re-identified with a multigene sequence analysis. The strains are stored in 20% glycerol at −80 C in the Seoul National University Fungus Collection (SFC), Seoul, Korea. The ex-type also was deposited in the Korean Collection for Type Culture (KCTC), Daejeon, Korea, and the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, the Netherlands.

DNA extraction, amplification and sequencing.—Genomic DNA was extracted with a modification of the cetyltrimethylammonium bromide (CTAB) extraction protocol of Rogers and Bendich (1994). Four loci from the three strains were amplified and sequenced: nuc rDNA internal transcribed spacer barcodes (ITS; ITS1–5.8S–ITS2), portions of genes for β-tubulin (*BenA*), calmodulin (*CaM*) and RNA polymerase II second largest subunit (*RPB2*). The respective PCR were performed according to described methods using the primers ITS1F and ITS4 (White et al. 1990), Bt2a and Bt2b (Glass and Donaldson 1995), CF1 and CF4 (Peterson et al. 2005) and RPB2-5F_Eur and RPB2-7CR_Eur (Houbraken and Samson 2011), respectively. Each PCR reaction was performed on a C1000™ thermal-cycler (Bio-Rad, Richmond, California) using Maxime PCR PreMix with StarTaq™ (Intron Biotechnology Inc., Seoul, Korea) in a final volume of 20 μL, containing 10 pmol each primer and 10 ng DNA. The PCR products were electrophoresed through a 1% agarose gel stained with loading STAR (Dyne Bio, Seoul, Korea) and purified with the Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Sequencing was performed in both forward and reverse directions with the corresponding PCR primers at Macrogen (Seoul, Korea), using an ABI Prism 3700 genetic analyzer (Life Technologies, Gaithersburg, Maryland).

Phylogenetic analyses.—Sequences were assembled, proofread and edited with MEGA 5 (Tamura et al. 2011). The resulting consensus sequences were deposited in GenBank. Multiple sequence alignments were performed with the default settings of MAFFT 7 (Katoh and Standley 2013) and optimized by eye, with ambiguously aligned positions adjusted manually. Maximum likelihood (ML) phylogenetic analyses were performed on four individual gene datasets (ITS, *BenA*, *CaM*, *RPB2*) and one combined dataset (*BenA* + *CaM* + *RPB2*) with RAxML (Stamatakis 2006), using the GTR + G model of evolution and 1000 bootstrap replicates. Alignments and phylogenetic trees are all available in TreeBASE (study 16285).

Morphological analysis.—To observe macroscopic culture characters, the new species was inoculated at three points on Czapek yeast autolysate agar (CYA, yeast extract, Difco), yeast extract sucrose agar (YES, yeast extract, Difco), malt extract agar (MEA, Oxoid) and creatine sucrose agar (CREA) and incubated 7 d at 25 C. All media were prepared as described in Frisvad and Samson (2004). Additional CYA plates were incubated at 30 C and 37 C. After incubation, the culture characters were recorded using the models of Pitt (1979) and Frisvad et al. (2004). All color names and alphanumeric codes were based on 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 with an Eclipse 80i light microscope (Nikon, Tokyo, Japan).

Halotolerance assays.—Halotolerance of the three strains was tested. Growth in the presence of sea salt was determined by inoculating strains on MEA with 3.3% (w/v) sea salt (Sigma-Aldrich, St Louis, Missouri), while salt tolerance was determined by growing the strains on MEA supplemented with 5, 10, 15, and 20% NaCl. Colony diameter was measured after the cultures were incubated 7 d at 25 C.

RESULTS

Phylogenetic analysis.—Sequencing of ITS, *BenA*, *CaM* and *RPB2* from the three strains was successful (GenBank KF818459–70). Additional taxon sampling for each of the four genes differed. For ITS, we used a broad taxonomic sampling across *Penicillium* to demonstrate the placement of the new species in section *Aspergilloides*. Next, for *BenA*, *CaM* and *RPB2*, we focused sampling on section *Aspergilloides* to determine the sister taxon of the new species. The alignment length and number of taxa sampled for each of the five datasets varied: ITS (500 bp, 67 taxa), *BenA* (431 bp, 18 taxa), *CaM* (627 bp, 19 taxa), *RPB2* (917 bp, 18 taxa), and *BenA* + *CaM* + *RPB2* (1975 bp, 18 taxa).

