Journal of Species Research 12(Sepcial Edition 2):54-59, 2023

A report on 10 unrecorded bacterial species isolated from the Korean islands in 2022

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To obtain unrecorded bacterial species from Korean islands, various samples were collected from the islands in 2022. After plating the samples on marine agar or Reasoner's 2A, and incubating aerobically, approximately 1,200 bacterial strains were isolated and identified using 16S rRNA gene sequences. A total of 10 strains showed ≥98.7% 16S rRNA gene sequence similarity with the bacterial species that were validly published but not reported in Korea. The unrecorded bacterial strains belong to three phyla, five classes, 10 orders, 10 families, and 10 genera, which are assigned to *Sphingomonas*, *Falsirhodobacter* and *Asticcacaulis* of the class *Alphaproteobacteria*; *Colwellia* and *Halomonas* of the class *Gammaproteobacteria*; *Chitinophaga* of the class *Chitinophagia*; *Chryseobacterium* of the class *Flavobacteriia*; *Microlunatus*, *Zhihengliuella*, and *Streptomyces* of the class *Actinomycetia*. The details of the unreported species including Gram reaction, colony and cell morphology, biochemical characteristics, and phylogenetic position are also provided in the description of the strains.

Keywords: 16S rRNA, anaerobic bacteria, islands, lichens, seawater, unrecorded bacterial species

DOI:10.12651/JSR.2023.12(S2).054

Introduction

All islands in the world take up approximately an area of 9.9×10^6 km^2, and their coastlines are 1.5×10^6 km^2, constituting 6.7% of Earth's land area (Sayre *et al*., 2019). Korea has 3,358 islands with an area of about 3,757 km^2, which takes up 3.5% of Korea's total land area (Ministry of Environment, 2014). The Korean islands are characterized by diverse ecosystems such as mountain, seashore, tidal flat, and sand beach, contributing to encouraging high biodiversity of indigenous species. In terms of plants, 50% of the novel species in Korea over the past 30 years have been discovered on the islands (Lim *et al*., 2012; National Institute of Biological Resources, 2020). Numerous new and unrecorded bacterial species, as well as plant species, have also been discovered on the islands (Yoon *et al*., 2005; Kim *et al*., 2020; Shin *et al*., 2022).

In 2021, the Honam National Institute of Biological Resources (HNIBR) was newly established to conduct biological survey on the islands and coastal areas, and has launched research programs focusing on discovering unrecorded species (Lee *et al*., 2022; Shin *et al*., 2022). This study is a part of the research programs supported by the HNIBR in 2022. We have isolated previously unrecorded bacterial species in seawater, soil, root of halophytes and lichens collected from the Korean islands. Phylogenetic analyses based on the 16S rRNA gene sequences, 10 bacterial strains in the classes of *Actinomycetia*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Chitinophagia*, and *Flavobacteriia* were identified as unreported bacterial species in Korea and their taxonomic information and phenotypic characteristics are reported.

Materials and Methods

Various samples were collected from lichens, halophytes, seawater and soil of five Korean islands in 2022 (For more information, visit the Island Bioresource total Information System (IBIS) website (https://ibis.hnibr.re.kr). Using the spread plating technique on agar media, an aliquot (100 μL) of the seawater samples was directly spread onto

Marine agar (BD Diagnostics) and aerobically incubated at 20°C for seven days. Bacterial strains were purified as single colony and the pure cultures were preserved at −80°C in 20% (v/v) glycerol suspension, as well as lyophilized ampoules. The lichen specimens were examined under a microscope to remove contaminants, washed for 10 min in 1 mL of 0.85% NaCl in Multi-EP tube vortexer (FinePCR) four times, and crushed in a TissueLyser II (Qiagen) containing stainless beads (Qiagen) twice (Noh *et al*., 2021). The final suspension (100 µL) was spread on 0.1x Reasoner's 2A (R2A) media. The roots of halophytes were cut off by using sterile scissors. And then, the samples were washed with 1% Phosphate-buffered Saline (PBS) to remove the soil attached to the root. Using a homogenizer (IKA), 0.1 g of the sub-sample was thoroughly mixed with 1 mL of 1% PBS. The final suspension (100 µL) of the homogenized sample was spread onto MA agar or R2A agar and aerobically incubated at 20°C for seven days. Using a homogenizer, 1 g of the soil sample was thoroughly mixed with 100 mL of distilled. An aliquot (100 µL) of the homogenized sample was spread onto R2A agar and aerobically incubated at 20°C for seven days. The plates were incubated at 15°C for 14 days. Details on the strains are shown in Table 1.

