

## *Penicillium ulleungdoense* sp. nov. from Ulleung Island in Korea

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### ABSTRACT

In a study of the fungal diversity on Ulleung Island in Korea, three novel strains of *Penicillium* were isolated. Different sites on Ulleung Island were selected for collecting endophytic fungi, and three endophytic fungal strains showed unique morphological characteristics. DNA sequence of the internal transcribed spacer,  $\beta$ -tubulin, calmodulin, and RNA polymerase II second largest subunit regions of the strains were analyzed and they showed unique taxonomic position from the other species of *Penicillium* section *Sclerotiora*. The new strains were named *Penicillium ulleungdoense* sp. nov. As the novel endophytic *Penicillium* taxa were discovered in a unique environment, the data could be meaningful for understanding the geographical distribution of Ascomycetes on Ulleung Island.

### ARTICLE HISTORY

Received 7 May 2020  
Revised 4 August 2020  
Accepted 14 November 2020

### KEYWORDS

*Penicillium ulleungdoense* sp. nov.; endophytic  
Ulleung Island

## 1. Introduction

The genus *Penicillium* classified in the order Eurotiales within the family Trichocomaceae [1], was first introduced as *P. candidum*, *P. glaucum*, and *P. expansum* by Johann Heinrich Friedrich Link in 1809 [2]. With association of 26 sections, this genus is subdivided into *Aspergilloides*, and *Penicillium* [3,4]. As one of the most common genera in earth, many species belong to this genus could be isolated from various environments including air, soil, food product, and organism [5,6]. *Penicillium* was originally part of family Trichocomaceae. Since 1990s however, not only morphological characterization but also molecular analysis has been used for identification. Due to the new identification of fungi, *Penicillium* was redefined as part of family Aspergillaceae [7], and all names of *Penicillium* were well arranged recently based on polyphasic taxonomy [8].

Based on the color like yellow, and/or orange mycelia, orange, and/or reddish colony reverses, Houbraken and Samson established the *Penicillium* section *Sclerotiora* [9]. As the fungi were isolated from soil, plants and insects, the type of host also becomes a standard for identification [3]. With combination of morphological features including colony pattern, conidiophore structure and sclerotia production, the species in section *Sclerotiora* is identified [10]. The molecular analysis with sequence of

internal transcribed spacer (ITS) region and additional DNA marker;  $\beta$ -tubulin (*BenA*), calmodulin (*CaM*) and the RNA polymerase II second largest subunit (*RPB2*) is added to increase the reliability of analysis [8,11]. In worldwide, over 350 species were named as *Penicillium* [11], and among them, about 100 species of *Penicillium* were discovered in Korea [12]. Including reporting of *P. daejeonium* as new kind species by doctor Lim's team, 12 species were identified as new from Korea [13,14].

In this study, three specific plants (*Phedimus takesimensis*, *Sedum oryzifolium*, and *Aster spathulifolius*) isolated from Ulleung Island were chosen for the collection of novel endophytic fungal strains [15,16]. Ulleung Island is a volcanic island located in the Ulleung Basin in the East Sea (E 131°52'07", N 37°14'12"). The annual average temperature in the Ulleung Basin is 12 °C with a high humidity; most parts of the island have steep slopes, and strong winds make the inhabitation of plants difficult [17]. Ulleung Island is composed of 89 eastern and western islands, and 65.4% of the land area is made of steep slopes over 40° [18]. Owing to a predominantly dry and salty environment, Ulleung Island has a unique biosystem that includes 48 species of plants [15,19]. Endophytic fungi from *Phedimus takesimensis*, *Sedum oryzifolium*, and *Aster spathulifolius* on Ulleung Island were screened

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

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morphologically and molecularly. This study describes a novel strain of *P. ulleungdoense* sp. nov., isolated from the roots of the three plants on Ulleung Island.