DNA sequences of the three strains of the new species were identical for all genes. For ITS, the sequences were identical to those of *P. crocicola* (CBS 745.57^T) and *P. spinulosum* (CBS 374.48^T). Phylogenetic analysis of this dataset recovered a strongly supported monophyletic group (ML bootstrap = 99) consisting of these three isolates and *P. aurantioviolaceum*, *P. crocicola, P. glabrum, P. patens, P. spinulosum, P. thomii, P. valentinum* and *P. yezoense* (FIG. 1A) representing section *Aspergilloides*. Resolution within this clade with ITS was poor, so we were unable to determine the interspecies relationships. For *BenA*, *CaM* and *RPB2* datasets, we focused taxon sampling on these closely related species. Phylogenetic analyses of the individual datasets recovered *P. crocicola* as the sister taxon of the new species, with varying levels of support (ML bootstraps *BenA* = 55, *CaM* = 96, *RPB2* = 80; FIG. 1B–D). Although *P. crocicola* and *P. spinulosum* had identical ITS sequences to the new species, *BenA*, *CaM* and *RPB2* analyses showed these species were distinct. To understand the overall phylogenetic signal of the data, we analyzed the combined dataset of *BenA*, *CaM* and *RPB2*. Analysis of the combined dataset recovered a monophyletic group of the three isolates and its relationship to known *Penicillium* species (FIG. 2).

