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ORIGINAL PAPER

Marine-derived *Penicillium* in Korea: diversity, enzyme activity, and antifungal properties

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Abstract The diversity of marine-derived *Penicillium* from Korea was investigated using morphological and multigene phylogenetic approaches, analyzing sequences of the internal transcribed spacer region, β-tubulin gene, and RNA polymerase subunit II gene. In addition, the biological activity of all isolated strains was evaluated. We tested for the extracellular enzyme activity of alginase, endoglucanase, and β-glucosidase, and antifungal activity against two plant pathogens (*Colletotrichum acutatum* and *Fusarium oxysporum*). A total of 184 strains of 36 *Penicillium* species were isolated, with 27 species being identified. The most common species were *Penicillium polonicum* (19.6 %), *P. rubens* (11.4 %), *P. chrysogenum* (11.4 %), and *P. crustosum* (10.9 %). The diversity of *Penicillium* strains isolated from soil (foreshore soil and sand) and marine macroorganisms was higher than the diversity of strains isolated from seawater. While many of the isolated strains showed alginase and β-glucosidase activity, no endoglucanase activity was found. More than half the strains (50.5 %) showed antifungal activity against at least one of the plant pathogens tested. Compared with other strains in this study, *P. citrinum* (strain SFC20140101-M662) showed high antifungal activity against both plant pathogens. The results reported here expand our knowledge of marine-derived *Penicillium* diversity. The relatively high proportion of strains that showed antifungal and enzyme activity demonstrates that marine-derived *Penicillium* have great potential to be used in the production of natural bioactive products for pharmaceutical and/or industrial use.

Keywords Marine-derived fungi · *Penicillium* · Multi-gene phylogenetic approach · Alginase · β-Glucosidase · Antifungal activity

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Introduction

Fungi are frequently found in marine environments on substrates such as plants, animals, mud, sand, and seawater. These fungi play important ecological roles as decomposers of organic material and symbionts or pathogens of other marine organisms (Hyde et al. 1998). Marine-derived fungi are not restricted to specific clades, but rather are found throughout the fungal tree of life, with an estimated 1,500 species (Hyde et al. 1998; Kohlmeyer and Kohlmeyer 1979).

These fungi can be categorized into two distinct groups based on a broad ecological definition: obligate and facultative marine fungi (Kohlmeyer and Kohlmeyer 1979). Obligate marine fungi grow and sporulate exclusively in marine environments and include *Asteromyces cruciatus*, *Dendriphiella* spp., and *Phialophorophoma litoralis* (Kohlmeyer and Volkmann-Kohlmeyer 1991; Khudyakova et al. 2000). Facultative marine fungi are terrestrial fungi that have adapted to marine environments and commonly belong to Ascomycota genera such as *Penicillium*, *Aspergillus*, *Trichoderma*, and *Cladosporium* (Khudyakova et al. 2000; Cantrell et al. 2006). The unique physiochemical conditions of marine environments result in marine-derived fungi producing a variety of novel bioactive compounds that have the potential to be applied to pharmaceutical and industrial uses (Jones 2000; Raghukumar 2008).

Many marine-derived fungi are found in the genus *Penicillium*. *Penicillium* species can be isolated from various substrates in outdoor and indoor environments (Pitt 1979; Samson et al. 2010), and inhabit extreme environments such as polar regions (Vishniac 1996; Ivanushkina et al. 2005), high altitude soils (Petrovič et al. 2000), and marine habitats (Kagata et al. 2000; Lin et al. 2000; Edrada et al. 2002). Numerous bioactive compounds have been isolated from *Penicillium* species, including mycotoxins, antibiotics, herbicides, antioxidants, insecticides, and anticancer compounds (Frisvad et al. 2004). In particular, marine-derived *Penicillium* species are known to be producers of secondary metabolites such as the anticancer compound citrinadin A, DNA polymerase inhibitors sculezonone-A and -B, and antifungal xestodecalactones A–C (Edrada et al. 2002; Komatsu et al. 2000; Tsuda et al. 2004). For example, *P. dravuni* isolated from algae exhibited antibacterial activity against methicillin-resistant *Staphylococcus aureus*—a bacterial strain that causes difficult-to-treat infections in humans (Bugni et al. 2004).

In Korea, approximately 100 *Penicillium* species have been recorded (Lee et al. 2003; Yu et al. 1997; Kim et al. 2009; Min et al. 2014). Many of these species were isolated from soil, and some were found to be associated with post-harvest diseases of plant products (Lee et al. 2003; Yu et al. 1997; Kim et al. 2009). However, the diversity of marine-derived *Penicillium* in Korea is poorly understood relative to terrestrial species. 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 current study forms part of the Ministry's long-term project to study marine-derived microbes in Korea, and had two main goals. First, we explored the diversity of marine-derived *Penicillium* in Korea by isolating *Penicillium* species from various marine substrates and identifying them using a multigene phylogenetic approach. In this approach, we sequenced three commonly used markers for species identification in *Penicillium*: the internal transcribed spacer region (ITS) (Peterson 2000), β-tubulin (benA) (Samson et al. 2004), and RNA polymerase subunit II (RPB2) (Houbraken and Samson 2011). Second, we evaluated the biological activity of the strains. Extracellular enzyme activity was evaluated in the presence of the carbon sources alginate, carboxymethyl cellulose, and cellobiose, and antifungal activity was evaluated against the plant pathogens *Colletotrichum acutatum* and *Fusarium oxysporum*.