For determination of colony morphology, bacterial colonies reaching the stationary phase on the agar plates were observed. Cellular morphology including cell shape, presence of flagella, and cell size was examined by a transmission electron microscopy (CM200; Philips) after staining with 2% (w/v) uranyl acetate. Gram staining was performed using a Gram-staining kit (bioMérieux). Catalase and oxidase activities were examined using 3% hydrogen peroxide and oxidase reagent (bioMérieux), respectively. API 20NE galleries (bioMérieux) were employed for additional biochemical characterization. In API 20NE, the strains were tested according to the manufacturer's instructions, but 2% NaCl API AUX medium and 1% L-cysteine were added for anaerobic cultivation.

For determination of the phylogenetic position of the isolated strains, bacterial DNA extraction, PCR amplification, and 16S rRNA gene sequencing were performed according to the standard procedures as previously described (Yang and Cho, 2008). The 16S rRNA gene sequences were obtained using the primers 518F and 800R. The resultant 16S rRNA gene sequences were initially compared with those of other bacterial strains with the validly published names using the EzBioCloud (Yoon *et al*., 2017) and the NIBR database. The 16S rRNA gene sequence similarity of 98.7% was used as the cut-off value for bacterial species demarcation (Chun *et al*., 2018). Therefore, the bacterial strains exhibiting ≥98.7% 16S rRNA gene sequence similarities with the species that were validly published but have never reported in Korea. For determining the phylogenetic position, multiple se-

Table 1. Summary of strains isolated and their taxonomic affiliations.

Class	Order	Family	Strain ID	Accession number	Closest species	Similarity (%)	Source	Sampling site	Medium	Incubation condition
Actinomycetia	*Propionibacteriales*	*Propionibacteriaceae*	HNIBRBA1272	OQ186550	*Microlunatus spumicola*	99.10	Lichens	Wan-do	R2A	15°C, 14d
	Micrococcales	*Micrococcaceae*	HNIBRBA3048	OQ186555	*Zhihengliuella halotolerans*	99.86	Halophytes	Goha-do	MA	20°C, 7d
	Streptomycetale	*Streptomycetaceae*	HNIBRBA4518	OQ186559	*Streptomyces bobili*	99.26	Soil	Jeju-do	R2A	25°C, 7d
Alphaproteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	HNIBRBA1398	OQ186551	*Sphingomonas palmae*	99.15	Lichens	Anmyeon-do	R2A	15°C, 14d
	Rhodobacterales	*Rhodobacteraceae*	HNIBRBA1909	OQ186554	*Falsirhodobacter halotolerans*	99.11	Halophytes	Goha-do	MA	20°C, 7d
	Caulobacterales	*Caulobateraceae*	HNIBRBA4515	OQ186556	*Asticcacaulis excentricus*	99.49	Soil	Jeju-do	R2A	25°C, 7d
Gammaproteobacteria	*Alteromonadales*	*Colwelliaceae*	HNIBRBA1717	OQ186552	*Colwellia echini*	99.58	Sea water	Anmyeon-do	MA	20°C, 7d
	Oceanospirillales	*Halomonadaceeae*	HNIBRBA1879	OQ186553	*Halomonas radicis*	99.92	Halophytes	Amtae-do	MA	20°C, 7d
Chitinophagia	*Chitinophagales*	*Chitinophagaceae*	HNIBRBA4516	OQ186557	*Chitinophaga pinensis*	98.76	Soil	Jeju-do	R2A	25°C, 7d
Flavobacteriia	*Flavobacteriales*	*Weeksellaceae*	HNIBRBA4517	OQ186558	*Chryseobacterium bernardetii*	99.72	Soil	Jeju-do	R2A	25°C, 7d