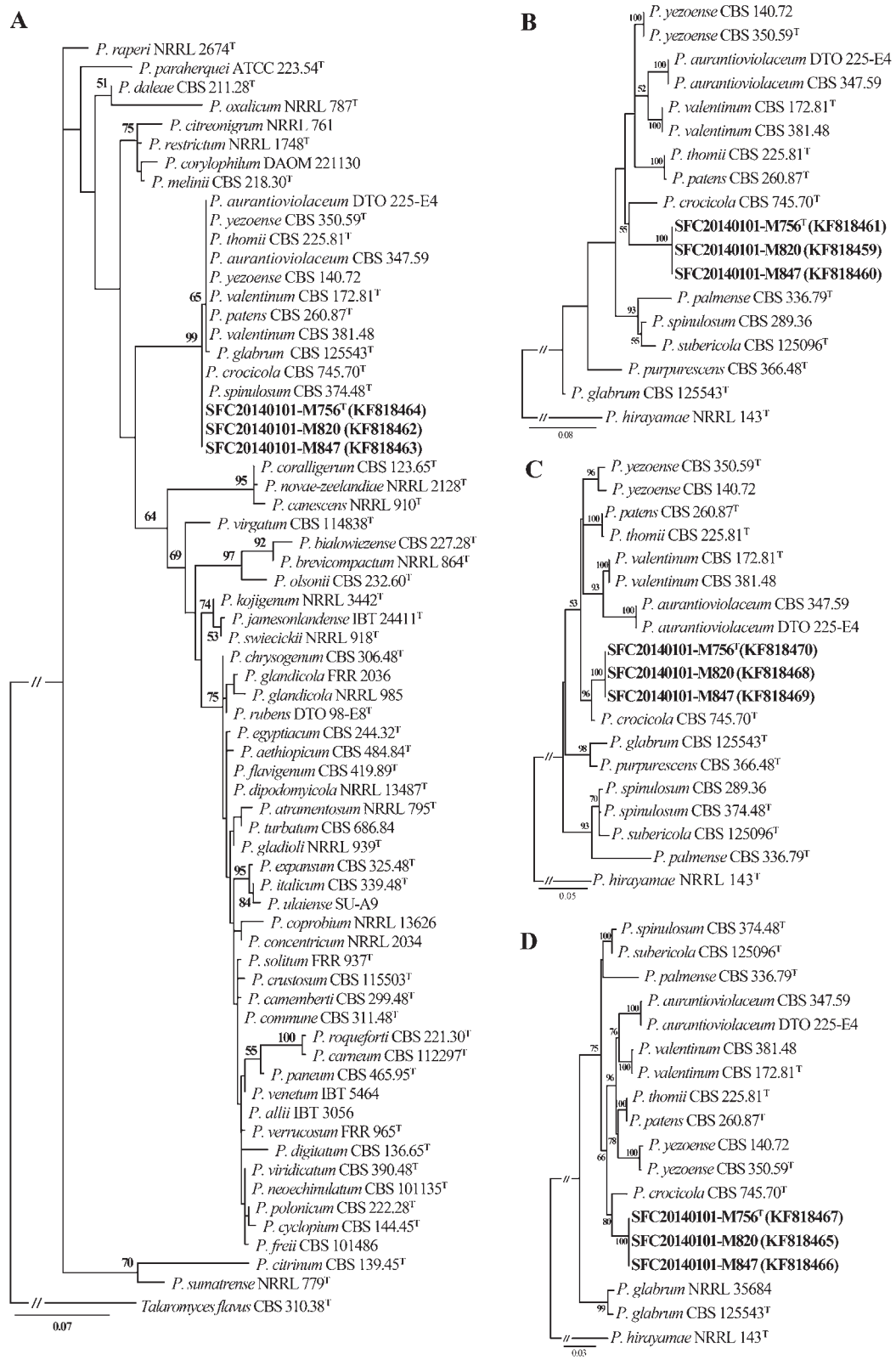

FIG. 1. Phylogenetic tree for *Penicillium jejuense* and related species based on maximum likelihood (ML) analysis of (A) nuc rDNA internal transcribed spacer barcode, (B) partial β-tubulin (*BenA*) gene sequences, (C) partial calmodulin (*CaM*) gene sequences and (D) partial RNA polymerase II second largest subunit (*RPB2*) gene sequences. Bootstrap scores are presented at the nodes only if > 50. The scale bar indicates the number of nucleotide substitutions per site and the letter T indicates ex-type strains.