Materials and methods

Materials studied

Soil (foreshore soil and sand), seawater, and macroorganisms (algae, crab, sponge, and mussel) were collected from seven sites in Korea between 2007 and 2011. Six of the collection sites were along the southern coast of Korea (Wando, Namhae, Geoje, Dadaepo, Ilgwang, and Jeju) and one site on the eastern coast (Uljin). Macroorganisms were processed for culturing by the addition of two volumes of sterile seawater followed by thorough homogenization using a blender. Before culturing, all samples were diluted tenfold with sterile seawater to reduce the density of colonies for improved strain recovery. For fungal cultures, 100 μL of each dilution was plated on potato dextrose agar [PDA; 4 g L^{-1} potato infusion (Difco-Becton, Sparks, MD, USA), 20 g L^{-1} glucose (Difco-Becton, Sparks, MD, USA), 18 g L^{-1} agar (Difco-Becton, Sparks, MD, USA), 750 mL L^{-1} seawater, 250 mL L^{-1} distilled water], yeast extract peptone glucose agar [5 g L^{-1} yeast extract (Difco-Becton, Sparks, MD, USA), 5 g L^{-1} peptone (Difco-Becton, Sparks, MD, USA), 10 g L^{-1} glucose, 18 g L^{-1} agar, 750 mL L^{-1} seawater, 250 mL L^{-1} distilled water], and glucose yeast

Table 1 Information of *Penicillium* strains isolated from marine environments and GenBank accession number

Section	Species	Representative strain	No. of isolate(s)	Number of fungal strains			Accession number		
				Soil	Seawater	Macroorganisms	ITS	benA	RPB2
Aspergilloides	*P. glabrum*	SFC20140101-M828	11	2	1	8	KJ527446	KJ527411	KJ527376
	Penicillium sp. 5	SFC20140101-M756	3	1	0	2	KF818464	KF818461	KF818467
Brevicompacta	*P. bialowiezense*	SFC20140101-M712	2	0	0	2	KJ527438	KJ527403	KJ527368
	P. brevicompactum	SFC20140101-M641	2	1	0	1	KJ527439	KJ527404	KJ527369
Canescentia	*P. antarcticum*	SFC20140101-M746	10	0	1	9	KJ527436	KJ527401	KJ527366
	P. yarmokense	SFC20140101-M833	1	1	0	0	KJ527468	KJ527433	KJ527398
	Penicillium sp. 6	SFC20140101-M744	1	1	0	0	KJ527463	KJ527428	KJ527393
Chrysogena	*P. aethiopicum*	SFC20140101-M675	2	1	1	0	KJ527434	KJ527399	KJ527364
	P. chrysogenum	SFC20140101-M647	21	9	5	7	KJ527440	KJ527405	KJ527370
	P. rubens	SFC20140101-M682	21	11	1	9	KJ527454	KJ527419	KJ527384
	Penicillium sp. 4	SFC20140101-M777	3	2	0	1	KJ527457	KJ527422	KJ527387
Citrina	*P. citrinum*	SFC20140101-M662	12	8	3	1	KJ527441	KJ527406	KJ527371
	P. sumatrense	SFC20140101-M747	4	4	0	0	KJ527465	KJ527430	KJ527395
Digitata	*P. digitatum*	SFC20140101-M667	3	0	1	2	KJ527443	KJ527408	KJ527373
	Penicillium sp. 3	SFC20140101-M836	1	0	0	1	KJ527464	KJ527429	KJ527394
Exilicaulis	*P. rubefaciens*	SFC20140101-M799	2	1	1	0	KJ527453	KJ527418	KJ527383
	Penicillium sp. 7	SFC20140101-M762	1	1	0	0	KJ527458	KJ527423	KJ527388
Fasciculata	*P. allii*	SFC20140101-M742	2	2	0	0	KJ527435	KJ527400	KJ527365
	P. crustosum	SFC20140101-M781	20	7	2	11	KJ527442	KJ527407	KJ527372
	P. freii	SFC20140101-M815	2	0	0	2	KJ527445	KJ527410	KJ527375
	P. nordicum	SFC20140101-M802	3	2	0	1	KJ527448	KJ527413	KJ527378
	P. polonicum	SFC20140101-M733	36	21	8	7	KJ527451	KJ527416	KJ527381
	P. radicicola	SFC20140101-M697	1	1	0	0	KJ527452	KJ527417	KJ527382
	P. solitum	SFC20140101-M780	2	2	0	0	KJ527455	KJ527420	KJ527385
	Penicillium sp. 8	SFC20140101-M767	2	1	1	0	KJ527456	KJ527421	KJ527386
Lanata-divaricata	*P. oxalicum*	SFC20140101-M839	1	0	0	1	KJ527449	KJ527414	KJ527379
	P. daleae	SFC20140101-M660	1	0	0	1	KJ527459	KJ527424	KJ527389
	P. raperi	SFC20140101-M477	1	1	0	0	KJ527462	KJ527427	KJ527392
	Penicillium sp. 9	SFC20140101-M639	1	0	0	1	KJ527450	KJ527415	KJ527380
Paradoxa	*P. atramentosum*	SFC20140101-M769	4	0	2	2	KJ527437	KJ527402	KJ527367
Penicillium	*P. expansum*	SFC20140101-M737	2	1	0	1	KJ527444	KJ527409	KJ527374