## 2. Materials and methods

### 2.1. Isolation of endophytes

The roots of *Phedimus takesimensis* (E 130°54'32.76", N 37°28'54.97"), *Sedum oryzifolium* (E 130°47'55.45", N 37°31'07.27"), and *Aster spathulifolius* (E 130°53'15.88", N 37°32'29.09") were collected from Ulleung Island in 4 April 2017. The root samples were washed with distilled water to remove sand particles and treated with Tween-80 solution for 5 min. The surface was sterilized using 1% perchloric acid solution and subsequently washed with distilled water. After washing, the roots were cut, and the pieces were incubated at 25 °C in Hagem minimal medium containing 80 ppm of streptomycin for pure culture [20,21]. The isolated fungal strains from the roots were subcultured on potato dextrose agar (PDA) and incubated at 25 °C for a week in the dark to allow fungal growth [22]. The isolates KMG401, KMG402, and KMG403 were deposited to the Korean Agricultural Culture Collection (KACC) with allotted no. KACC 48990, KACC 48991, and KACC 48992, respectively.

### 2.2. Morphological analysis

Agar plugs were cultured on PDA and then transferred to malt extract agar (MEA), Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YESA), czapek yeast autolysate agar with 5% NaCl (CYAS), czapek's agar (CZ), oatmeal gar (OA), and Creatine sucrose agar (CREA) for morphological analysis [11,23]. Plates were incubated at 25 °C in the dark for 7 days, and plates with CYA were additionally incubated at 30 °C and 37 °C in the dark for 7 days. After incubation, diameters, density of sporulation, obverse and reverse colony colors, and the existence of soluble pigments were recorded. Fungal morphological characterization was identified by using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) [5,11]. With 85% lactic acid and 99% ethanol, fixed specimen images were acquired.

### 2.3. DNA extraction, PCR, and sequencing

Strains of the sample were grown on PDA, and DNA was extracted using the Accuprep® Genomic DNA Extraction Kit (Bioneer Corp., Daejeon, Korea). DNA preps were stored at -20 °C until use for PCR. Four regions, internal transcribed spacer (ITS),  $\beta$ -tubulin (*BenA*), calmodulin (*CaM*), and

RNA polymerase II second largest subunit (*RPB2*), were amplified with the primer pairs ITS1-ITS4, Bt2a-Bt2b, CMD5-CMD6, and 5Feur-7CReur, respectively [9,11]. Amplifications were performed with different primer sets in a 20  $\mu$ l reaction mixture [23,24]. The products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster, CA, USA) with the ABI 310 DNA sequencer (PE Biosystems) [8].

### 2.4. Phylogenetic analysis

Under manual, the obtained nucleotide sequences were aligned, and submitted for phylogenetic analysis using BioEdit v7.2.5 (Clustal W) [25]. The similarities of fungal sequences were calculated by using Molecular Evolutionary Genetics Analysis (MEGA) 7 software [26]. A maximum likelihood (ML) phylogenetic tree was constructed based on a combination of the markers of ITS, *BenA*, *CaM*, and *RPB2* for three strains [11]. While constructing phylogenetic tree, *RPB2* was not included in the combination of ITS, *BenA*, and *CaM* [27]. This is because that some *Penicillium* species showed no primer sequences of the *RPB2*. With Tamura 3-parameter model, the ML heuristic method was set as the level 3 of Subtree Pruning Regrafting (SPR), and number of bootstrap replicates was 1,000. To set the outgroup for section *Sclerotiora*, *P. levitum* CBS 45.48 of section *Lanata-Divaricata* was used [28]. Through this manual, the initial ML tree was set automatically. The percentage of sequence identity was obtained from a National Center for Biotechnology Information (NCBI) BLASTn search [29,30]. In phylogenetic analysis, the obtained sequences were compared with *Penicillium* species based on the study of Wang et al. [20].

## 3. Results

### 3.1. Phylogenetic analysis

The DNA sequences of the new species were registered in the GenBank database of the NCBI (MN640087–MN640089 for rDNA-ITS, MN737487–MN737489 for *BenA*, MN745074–MN745076 for *CaM*, and MN756007–MN756009 for *RPB2*) (Table 1). The target strains KACC 48990, KACC 48991, and KACC 48992 were positioned using the sequences of the ITS region, *BenA* region, *CaM* region, and *RPB2* region (Figures 1 and 2). Based on these four genes, the target strains were compared with other strains available in the NCBI database. A phylogenetic tree was constructed following the bootstrap analysis of 1,000 replicates, and all three strains were the most