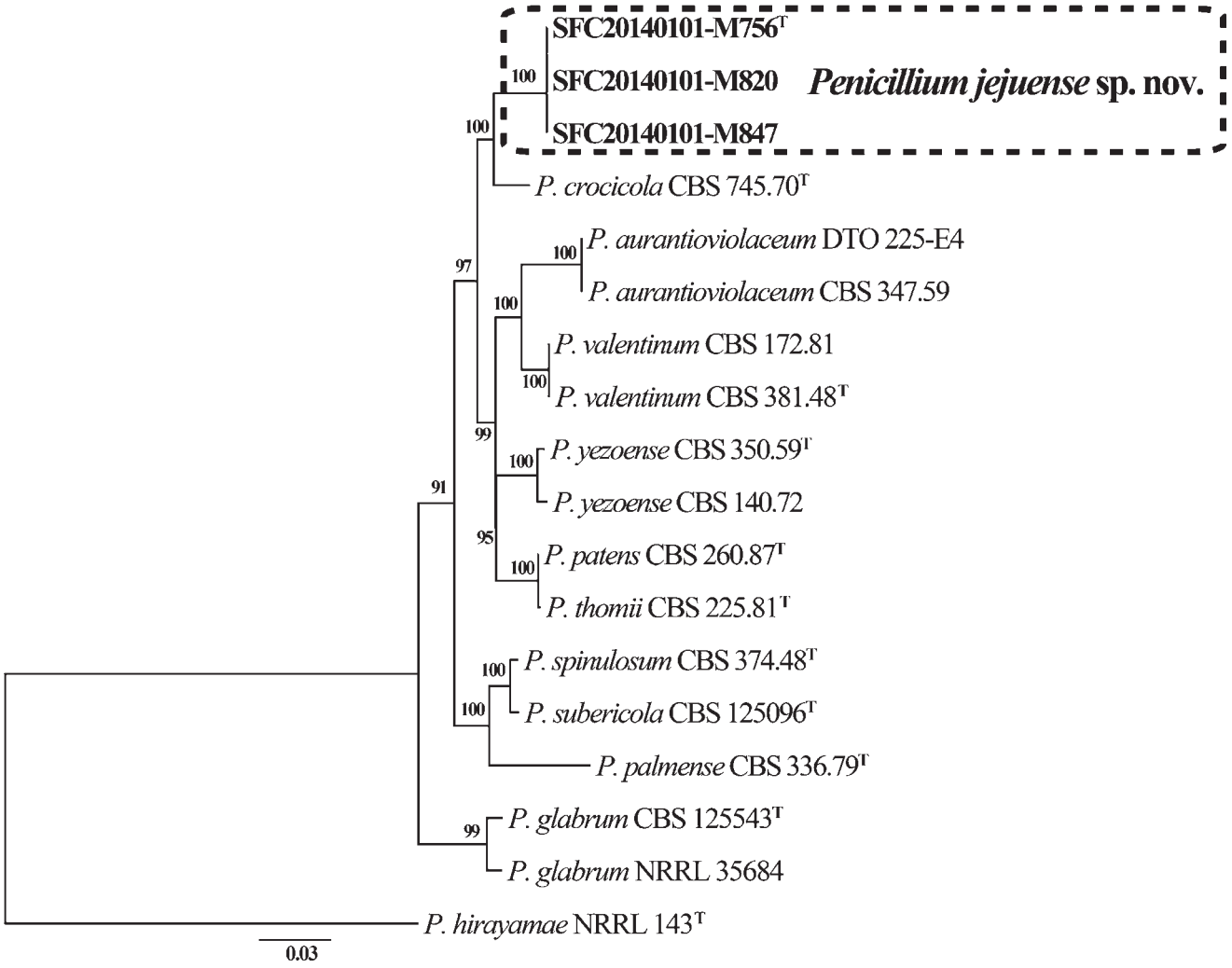

FIG. 2. Phylogenetic tree for *Penicillium jejuense* and related species based on maximum likelihood (ML) analysis of the combined dataset (*BenA* + *CaM* + *RPB2*). Bootstrap scores are presented at the nodes only if > 50. The scale bar indicates the number of nucleotide substitutions per site and the letter T indicates ex-type strains.

Halotolerance.—We analyzed the salt tolerance of the new species because it was isolated from the marine environment. The new species exhibited strong growth in the presence of sea salt and NaCl (TABLE I). The growth rates of the strains on MEA lacking salt, with 3.3% sea salt and with 5% NaCl were similar. Increasing the NaCl concentration to 10% and 15% successively decreased growth rates. The strains did not grow at 20% NaCl concentration (TABLE I).

TAXONOMY

Penicillium jejuense M.S. Park & Y.W. Lim, sp. nov. FIG. 3

MycoBank MB808392

Typification: REPUBLIC OF KOREA. Jeju Island, on *Pollicipes mitella*, Feb 2011, *Jae Hak Sohn* (holotype SFC 20140101-M756T, culture permanently preserved in a metabolically inactive state in 20% glycerol at −80 C). Ex-type cultures KCTC 46212, CBS 138646.

Etymology: Named for Jeju Island where the type strain was collected.

Diagnosis: Phylogenetically closely related to *P. crocicola*; differentiated morphologically by reverse color on CYA, abundant production of sclerotia on MEA and colony characters on YES (TABLE II).

Colony diameters, 7 d, 25 C (unless stated otherwise), in millimeters: CYA 55–60; CYA at 30 C 33–37; CYA at 37 C no growth; MEA 45–48; YES 48–52; CREA 23–26.