Table 1 continued

Section	Species	Representative strain	No. of isolate(s)	Number of fungal strains			Accession number		
				Soil	Seawater	Macroorganisms	ITS	benA	RPB2
	P. italicum	SFC20140101-M724	2	0	1	1	KJ527447	KJ527412	KJ527377
	P. ulaiense	SFC20140101-M717	1	0	0	1	KJ527466	KJ527431	KJ527396
	Penicillium sp. 2	SFC20140101-M734	1	1	0	0	KJ527461	KJ527426	KJ527391
Ramosa	*P. virgatum*	SFC20140101-M659	1	0	0	1	KJ527467	KJ527432	KJ527397
	Penicillium sp. 1	SFC20140101-M830	1	0	1	0	KJ527460	KJ527425	KJ527390
	Total species/strains		36/184	23/82	14/29	23/73			

Strain numbers are from the Seoul National University Fungal Collection (SFC)

extract agar (0.1 g L^{-1} yeast extract, 5 g L^{-1} glucose, 18 g L^{-1} agar, 750 mL L^{-1} seawater, 250 mL L^{-1} distilled water). The plates were incubated at 25 °C for 7–15 days until the morphology of the culture could be distinguished, and then each *Penicillium* strain was picked and transferred onto a new PDA plate. The strains isolated in this study were stored in 20 % glycerol at −80 °C in the Seoul National University Fungus Collection (SFC) (Table 1).

DNA extraction and PCR amplification

A small amount of fungal material was placed in a 2× cetyltrimethylammonium bromide buffer and ground with a plastic pestle. Genomic DNA was extracted using the modified extraction protocol published by Rogers and Bendich (1994). The PCR amplifications of ITS, benA, and RPB2 were performed using primers ITS1F and ITS4 (White et al. 1990), Bt2a and Bt2b (Glass and Donaldson 1995), and RPB2-5F_Eur and RPB2-7CR_Eur (Houbraken and Samson 2011), respectively. Each PCR reaction was performed on a C1000^{TM} thermal cycler (Bio-Rad, Richmond, CA, USA) using Maxime PCR PreMix with StarTaq^{TM} (Intron Biotechnology Inc., Seoul, Korea) in a final volume of 20 μL, containing 10 pmol of each primer and 1 μL of DNA (10 ng μL^{-1}). PCR amplification of each gene was performed as described in Park et al. (2013). PCR products were electrophoresed through a 1 % agarose gel stained with loading STAR (Dyne Bio, Seoul, Korea) and purified using the Expin^{TM} PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

DNA sequencing using the appropriate PCR primers for each gene was performed at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea) using an ABI Prism 3700 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and proofread using MEGA 5 (Tamura et al. 2011). The resulting consensus sequences were deposited in GenBank (accession numbers in Table 1). Multiple DNA sequence alignments were performed using the default settings of MAFFT v7 (Katoh and Standley 2013) and were checked by eye, with ambiguously aligned positions adjusted

manually. Maximum Likelihood (ML) phylogenetic analyses were conducted with RAxML 8.0.2 (Stamatakis 2014) using the GTRGAMMA model of evolution and 1,000 bootstrap replicates.

Identification

We combined molecular and morphological methods to identify marine-derived *Penicillium* species. For molecular analyses, we compared ITS, benA, and RPB2 sequences to type strains available on the RefSeq database of GenBank (release 64), supplemented with BLAST searches of Genbank (type and non-type strains) and the CBS *Penicillium* database (http://www.cbs.knaw.nl/Collections). ITS was sequenced for all strains, and phylogenetic analysis was performed to determine the species clusters. From each ITS group, 1–10 representatives were selected for benA and RPB2 sequencing and subsequent phylogenetic analyses. Molecular identification of species was done using a section-by-section approach. Preliminary phylogenetic analyses were performed for each gene to determine the *Penicillium* section for the unknown strains. Next, for each gene, separate phylogenetic analyses were done for each section.

For morphological analyses, all strains were inoculated at three points onto Czapek yeast extract agar (CYA) and malt extract agar (MEA, Oxoid, UK), then incubated at 25 °C for 7 days. Additional mounts of unidentifiable strains were made in lactic acid from MEA colonies. Microscopic observations were made using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan).