**Table 1.** Details of the strains used in phylogenetic analyses.

Species	Strain numbers	Sequence accession numbers			
		ITS	BenA	CaM	RPB2
<i>P. adametzii</i>	CBS 209.28 <sup>T</sup>	JN714929	JN625957	KC773796	JN121455
<i>P. adametzioides</i>	CBS 313.59 <sup>T</sup>	JN686433	JN799642	JN686387	JN406578
<i>P. alexiae</i>	CBS 134558 <sup>T</sup>	KC790400	KC773778	KC773803	
<i>P. amaliae</i>	CBS 134209 <sup>T</sup>	JX091443	JX091563	JX141557	
<i>P. angulare</i>	CBS 130293 <sup>T</sup>	AF125937	KC773779	KC773804	JN406554
<i>P. arianae</i>	CBS 134559 <sup>T</sup>	KC773833	KC773784	KC773811	
<i>P. austrosinicum</i>	HMAS 248734 <sup>T</sup>	KX885061	KX885041	KX885051	KX885032
<i>P. bilaiae</i>	CBS 221.66 <sup>T</sup>	JN714937	JN625966	JN626009	JN406610
<i>P. brocae</i>	CBS 116113 <sup>T</sup>	AF484398	KC773787	KC773814	JN406639
<i>P. cainii</i>	DAOM 239914 <sup>T</sup>	JN686435	JN686366	JN686389	
<i>P. citrinum</i>	CBS 139.45 <sup>T</sup>	AF033422	GU944545	GU944638	JF417416
<i>P. coffeae</i>	CBS 119387 <sup>T</sup>	AY742702	KJ834443	AY741747	JN121436
<i>P. guanacastense</i>	DAOM 239912 <sup>T</sup>	JN626098	JN625967	JN626010	
<i>P. herquei</i>	CBS 336.48 <sup>T</sup>	JN626101	JN625970	JN626013	JN121494
<i>P. hirayamae</i>	CBS 229.60 <sup>T</sup>	JN626095	JN625955	JN626003	JN121459
<i>P. jacksonii</i>	DAOM 239937 <sup>T</sup>	JN686437	JN686368	JN686391	
<i>P. janthinellum</i>	CBS 340.48 <sup>T</sup>	GU981585	GU981625	KF296401	JN121497
<i>P. johnkrugii</i>	DAOM 239943 <sup>T</sup>	JN686447	JN686378	JN686401	
<i>P. jugoslavicum</i>	CBS 192.87 <sup>T</sup>	KC773836	KC773789	KC773815	JN406618
<i>P. levitum</i>	CBS 345.48 <sup>T</sup>	GU981607	GU981654	KF296394	KF296432
<i>P. lilacinoechinulatum</i>	CBS 454.93 <sup>T</sup>	AY157489	KC773790	KC773816	
<i>P. malachiteum</i>	CBS 647.95 <sup>T</sup>	KC773838	KC773794	KC773820	
<i>P. mallochii</i>	DAOM 239917 <sup>T</sup>	JN626104	JN625973	JN626016	
<i>P. maximae</i>	CBS 134565 <sup>T</sup>	EU427298	KC773795	KC773821	
<i>P. multicolor</i>	CBS 501.73 <sup>T</sup>	JN799647	JN799645	JN799646	EU427262
<i>P. paxilli</i>	CBS 360.48 <sup>T</sup>	GU944577	GU944577	JN606844	JN606610
<i>P. sclerotiorum</i>	CBS 287.36 <sup>T</sup>	JN626132	JN626001	JN626044	JN406585
<i>P. simplicissimum</i>	CBS 372.48 <sup>T</sup>	GU981588	GU981632	KF296368	JN121507
<i>P. ulleungdoense</i> sp. nov.	KACC 48990 <sup>T</sup>	MN640087	MN737487	MN745074	MN756007
	KACC 48991	MN640088	MN737488	MN745075	MN756008
	KACC 48992	MN640089	MN737489	MN745076	MN756009
<i>P. vanoranjei</i>	CBS 134406 <sup>T</sup>	KC695696	KC695686	KC695691	
<i>P. viticola</i>	JCM 17636 <sup>T</sup>	AB606414	AB540174		

The accession numbers of the strains KACC 48990, KACC 48991, KACC 48992, and others are available in the NCBI database.