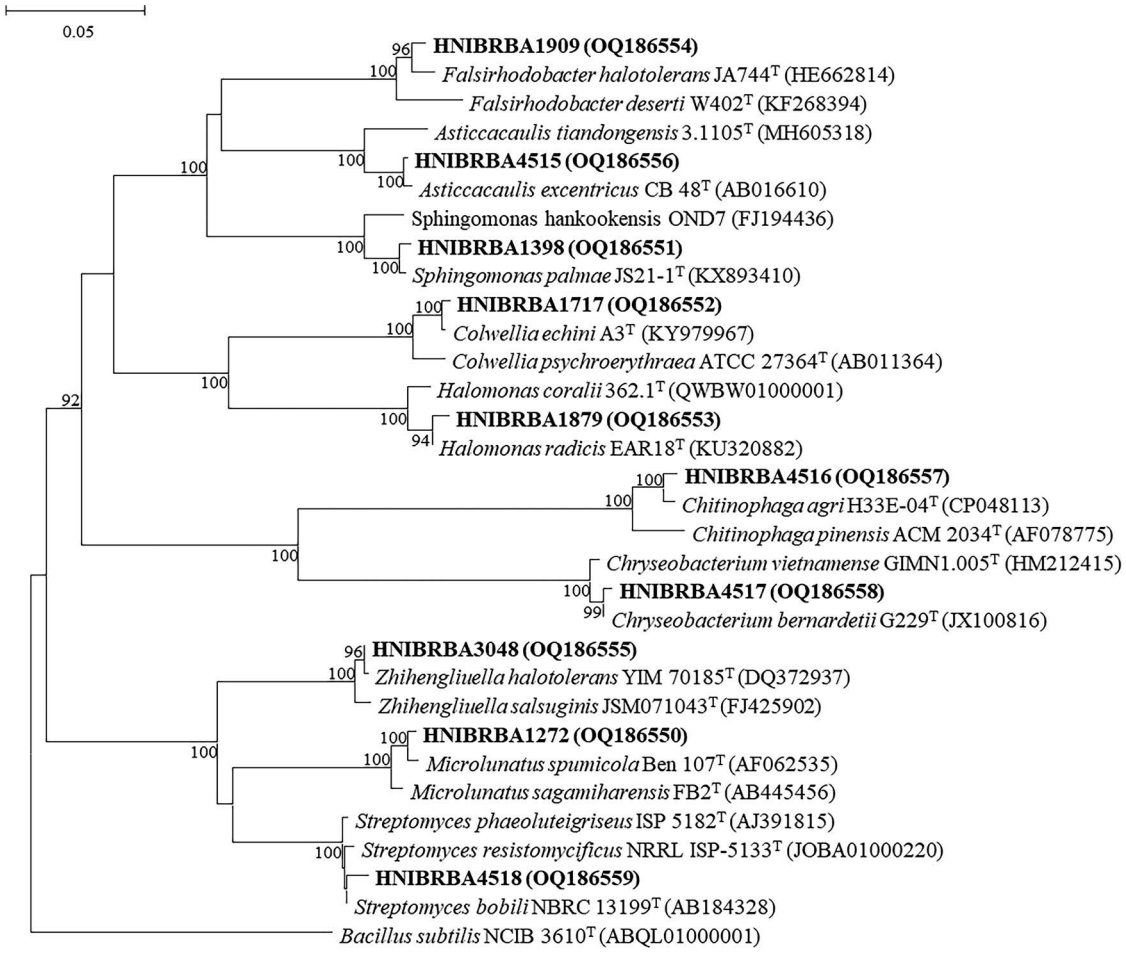

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their closest related bacterial species. Bootstrap values over 70% are shown. *Bacillus subtilis* NCIB 3610^T (ABQL01000001) was used to an outgroup. Bar, 0.05 substitutions per nucleotide position.

quence alignments between the 16S rRNA gene sequences of 10 strains and those of the unreported species were performed using ClustalW, which was implemented in MEGA X (Kumar *et al*., 2018). Using the unambiguously aligned 16S rRNA gene sequences, phylogenetic trees based on the neighbor-joining method were reconstructed (Saitou and Nei, 1987). The robustness of the inferred phylogenetic trees was evaluated by the bootstrap analyses based on 1,000 random re-samplings (Felsenstein, 1985).