Enzyme and antifungal activity assays

Extracellular alginase, endoglucanase, and β-glucosidase activities were assessed for each strain using plate screening methods, in which enzyme activity was identified by the formation of a clear zone surrounding the colony (Gacesa and Wusteman 1990). 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 (Kim et al. 2010). 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 (Pointing 1999). After incubation for 5 days, the plates were flooded with 0.5 % Congo red (Sigma-Aldrich) for 10 min, and this 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 (Yoon et al. 2007). Next, the plates were flooded with 0.5 % Congo red (Sigma-Aldrich) for 10 min, and this was then replaced by 1 M NaCl.

To test for antifungal activity, strains were screened using a dual culture method with two plant pathogens, *C. acutatum* (SFC20130816-01) and *F. oxysporum* (SFC20130816-02). Mycelial disks (5 mm diameter) from 5- to 7-day-old cultures were inoculated at three points on PDA, after which the pathogen was placed on the center of the plate and incubated at 25 °C for 5–10 days. Antifungal activity was assessed by measuring the inhibition zone (Paul et al. 2012), and all dual culture experiments were performed in triplicate.

Results

A total of 184 *Penicillium* strains were isolated from three different marine substrates (soil, seawater, and macroorganisms) at seven sites in South Korea. Of all regions assessed, Jeju had the largest number of strains (83), followed by Wando (51), Ilgwang (24), and Geoje (22) (Fig. 1). The most strains were isolated from soil (82), followed by macroorganisms (73), and seawater (29) (Table 1).

Marine-derived *Penicillium* identification

ITS sequence analysis and morphological comparisons were performed on all of the 184 *Penicillium* strains, resulting in 36 groups. Representatives of each species group were selected for benA and RPB2 sequencing, with a total of 96 and 36 strains chosen, respectively. Comparing results from the three markers, 27 groups were confidently identified to the species level (Figs. 2, 3, 4). The species identification recovered from section-by-section phylogenetic analyses was identical to results from the complete datasets, so we only show phylogenies from the complete datasets. The remaining nine groups could

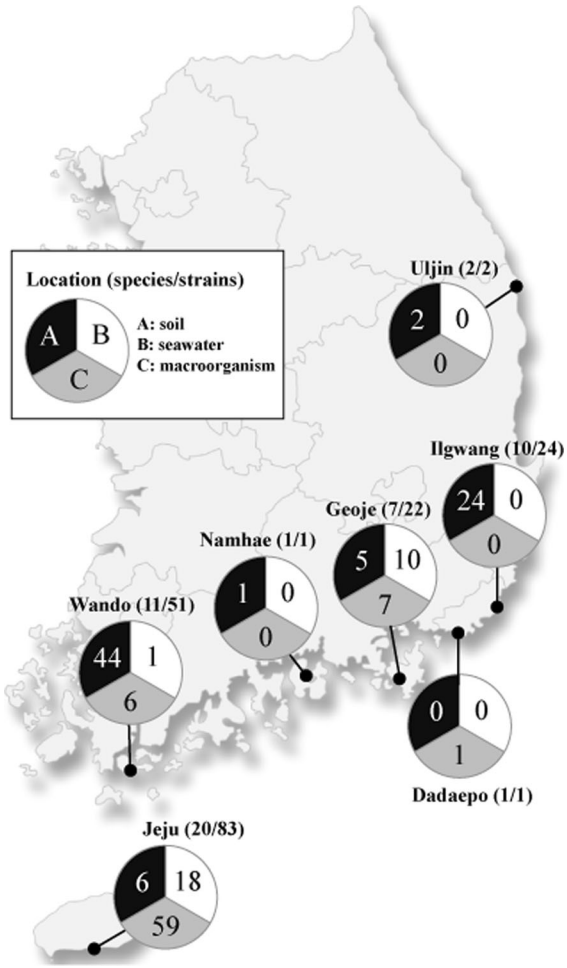


Fig. 1 Map showing the location of the sampling sites along the southern and eastern coasts of Korea. Circles for each site indicate the number of the *Penicillium* strains from *A* soil, *B* seawater, and *C* macroorganism

not be confidently identified because of unclear relationships in the phylogenies and the absence of distinguishing morphological characteristics. These groups were designated *Penicillium* spp. 1–9. Since some *Penicillium* type strains have not been sequenced, we cannot determine whether these unidentified taxa are new species or conspecific with known species with no available DNA data. Additional examination will be required to identify these unknown taxa.

Diversity comparisons

Penicillium polonicum (19.6 %) was the dominant species, followed by *Penicillium rubens* (11.4 %), *Penicillium chrysogenum* (11.4 %), and *Penicillium crustosum* (10.9 %) (Table 1). Of the locations surveyed, Jeju had the highest *Penicillium* diversity (20 species), followed by Wando (11 species), Ilgwang (10 species), and Geoje (7 species) (Fig. 1). The remaining three sites, Uljin, Namhae, and Dadaepo, exhibited extremely low fungal diversity, with only one or two species isolated from each site (Fig. 1).