similar to the type strain of *Penicillium hirayamae*. In addition, BLASTn search was performed against the strains KACC 48990, KACC 48991, and KACC 48992. ITS gene sequence analysis showed that KACC 48990, KACC 48991, and KACC 48992 were the most similar to *P. hirayamae* with 97% sequence identity. *BenA* gene sequence analysis showed that KACC 48990, KACC 48991, and KACC 48992 were the most similar to *P. hirayamae* with 88% sequence identity. *CaM* gene sequence analysis showed that KACC 48990, KACC 48991, and KACC 48992 were the most similar to *P. hirayamae* with 91% sequence identity. *RPB2* gene sequence analysis showed that KACC 48990, KACC 48991, and KACC 48992 were the most similar to *P. hirayamae* with 91% sequence identity. Gene sequence analysis with a combination of four primer pairs showed that KACC 48990, KACC 48991, and KACC 48992 were the most similar to *P. hirayamae* with 93% sequence identity [8,31].

### 3.2. Morphological feature

The colony of various plates of the KACC 48990 strain are shown, and the photomicrographs of morphological structures are shown in Figure 3. The detailed fungal morphological descriptions are in the Taxonomy section. Distinct morphological features

between *P. ulleungdoense*, and its related species are summarized in Table 2.

### 3.3. Taxonomy

*Penicillium ulleungdoense* D.H. Choi & J.G. Kim, sp. nov. (Figure 3).

**Fungal Names:** KMG 401.

**Typus:** KACC 48990.

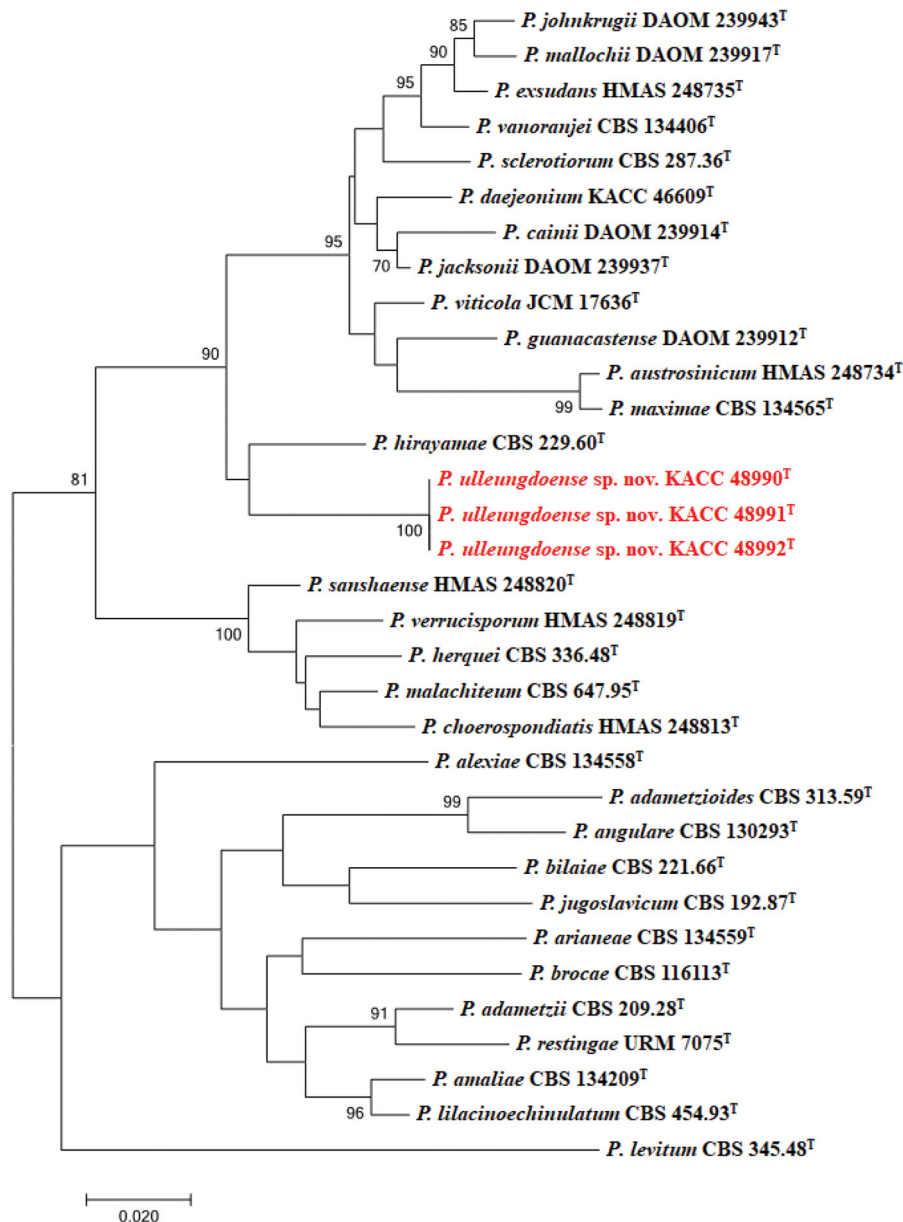
**Mycobank:** MB835474.