Results and Discussion

The 16S rRNA gene sequence analyses on the approximately 1,200 bacterial strains obtained here revealed that many of the strains belong to novel species, or previously unreported species in Korea. Of these, 10 strains showed $\geq$98.7% 16S rRNA gene sequence similarities with unrecorded bacterial species in Korea. The strain information, identification, taxonomic assignment from species to classes, isolation source and sequence accession numbers including the HNIBR and GenBank are listed in Table 1. Phylogenetic assignment of the strains to the established bacterial species based on the 16S rRNA gene sequence similarity was confirmed by the phylogenetic tree analysis (Fig. 1). All strains identified as unrecorded species formed a robust clade with the type strains of each corresponding species with high bootstrap values.

The 10 unrecorded bacterial species were phylogenetically diverse, belonging to three phyla, five classes, 10 orders, 10 families, and 10 genera (Table 1). At the generic level, the unreported species were assigned to *Sphingomonas*, *Falsirhodobacter* and *Asticcacaulis* of the class *Alphaproteobacteria*; *Colwellia* and *Halomonas* of the class *Gammaproteobacteria*; *Chitinophaga* of the class *Chitinophagia*; *Chryseobacterium* of the class *Flavobacteriia*; *Microlunatus*, *Zhihengliuella*, and *Streptomyces* of the class *Actinomycetia*.

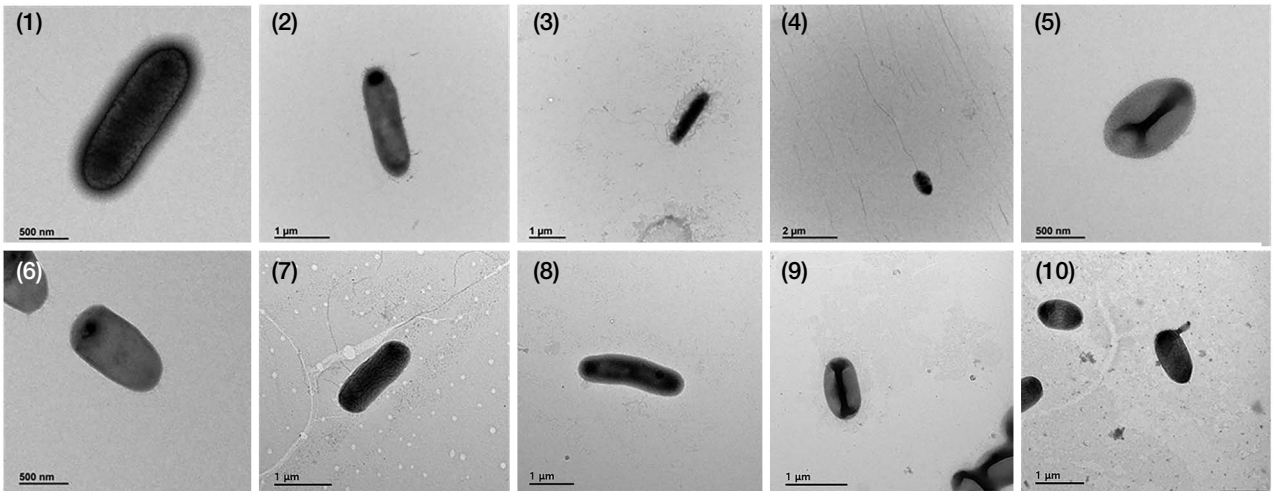

Fig. 2. Transmission electron micrographs of cells in this study. Strains: 1, HNIBRBA1272; 2, HNIBRBA1398; 3, HNIBRBA1717; 4, HNIBRBA1879; 5, HNIBRBA1909; 6, HNIBRBA3048; 7, HNIBRBA4515; 8, HNIBRBA4516; 9, HNIBRBA4517; 10, HNIBRBA4518.