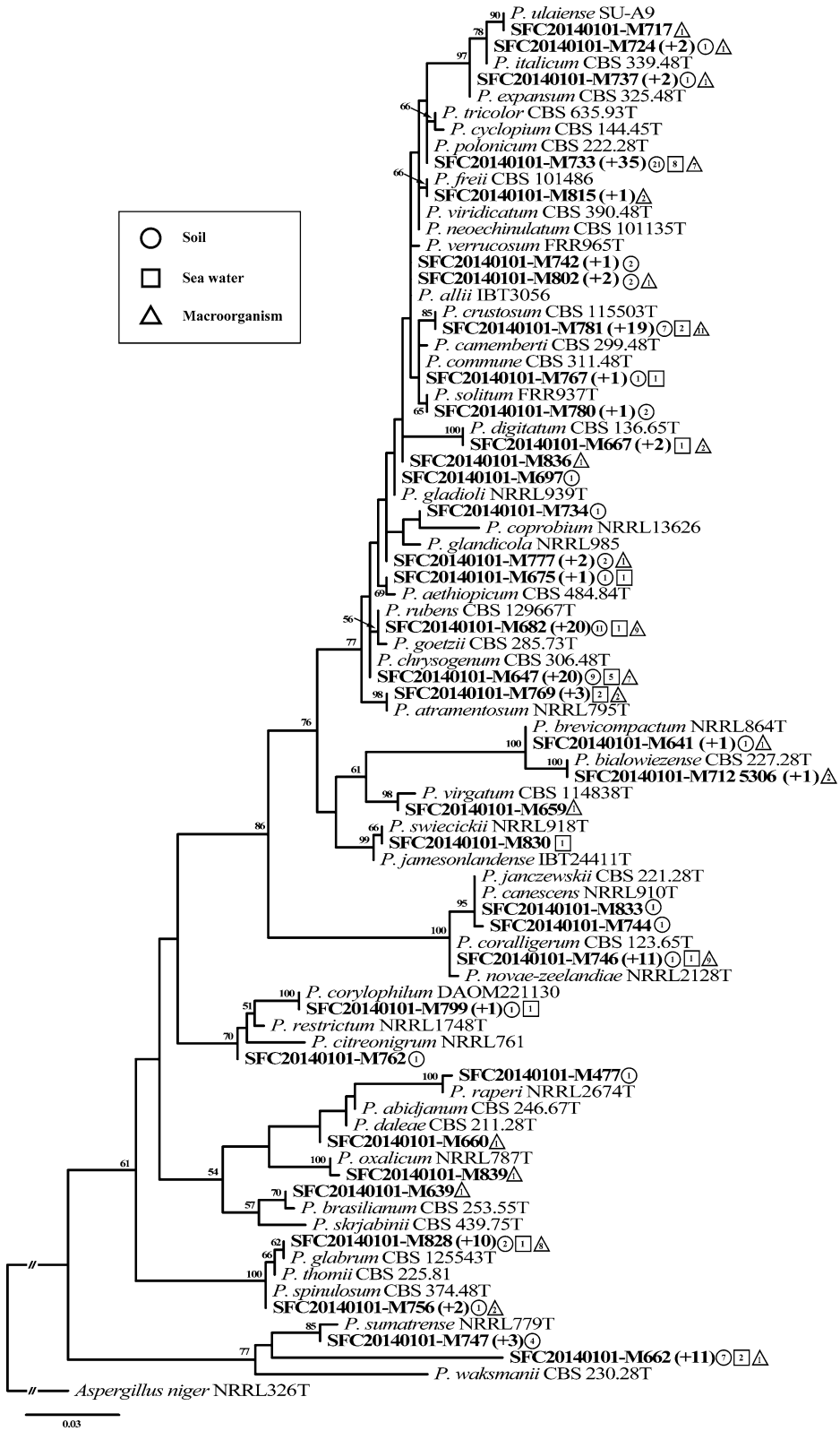
The number of species isolated from each substrate differed, with *Penicillium* diversity being higher in soil and macroorganisms (23 species each) compared with seawater (14 species). Six species were shared across the three substrates: *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. glabrum*, *P. polonicum*, and *P. rubens*. Furthermore, unique species were isolated from each of the substrates—nine from soil, eight from macroorganisms, and one from seawater (Table 1). *Penicillium citrinum* and *P. polonicum* were more commonly isolated from soil, whereas *P. antarcticum* and *P. glabrum* were more commonly isolated from macroorganisms. *P. chrysogenum* was commonly isolated from all three substrates.