**Etymology:** The specific epithet refers the collected space: Korean, Ulleung Island in 4 April 2017, collector D.H. Choi.

**DNA barcodes:** ITS MN640087, *BenA* MN737487, *CaM* MN745074, *RPB2* MN756007.

**Colony diam, 7d, 25 °C (unless stated otherwise):** CYA 16–18 mm; CYA 30 °C 25–28 mm; CYA 37 °C 4–7 mm; MEA 12–17 mm; YES 19–24 mm; CREA 6–10 mm; CZ 8–9 mm; OA 13–16mm.

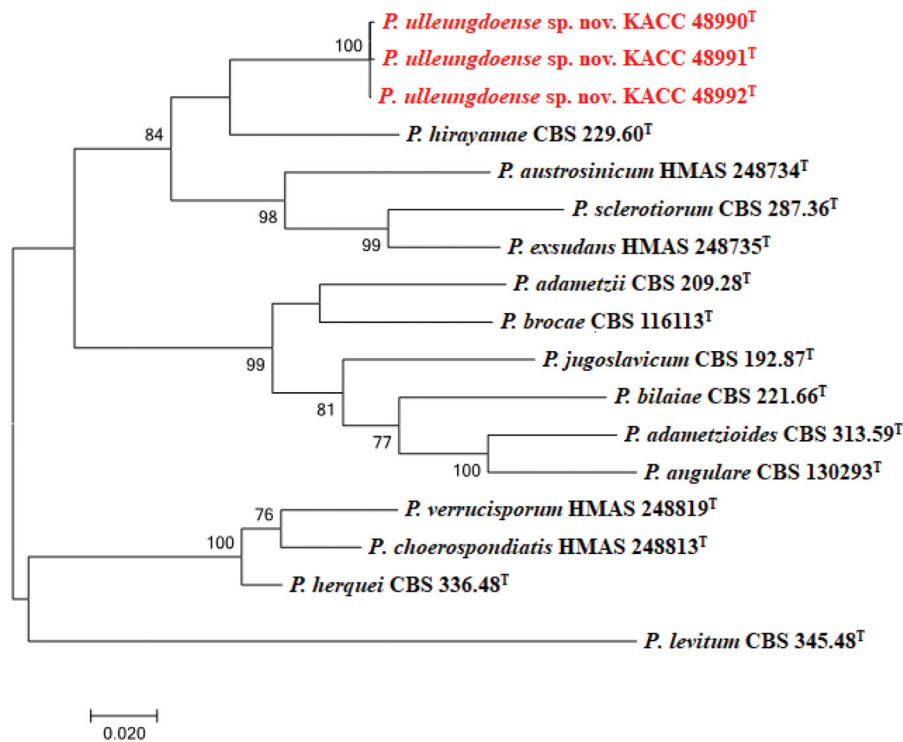
**Colony characteristics:** On CYA 25 °C, 7 days: Colonies nearly circular, convex, concentrically sulcate, cobblestone gray mycelia appeared in centers; margins narrow, entire; mycelia white near margin, bright ivory elsewhere; texture floccose; sporulation moderately dense; conidial color light ivory; soluble pigments absent; exudates present; reverse conspicuously, and radially sulcate, generally silk gray (Figure 3(A)). On CYA 30 °C, 7 days: Colonies



**Figure 1.** Phylogenetic tree based on the maximum likelihood analysis of the combined ITS, *BenA* and *CaM* dataset for species classified in the *Penicillium* section. *Penicillium levitum* was included as an outgroup. Bootstrap analysis was performed with 1,000 replications. Bootstrap support values of  $\geq 70\%$  are indicated at the nodes. The bar indicates the number of substitutions per position. T indicates the type strains of the species. Bar, 0.02 substitutions per nucleotide position. The isolates KACC 48990, KACC 48991 and KACC 48992 are marked in red.