Colonies on CYA with 30–35 sulcae; conidia dull green (27E3); colony texture velvety; floccose in center; sporulation strong, absent toward the margins; nonsporulating margins 3–4 mm; exudate produced as a few small, clear droplets; reverse color grayish yellow (4B3). Colonies on MEA with 11–15

TABLE I. Growth rate of *Penicillium jejuense* on media with sea salt and varying NaCl concentrations

Strain[a]	NaCl (% w/v)					Sea salt (3.3%, w/v)
	0	5	10	15	20	
SFC20140101-M820	48.4 ± 0.4	49.7 ± 0.3	30.3 ± 0.3	13.0 ± 0.2	0.0 ± 0.0	53.1 ± 0.4
SFC20140101-M847	48.2 ± 0.3	49.8 ± 0.3	30.7 ± 0.3	12.8 ± 0.3	0.0 ± 0.0	50.9 ± 0.4
SFC20140101-M756	48.3 ± 0.8	47.4 ± 2.2	30.3 ± 0.3	12.6 ± 0.2	0.0 ± 0.0	51.2 ± 1.6

[a] All strains were grown on MEA supplemented with NaCl for 7 d at 25 C.

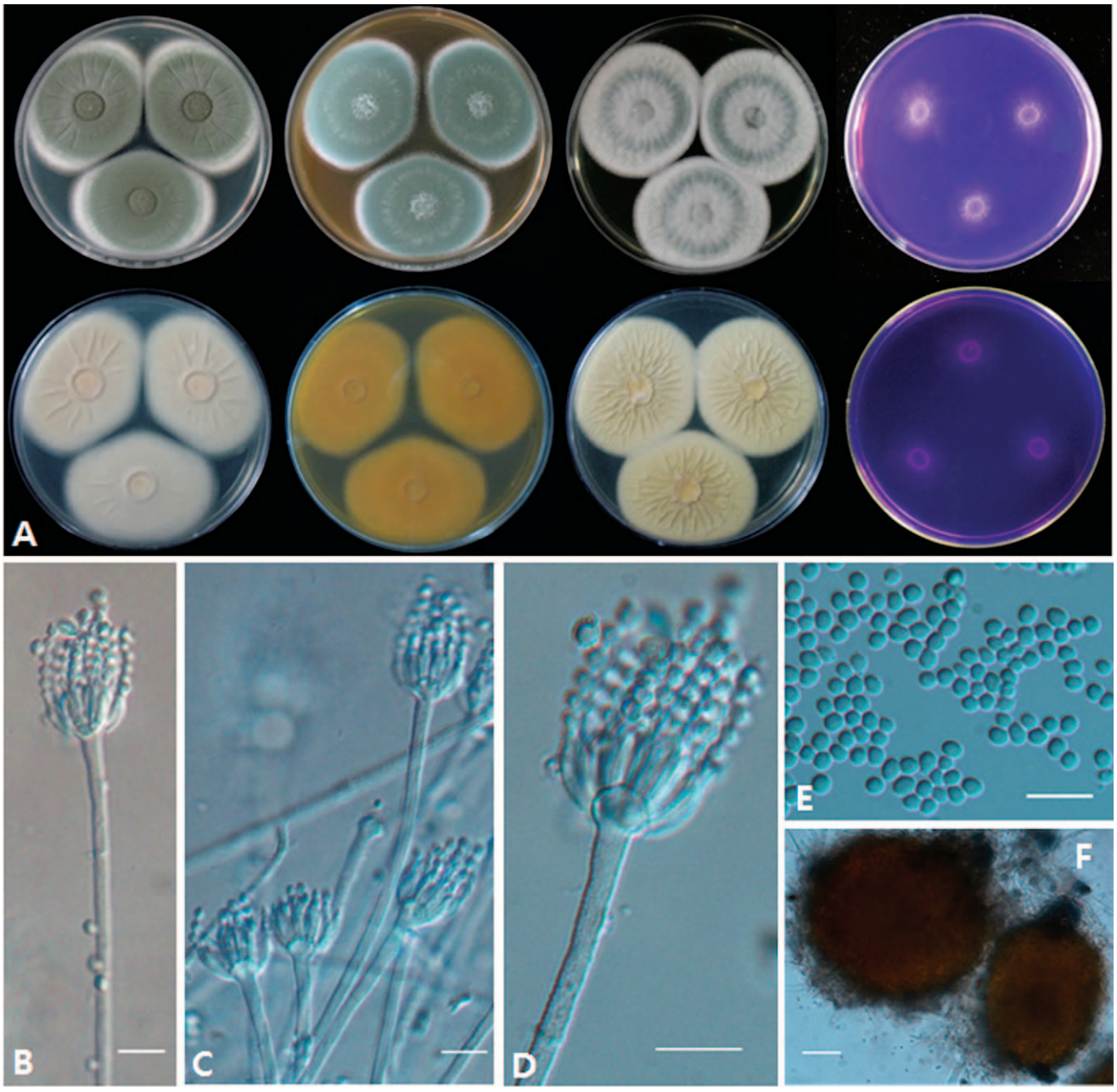

FIG. 3. *Penicillium jejuense* SFC20140101-M756. A. Seven d old cultures, at 25 C, left to right; first row, all obverse, CYA, MEA, YES, CREA; second row, CYA reverse, MEA reverse, YES reverse, CREA reverse. B–D. Conidiophores. E. Conidia. F. Sclerotia. Scale bars: A–E = 10 μm, F = 25 μm.

TABLE II. Comparison of morphological characters of *Penicillium jejuense* and *Penicillium crocicola*

Character		*P. jejuense* sp. nov. SFC20140101-M756^T	*P. crocicola* CBS 745.70^T
CYA	Colony	30–35 sulcae	5–8 weak sulcae
	Color	Dull green	Greenish gray
	Texture	Velvety and floccose in center	Floccose
	Sporulation	Strong	Moderate
	Exudates	Few small clear exudate	Absent
	Reverse color	Grayish yellow	Brownish orange
	Sclerotia	Few	Few
MEA	Colony	11–15 sulcae	3–4 weak sulcae
	Color	Grayish green	Grayish green