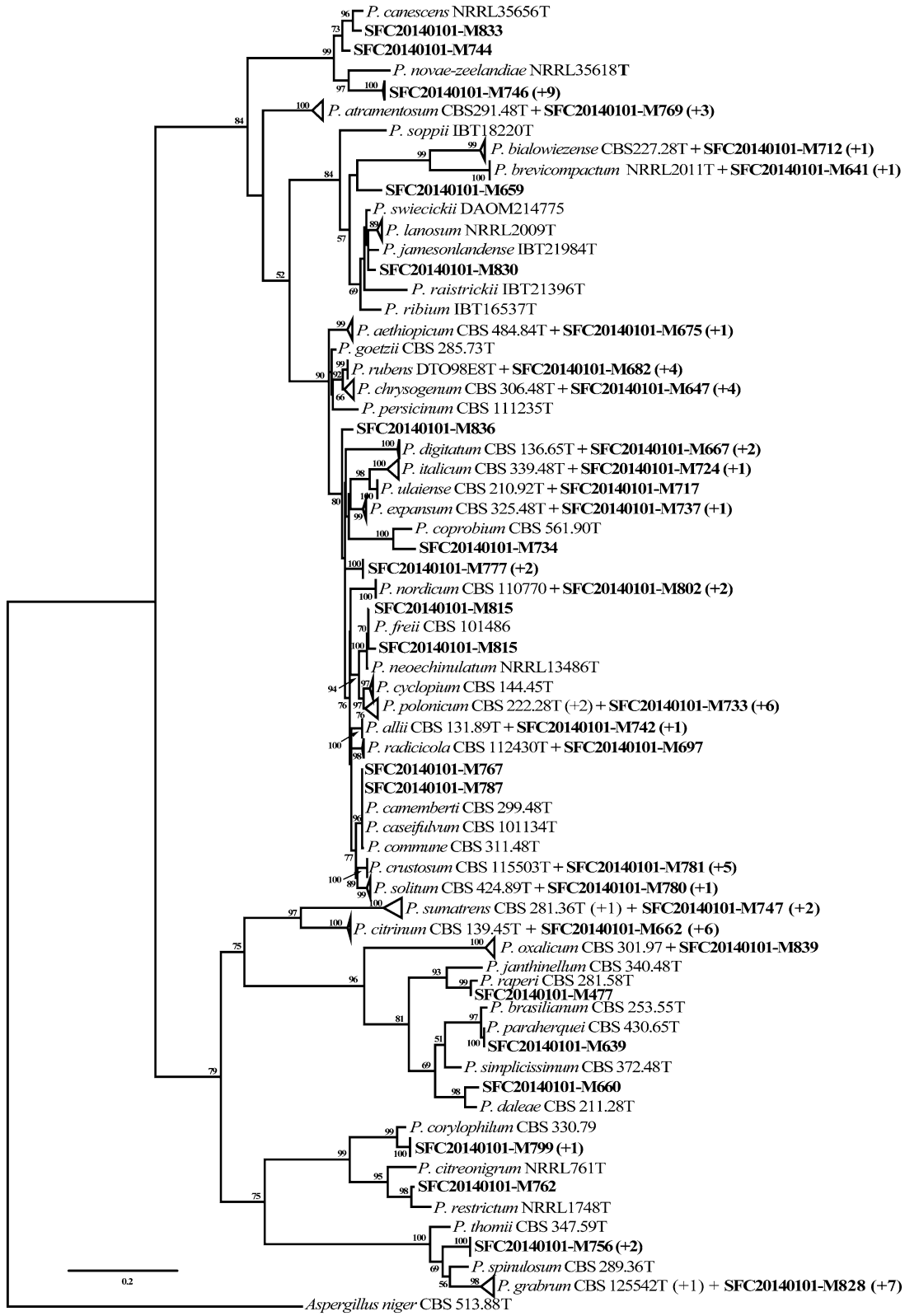
Fig. 2 Phylogenetic tree for *Penicillium* and related species based on ML analysis of the ITS. Bootstrap scores are presented at the nodes only if >50. The *scale bar* indicates the number of nucleotide substitutions per site and the *letter T* indicates ex-type strains. Phylogeny has been pruned of distantly-related taxa to simplify viewing of the results ▶

Enzyme and antifungal activity

All strains were screened for the extracellular enzyme activity of alginase, endoglucanase, and β-glucosidase (Tables 2, 3). Twenty-two strains of 11 species showed alginase activity and 132 strains of 27 species demonstrated β-glucosidase activity, but no endoglucanase activity was identified for any strain. *Penicillium chrysogenum* (strain SFC20140101-M797) showed the strongest alginase activity; its clear zone was approximately double the size of those formed by the other strains (Table 3). *Penicillium oxalicum* (strain SFC20140101-M839) and *Penicillium* sp. 5 (strains SFC20140101-M820, SFC20140101-M847, and SFC20140101-M756) showed strong β-glucosidase activity, with clear zones approximately 20 mm in diameter (Table 3).

All strains were screened for antifungal activity against the plant pathogens *C. acutatum* and *F. oxysporum* (Tables 2, 3; Fig. 5). Among them, 90 strains of 32