similar to those on CYA 25 °C, 7 days but larger scale; margins moderate, entire; mycelium white near margin, brown beige elsewhere; reverse generally silk gray or beige red in center but at beige periphery (Figure 3(B)). On CYA 37 °C, 7 days: Colonies irregular, plane; margins low, narrow, undulate; mycelia ivory; texture floccose; sporulation dense; conidial color light ivory; soluble pigments absent; exudates present; reverse generally silk gray (Figure 3(C)). On MEA 25 °C, 7 days: Colonies irregular, convex, agate gray mycelia appeared in centers; margins low, narrow, entire or undulate; mycelia yellow near margin, white elsewhere; texture floccose; sporulation dense; conidial color bright yellow; soluble pigments slight dull yellow; exudates

absent; reverse orange in centers but orangish white at periphery (Figure 3(D)). On YES 25 °C, 7 days: Colonies nearly circular, convex, dusty gray and pearl dark gray mycelia appeared in centers; margins undulate; mycelia yellowish white, and papyrus white; texture floccose; sporulation moderately dense; conidial color yellowish white; soluble pigments absent; exudates present; reverse conspicuously, and radially sulcate, beige red in center but at beige periphery (Figure 3(E)). On CYAS 25 °C, 7 days: Colonies nearly circular, highly convex in centers; margins low, narrow, entire; mycelia white near margin, beige elsewhere; texture floccose; sporulation dense; conidial color green beige; soluble pigments absent; exudates present; reverse



**Figure 2.** Phylogenetic tree based on the maximum likelihood analysis of *RPB2* dataset for species classified in the *Penicillium* section. *Penicillium levitum* was included as an outgroup. Bootstrap analysis was performed with 1,000 replications. Bootstrap support values of  $\geq 70\%$  are indicated at the nodes. The bar indicates the number of substitutions per position. T indicates the type strains of the species. Bar, 0.02 substitutions per nucleotide position. The isolates KACC 48990, KACC 48991 and KACC 48992 are marked in red.

conspicuously, and radially sulcate, traffic gray in center but at pearl light gray periphery (Figure 3(F)). On CZ 25 °C, 7 days: Colonies nearly circular or irregular, convex; margins low, narrow, entire; mycelia bright beige near margin, yellow elsewhere; texture floccose; sporulation dense; conidial color yellow; soluble pigments absent; exudates present; reverse generally bright ivory (Figure 3(G)). On OA 25 °C, 7 days: Colonies circular, convex; margins low, narrow, entire; mycelia bright ivory near margin, cobblestone gray elsewhere; texture floccose; sporulation dense; conidial color ivory; soluble pigments slight dull yellow; exudates absent (Figure 3(H)). On CREA 25 °C, 7 days: acid production moderately present (Figure 3(F)).

**Micromorphology:** Conidiophores strictly monovericillate; stipes septate, smooth-walled, 28.3–50.0  $\times$  2.1–2.8  $\mu\text{m}$ , vesticulate; phialides ampulliform, navicular, smooth, 2–8 per stipe, 5.5–7.0  $\times$  2.0–2.5  $\mu\text{m}$ ; conidia subglobose, smooth-walled, 2.3–2.5  $\times$  1.9–2.0  $\mu\text{m}$ ; sclerotia not observed.