The 10 unrecorded bacterial species observed in this study were Gram-staining-negative or positive, flagellated or non-flagellated, rod-shaped bacteria (Fig. 2). Detailed morphological, physiological, and biochemical characteristics of the unrecorded bacterial species are elucidated in the following strain descriptions.

Description of *Microlunatus spumicola* BN9

The cells are Gram-stain-positive, non-flagellated and rod shaped. The colonies are circular, convex, entire and yellow colored after incubation for 14 days on R2A agar at 15°C. Positive for arginine dihydrolase, urease, esculin hydrolysis, β-galactosidase and cytochrome oxidase; but negative for nitrates reduction, indole production, glucose fermentation and gelatin hydrolysis in API 20NE. D-Glucose, *N*-acetyl-glucosamine and D-maltose are utilized as sole carbon sources; but L-arabinose, D-mannose, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not. Strain BN9 (=HNIBRBA1272) was isolated from a lichen sample, Wan-do (34°23′17″N, 126°41′36″E), Jeollanam-do, Korea.

Description of *Sphingomonas palmae* BN246

The cells are Gram-stain-negative, non-flagellated and rod shaped. The colonies are circular, convex, entire and orange colored after incubation for 14 days on R2A agar at 15°C. Positive for arginine dihydrolase, urease, esculin hydrolysis, β-galactosidase and cytochrome oxidase; but negative for nitrates reduction, indole production, glucose fermentation and gelatin hydrolysis in API 20NE. D-Glucose, D-mannose, potassium gluconate, adipic acid and malic acid are utilized as sole carbon sources; but L-arabinose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, capric acid, trisodium citrate and phenylacetic acid are not. Strain BN246 (=HNIBRBA1398) was isolated from a lichen sample, Anmyeon-do (36°34′16″N, 126°18′50″E), Chungcheongnam-do, Korea.

Description of *Colwellia echini* YMSW20

The cells are Gram-stain-negative, flagellated and rod shaped. The colonies are circular, convex, mucoid and brown colored after incubation for seven days on MA at 20°C. Positive for nitrates reduction, esculin hydrolysis, β-galactosidase and cytochrome oxidase; but negative for indole production, glucose fermentation, arginine dihydrolase, urease and gelatin hydrolysis in API 20NE. D-Glucose, *N*-acetyl-glucosamine and adipic acid are utilized as sole carbon sources; but L-arabinose, D-mannose, D-mannitol, D-maltose, potassium gluconate, capric acid, malic acid, trisodium citrate and phenylacetic acid are not. Strain YMSW20 (=HNIBRBA1717) was isolated from a sea water sample, Anmyeon-do (36°24′5.23″N, 126°25′39.67″E), Chungcheongnam-do, Korea.

Description of *Halomonas radicis* JGD6

The cells are Gram-stain-negative, flagellated and rod shaped. The colonies are circular, convex, undulate and light orange colored after incubation for seven days on MA at 20°C. Positive for nitrates reduction and esculin hydrolysis; but negative for indole production, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis, β-galactosidase and cytochrome oxidase in API 20NE. D-Glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid and trisodium citrate are utilized as sole carbon

sources; but capric acid, adipic acid and phenylacetic acid are not. Strain JGD6 (=HNIBRBA1879) was isolated from a halophyte sample, Amtae-do (34°51′34.85″N, 126° 7′57.53″E), Jeollanam-do, Korea.

Description of *Falsirhodobacter halotolerans* GHBS15

The cells are Gram-stain-negative, non-flagellated and rod shaped. The colonies are circular, convex, entire and yellow colored after incubation for seven days on MA at 20°C. Positive for esculin hydrolysis; but negative for nitrates reduction, indole production, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis, β-galactosidase and cytochrome oxidase in API 20NE. D-Glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, malic acid and trisodium citrate are utilized as sole carbon sources; but potassium gluconate, capric acid, adipic acid and phenylacetic acid are not. Strain GHBS15 (=HNIBRBA1909) was isolated from a halophyte sample, Goha-do (34°45′50.19″N, 126°22′ 14.06″E), Mokpo-si, Jeollanam-do, Korea.