Soil
Sea water
Macroorganism
P. ulaiense SU-A9
SFC20140101-M717
SFC20140101-M724 (+2)
P. italicum CBS 339.48T
SFC20140101-M737 (+2)
P. expansum CBS 325.48T
P. tricolor CBS 635.93T
P. cyclopium CBS 144.45T
P. polonicum CBS 222.28T
SFC20140101-M733 (+35)
P. freii CBS 101486
SFC20140101-M815 (+1)
P. viridicatum CBS 390.48T
P. neoechinulatum CBS 101135T
P. verrucosum FRR965T
SFC20140101-M742 (+1)
SFC20140101-M802 (+2)
P. allii IBT3056
P. crustosum CBS 115503T
SFC20140101-M781 (+19)
P. camemberti CBS 299.48T
P. commune CBS 311.48T
SFC20140101-M767 (+1)
P. solitum FRR937T
SFC20140101-M780 (+1)
P. digitatum CBS 136.65T
SFC20140101-M667 (+2)
SFC20140101-M836
SFC20140101-M697
P. gladioli NRRL939T
SFC20140101-M734
P. coprobium NRRL13626
P. glandicola NRRL985
SFC20140101-M777 (+2)
SFC20140101-M675 (+1)
P. aethiopicum CBS 484.84T
P. rubens CBS 129667T
SFC20140101-M682 (+20)
P. goetzii CBS 285.73T
P. chrysogenum CBS 306.48T
SFC20140101-M647 (+20)
SFC20140101-M769 (+3)
P. atramentosum NRRL795T
P. brevicompactum NRRL864T
SFC20140101-M641 (+1)
P. bialowiezense CBS 227.28T
SFC20140101-M712 5306 (+1)
P. virgatum CBS 114838T
SFC20140101-M659
P. swiecickii NRRL918T
SFC20140101-M830
P. jamesonlandense IBT24411T
P. janczewskii CBS 221.28T
P. canescens NRRL910T
SFC20140101-M833
SFC20140101-M744
P. coralligerum CBS 123.65T
SFC20140101-M746 (+11)
P. novae-zeelandiae NRRL2128T
P. corylophilum DAOM221130
SFC20140101-M799 (+1)
P. restrictum NRRL1748T
P. citreonigrum NRRL761
SFC20140101-M762
SFC20140101-M477
P. raperi NRRL2674T
P. abidjanum CBS 246.67T
P. daleae CBS 211.28T
SFC20140101-M660
P. oxalicum NRRL787T
SFC20140101-M839
SFC20140101-M639
P. brasilianum CBS 253.55T
P. skrjabinii CBS 439.75T
SFC20140101-M828 (+10)
P. glabrum CBS 125543T
P. thomii CBS 225.81
P. spinulosum CBS 374.48T
SFC20140101-M756 (+2)
P. sumatrense NRRL779T
SFC20140101-M747 (+3)
SFC20140101-M662 (+11)
P. waksmanii CBS 230.28T
Aspergillus niger NRRL326T
0.03


P. canescens NRRL35656T
SFC20140101-M833
SFC20140101-M744
P. novae-zeelandiae NRRL35618T
SFC20140101-M746 (+9)
P. atramentosum CBS291.48T + SFC20140101-M769 (+3)
P. soppii IBT18220T
P. bialowiezense CBS227.28T + SFC20140101-M712 (+1)
P. brevicompactum NRRL2011T + SFC20140101-M641 (+1)
SFC20140101-M659
P. swiecickii DAOM214775
P. lanosum NRRL2009T
P. jamesonlandense IBT21984T
SFC20140101-M830
P. raistrickii IBT21396T
P. ribium IBT16537T
P. aethiopicum CBS 484.84T + SFC20140101-M675 (+1)
P. goetzii CBS 285.73T
P. rubens DTO98E8T + SFC20140101-M682 (+4)
P. chrysogenum CBS 306.48T + SFC20140101-M647 (+4)
P. persicinum CBS 111235T
SFC20140101-M836
P. digitatum CBS 136.65T + SFC20140101-M667 (+2)
P. italicum CBS 339.48T + SFC20140101-M724 (+1)
P. ulaiense CBS 210.92T + SFC20140101-M717
P. expansum CBS 325.48T + SFC20140101-M737 (+1)
P. coprobium CBS 561.90T
SFC20140101-M734
SFC20140101-M777 (+2)
P. nordicum CBS 110770 + SFC20140101-M802 (+2)
SFC20140101-M815
P. freii CBS 101486
SFC20140101-M815
P. neoechinulatum NRRL13486T
P. cyclopium CBS 144.45T
P. polonicum CBS 222.28T (+2) + SFC20140101-M733 (+6)
P. allii CBS 131.89T + SFC20140101-M742 (+1)
P. radicicola CBS 112430T + SFC20140101-M697
SFC20140101-M767
SFC20140101-M787
P. camemberti CBS 299.48T
P. caseifulvum CBS 101134T
P. commune CBS 311.48T
P. crustosum CBS 115503T + SFC20140101-M781 (+5)
P. solitum CBS 424.89T + SFC20140101-M780 (+1)
P. sumatrens CBS 281.36T (+1) + SFC20140101-M747 (+2)
P. citrinum CBS 139.45T + SFC20140101-M662 (+6)
P. oxalicum CBS 301.97 + SFC20140101-M839
P. janthinellum CBS 340.48T
P. raperi CBS 281.58T
SFC20140101-M477
P. brasilianum CBS 253.55T
P. paraherquei CBS 430.65T
SFC20140101-M639
P. simplicissimum CBS 372.48T
SFC20140101-M660
P. daleae CBS 211.28T
P. corylophilum CBS 330.79
SFC20140101-M799 (+1)
P. citreonigrum NRRL761T
SFC20140101-M762
P. restrictum NRRL1748T
P. thomii CBS 347.59T
SFC20140101-M756 (+2)
P. spinulosum CBS 289.36T
P. grabrum CBS 125542T (+1) + SFC20140101-M828 (+7)
Aspergillus niger CBS 513.88T
0.2