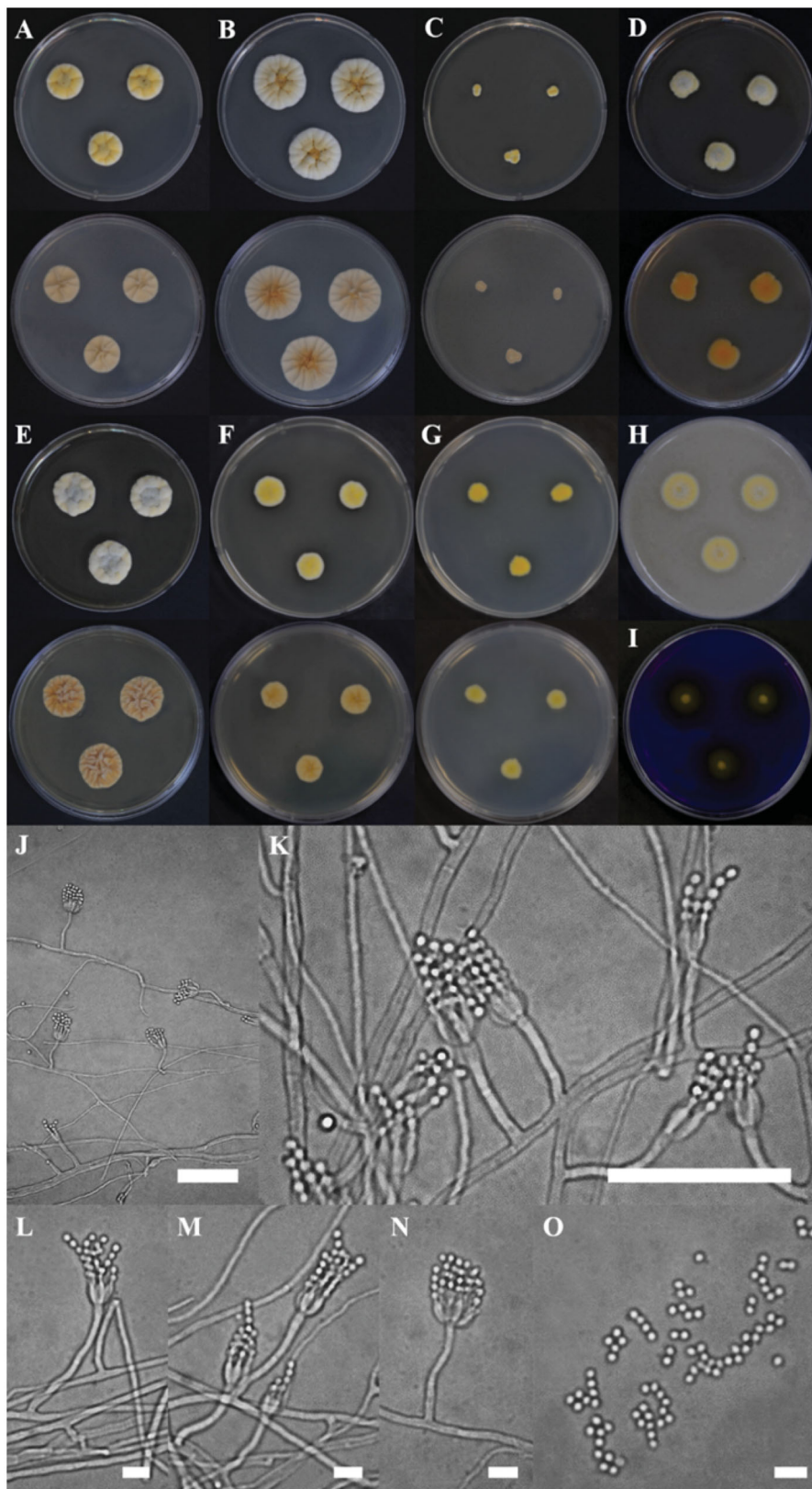
**Type strain:** KACC 48990, isolated from the root of *Phedimus takesimensis* in Ulleung Isl, and, Korea, 4 April 2017. The culture is preserved in Korean Agricultural Culture Collection (KACC) in Jeonju, Korea. Molecular markers for the species are MN640087 for rDNA-ITS, MN737487 for  $\beta$ -tubulin, MN745074 for calmodulin, and MN756007 for RNA polymerase II second largest subunit.

**Note:** In phylogenetic tree, and morphological analysis, KACC 48990 showed the most similarity with *P. hirayamae*. Though the similarity between KACC 48990, and *P. hirayamae*, KACC 48990 showed several differences with *P. hirayamae*; less growth on medium, better acid resistant, and lightly present of soluble pigments.

**Additional strains studied:** KACC 48991, Republic of Korea. Gyeongsang Province, Ulleung Island, 37°14'33.29"N 131°51'53.43"E, *Sedum oryzifolium*, 4 April 2017, D.H. Choi, J.G. Kim; KACC 48992, Republic of Korea. Gyeongsang Province, Ulleung Island, 37°14'20.10"N 131°52'08.50"E, *Aster spathulifolius*, 4 April 2017, D.H. Choi, J.G. Kim.