	Texture	Velvety and floccose in center	Floccose
	Sporulation	Strong	Moderate
	Exudates	Absent	Absent
	Reverse color	Dark blonde	Brownish orange
	Sclerotia	Abundantly produced	Absent
YES	Colony	Strongly wrinkled	4–5 weak sulcae but no wrinkles
	Color	White and grayish turquoise to yellowish-white with turquoise-gray	White with greenish white
	Texture	Velvety and floccose in center	Floccose
	Sporulation	Moderate	Weak
	Exudates	Absent	Absent
	Reverse color	Pale yellow	Grayish yellow
Strips	Size (μm)	30–230 × 2.5–3.5	35–155 × 2.5–3.5
Vesiculate		Nonvesiculate	Nonvesiculate
Phialide	Size (μm)	9.5–13.5 × 2.0–3.5	9.7–14.3 × 2.1–3.5
	per verticil	8–16	4–16
Conidia	Size (μm)	2.0–3.5 × 2.0–2.5	2.4–3.1 × 2.2–3.0
	Shape	Globose to ellipsoidal	Globose to subglobose
	Wall	Finely roughened	Finely roughened
Sclerotia	Size (μm)	160–340 × 125–210	140–320 × 130–255[a]
	Shape	Globose to ellipsoidal	Subglobose to ellipsoidal[a]
	Color	Grayish orange	Yellow-brown to dark brown[a]

[a] Not found in our study. Data from Yamamoto et al. 1955 on Czapek-Dox agar.

sulcae; conidia greyish green (26C3); colony texture velvety, floccose in the center; sporulation strong, absent toward the margins; nonsporulating edges 3–4 mm; exudates absent; reverse color dark blonde (5D4). Colonies on YES strongly wrinkled; conidia white and grayish turquoise (24C3) to yellowish white (2A2) with turquoise gray (24B2) in the center; colony texture velvety, floccose in center; sporulation moderate; non-sporulating margins 1–2 mm; exudates absent; reverse color pale yellow (3B3). Growth on CREA poor; no acid production.