Description of *Zhihengliuella halotolerans* GHGM7

The cells are Gram-stain-positive, non-flagellated and rod shaped. The colonies are circular, opaque, approximately and cream colored after incubation for seven days on MA at 20°C. Positive for nitrates reduction, arginine dihydrolase, urease, esculin hydrolysis, gelatin hydrolysis and β-galactosidase; but negative for indole production, glucose fermentation and cytochrome oxidase in API 20NE. D-Glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, trisodium citrate and phenylacetic acid are utilized as sole carbon sources; but capric acid and adipic acid are not. Strain GHGM7 (=HNIBRBA3048) was isolated from a halophyte sample, Goha-do (34°45′50.19″N, 126°22′14.06″E), Mokpo-si, Jeollanam-do, Korea.

Description of *Asticcacaulis excentricus* L3J1-30

The cells are Gram-stain-negative, flagellated and rod shaped. The colonies are circular, slightly convex, glistening and cream colored after incubation for seven days on R2A agar at 25°C. Positive for urease, esculin hydrolysis, β-galactosidase and cytochrome oxidase; but negative for nitrates reduction, indole production, glucose fermentation, arginine dihydrolase and gelatin hydrolysis in API 20NE. D-Glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, D-maltose and malic acid are utilized as sole carbon sources; but D-mannitol, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not. Strain L3J1-30 (=HNIBRBA4515) was isolated from a soil sample, Seogwipo-si (33°19′32.92″N, 126°50′23.71″E), Jeju-do, Korea.

Description of *Chitinophaga pinensis* L3R2-3

The cells are Gram-stain-negative, non-flagellated and rod shaped. The colonies are circular, slightly convex, glistening and yellow colored after incubation for seven days on R2A agar at 25°C. Positive for indole production, arginine dihydrolase, urease, esculin hydrolysis, gelatin hydrolysis and β-galactosidase; but negative for nitrates reduction, glucose fermentation and cytochrome oxidase in API 20NE. L-Arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, adipic acid, malic acid and trisodium citrate are utilized as sole carbon sources; but D-glucose, potassium gluconate, capric acid and phenylacetic acid are not. Strain L3R2-3 (=HNIBRBA4516) was isolated from a soil sample, Seogwipo-si (33°19′ 32.92″N, 126°50′23.71″E), Jeju-do, Korea.

Description of *Chryseobacterium bernardetii* L3J1-11

The cells are Gram-stain-negative, flagellated and rod shaped. The colonies are circular, slightly convex, glistening and yellow colored after incubation for seven days on R2A agar at 25°C. Positive for arginine dihydrolase, urease, esculin hydrolysis, gelatin hydrolysis and cytochrome oxidase; but negative for nitrates reduction, indole production, glucose fermentation and β-galactosidase in API 20NE. Potassium gluconate and malic acid are utilized as sole carbon sources; but D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not. Strain L3J1-11 (=HNIBRBA4517) was isolated from a soil sample, Seogwipo-si (33°19′32.92″N, 126°50′ 23.71″E), Jeju-do, Korea.

Description of *Streptomyces bobili* L3T2-23

The cells are Gram-stain-positive, non-flagellated and rod shaped. The colonies are circular, slightly convex, entire and orange colored after incubation for seven days on R2A agar at 25°C. Aerial mycelium was absent on R2A agar. Positive for nitrates reduction, urease, esculin hydrolysis, gelatin hydrolysis and β-galactosidase; but negative for in API 20NE. D-Glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, potassium gluconate and malic acid are utilized as sole carbon sources; but D-mannitol, D-maltose, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not. Strain YMSW20 (=HNIBR BA4518) was isolated from the soil, Seogwipo-si (33°19′ 32.92″N, 126°50′23.71″E), Jeju-do, Korea.

ACKNOWLEDGEMENTS

This study was supported by the research grant, "Survey of Indigenous Species in Korean Islands" (HNIBR

202101111) from the Honam National Institute of Biological Resources of the Ministry of Environment in Korea.

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Submitted: April 6, 2023
Revised: May 19, 2023
Accepted: May 26, 2023