#### 4. Discussion

With four primer sequences (ITS, *BenA*, *CaM*, and *RPB2*) [20,27], phylogenetic analysis was processed to study the relationship of *Penicillium* section *Sclerotiora*. As some of *Penicillium* species had no primer sequences of the *RPB2* region, the construction of phylogenetic tree was done with two kind of versions; combination of ITS, *BenA* and *CaM* regions; *RPB2* regions [20]. The result for individual markers showed a close relationship with *P. hirayamae* [8,13] and is confirmed by results of maximum-likelihood phylogenetic trees (Figure 1–2). Similar to many other Eurotiales species,



**Figure 3.** Morphological characteristics of *Penicillium ulleungdoense* (KACC 48990) grown on different media. (A) CYA 25 °C, (B) CYA 30 °C, (C) CYA 37 °C, (D) MEA 25 °C, (E) YESA 25 °C, (F) CYAS 25 °C, and (G) CZ 25 °C (top: obverse, bottom: reverse); (H) Colony on OA 25 °C; (I) Colony on CREA 25 °C; (J–N) Conidiophores; (O) Conidia (Scale bar = 50 μm in J–K. Scale bar = 10 μm in L–O).

*Penicillium* can be distinguished based on morphological features and growth temperature [11]. Similar to many other Eurotiales species, *Penicillium* can be distinguished based on morphological features and growth temperature [11]. *Penicillium*

strains have different growth patterns at different temperatures and in different media. *P. hirayamae* grew slightly faster on YESA and poorly on CREA. Conidiophores are mostly monoverticillate but rarely have a single metula form [9]. *Penicillium* species

**Table 2.** Morphological characteristics of KACC 48990 and the reference species *Penicillium hirayamae* on Czapek yeast autolysate agar (CYA) at 25 °C.

Characteristics	Study isolate, <i>Penicillium ulleungdoense</i> KACC 48990	<i>Penicillium hirayamae</i>
Colony color	bright ivory; reverse silk gray	yellowish brown; reverse orange
Colony diameter	16-18 mm in 7 days	25-33 mm in 7 days
Acid production	moderate	weak
Sclerotia	not observed	present; orange
Conidiophores	monoverticillate, stipe septate, smooth to rough, 2.1-2.8 × 28.3-50.0 µm	Monoverticillate, single metula rare branching, stipe septate, smooth to rough, 1.8-2.4 × 38.0-44.0 µm
Phialides	ampulliform, navicular shape, smooth, 3-8 in number, 2.0-2.5 × 5.5-7.0 µm	ampulliform, pear shape, smooth, 3-7 in number, 2-2.8 × 8.1-8.6 µm
Conidia	smooth to rough, globose, 1.9-2.0 × 2.3-2.5 µm	smooth to rough, globose to subglobose, 2.6-2.7 × 2.8-3.0 µm

aAccording to the description of Samson et al. [9].

could be distinguished according to the shape of the conidia, phialides, conidiophores, and vesicle. In the result of comparing with *Penicillium* species based on the study of Zhuang's team, three isolates KACC 48990, KACC 48991, and KACC 48992 were morphologically similar to *P. hirayamae* [9,20]. The conidia were globose, joined into chains, smooth to rough, and colorless. Similar to *P. hirayamae*, ampulliform phialides of conidiophores and yellowish colony color were recognized. Despite these similarities, the isolates could be distinguished from *P. hirayamae*. Not like *P. hirayamae*, the colony growth rates of isolates were much smaller and dull yellow soluble pigments were existed slightly on MEA. The reverse color on CYA of *P. hirayamae* was orange, whereas that of three isolates were silk gray. Another notable feature of three isolates was acid production because *P. hirayamae* had weak acid production, whereas the isolates had moderate acid production.

Since the initial study of endophytic fungal diversity, various species belonging to Ascomycota have been isolated from Ulleung Island [3,13]. As the novel endophytic *Penicillium* taxa were discovered in a unique environment, the data could be meaningful for understanding the geographical distribution of Ascomycetes on Ulleung Island. The diversity of endophytes on Ulleung Island is unclear. Further studies including the isolation and analysis of endophytes are important to identify unknown taxa on Ulleung Island. In the present study, a novel strain of *Penicillium* was identified.

### Disclosure statement

No potential conflict of interest was reported by the author(s).

### Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A1A05011910) and Research Institute for Dokdo and Ulleung-do Island of Kyungpook National University, Korea.

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