Sclerotia globose to ellipsoidal, 160–340 × 125–210 μm diam, Grayish orange (5B5) on MEA (FIG. 3F). Asci and ascospores not observed. Conidiophores strictly monoverticillate, inflated at the apex, with smooth or finely roughened walls, 30–230 × 2.5–3.5 μm; phialides ampulliform, 8–16 per stipe, 9.5–13.5 × 2.0–3.5 μm (FIG. 3B–D). Conidia globose to ellipsoidal, 2.0–3.5 × 2.0–2.5 μm, with smooth or finely roughened walls (FIG. 3E).

Additional strains examined: REPUBLIC OF KOREA. Jeju Island, on marine sponge, 2009, *Jae Hak Sohn,* SFC20140101-M820; same location, on sand, Feb 2011, *Jae Hak Sohn,* SFC20140101-M847 (strains available upon request).

Distribution: Marine habitat of Jeju Island, Republic of Korea.

Habitat: Pollicipes mitella (common stalked barnacle), marine sponge and sand.

DISCUSSION

To evaluate whether our three *Penicillium* strains represented a new species, we used both DNA and culture-based methods. Data from both approaches indicated that these three strains represent the same species and are distinct from all currently known *Penicillium* species. Here we discuss the phylogeny and morphological characters of this new species and compare it to the most closely related species, *P. crocicola*.

Phylogenetic analyses of four independent loci (ITS, *BenA*, *CaM*, *RPB2*) demonstrated a close relationship between *P. jejuense* and species of section *Aspergilloides*, being most closely related to *P. crocicola*. The classification of *P. jejuense* in this group is supported by similarities in morphological characters including fast growth, sclerotia production and predominantly monoverticillate, rough-walled conidiophores with an inflated apex (FIG. 3, TABLE II) (Yamamoto et al. 1956; Pitt 1979, 1985; Houbraken and Samson 2011). Despite these similarities, *P. jejuense* can be distinguished from *P. crocicola* by distinctive phenotypic characters, such as the reverse color on CYA, sclerotia production on MEA and colony features on YES (TABLE II). The reverse color on CYA of *P. jejuense* was grayish yellow, whereas *P. crocicola* is brownish orange. Cultures of *P. jejuense* grown 7 d on MEA produce abundant grayish orange sclerotia, while *P. crocicola* lacks sclerotia on MEA (note that *P. crocicola* produced sclerotia on CYA in our study and on Czapek-Dox agar in the study by Yamamoto et al. 1956). When grown on YES, colonies of *P. jejuense* are characterized by strongly wrinkled sulcate colonies while *P. crocicola* colonies are weakly radial sulcate and without wrinkles.

Penicillium species generally are considered terrestrial fungi, but it is not uncommon that some species can be isolated from the marine environment (Kagata et al. 2000, Lin et al. 2000, Edrada et al. 2002). Halophiles require saline conditions for growth and can be categorized into the following groups based on their salt requirements for optimal growth: (i) slight halophiles (1–5% NaCl), (ii) moderate halophiles (5–20% NaCl) and (iii) extreme halophiles (20–30% NaCl) (DasSarma and Arora 2001). We showed that our new species could grow at salt concentrations as high as 15% but had optimal growth on MEA supplemented with 3.3% (w/v) sea salt. Based on these observations, the new species should be classified as a slight halophile. However, there are no other indications that this species or these strains have adapted to its marine environment. Houbraken et al. (unpubl) studied the growth rate of species belonging to the *P. thomii* clade on CYA supplemented with 5% NaCl and showed that these terrestrial species grew well on this medium, although slightly slower than on regular CYA.

Marine-derived *Penicillium* species are important producers of secondary metabolites (Kagata et al. 2000, Komatsu et al. 2000, Lin et al. 2000, Edrada et al. 2002, Bugni et al. 2004), and some display antimicrobial (Bugni et al. 2004) and extracellular enzyme activity (Burtseva et al. 2010, Dubrovskaya et al. 2012). All three strains of *P. jejuense* exhibited alginase and β-glucosidase activity and had antifungal activity against the plant pathogens *Colletotrichum acutatum* and *F. oxysporum* (*Penicillium* sp. 5 in Park et al. 2014). Because of its enzyme and antifungal activity, *P. jejuense* should be further studied for its potential role as an enzyme producer and a biological control agent.

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