◀ **Fig. 3** Phylogenetic tree for *Penicillium* and related species based on ML analysis of β-tubulin (benA). 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

species exhibited antifungal activity against *C. acutatum*. *Penicillium bialowiezense* (strain SFC20140101-M642), *P. citrinum* (strains SFC20140101-M492, SFC20140101-M490, and SFC20140101-M662), *Penicillium* sp. 1 (strain SFC20140101-M830), and *Penicillium* sp. 4 (strains SFC20140101-M680 and SFC20140101-M810) showed relatively strong antifungal activity, with inhibition zones ≥5 mm (Table 3). Forty-one strains of 21 species showed antifungal activity against *F. oxysporum*. *Penicillium citrinum* (strain SFC20140101-M662) and *P. freii* (strain SFC20140101-M754) showed the strongest antifungal activity against *F. oxysporum* of all strains tested, with inhibition zones of 4 mm (Table 3).

Discussion

Penicillium diversity

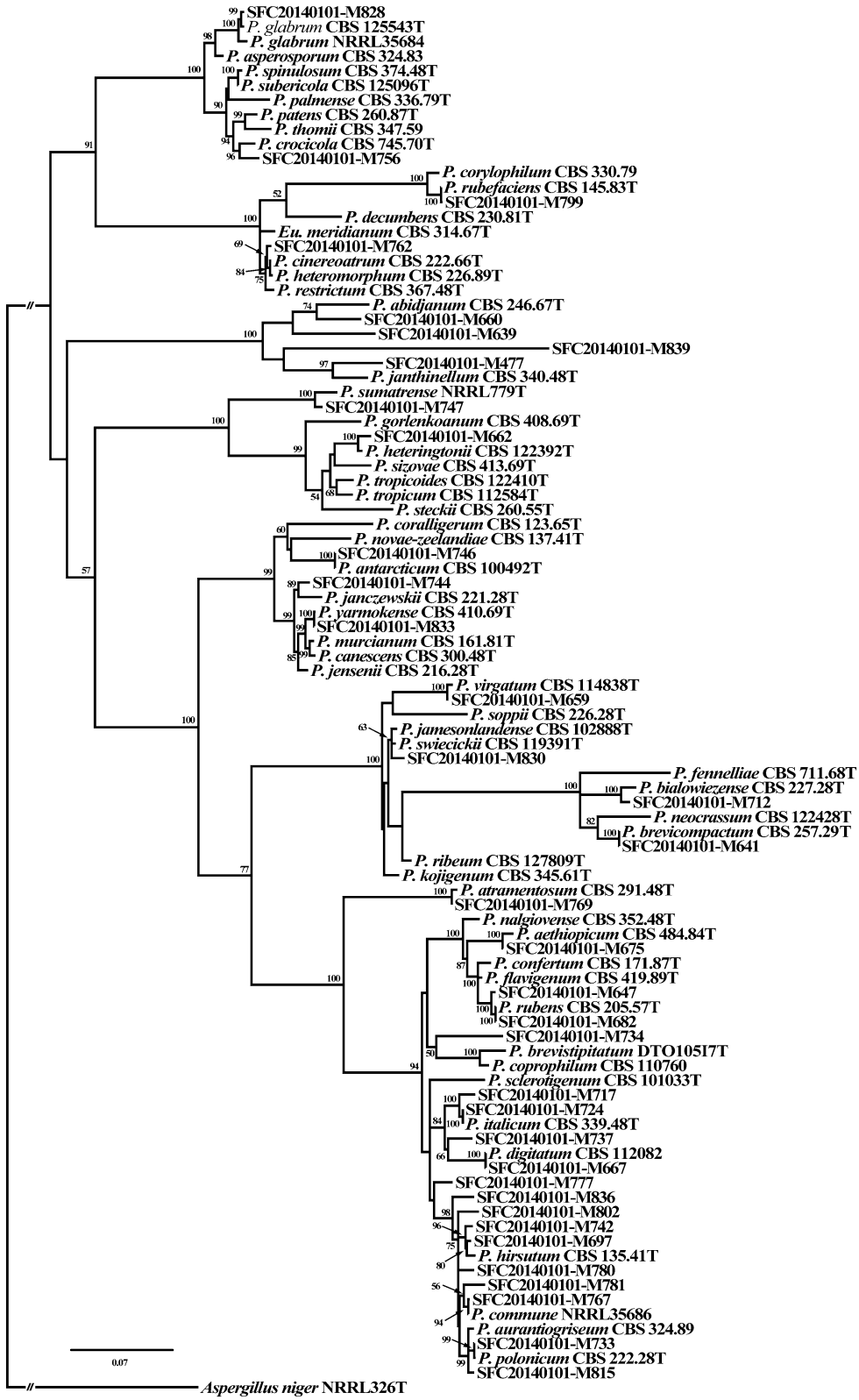
A total of 184 strains of 36 *Penicillium* species were identified in this study. Based on data from three independent molecular markers, 27 species were highly similar to type strains and were identified as such. The remaining species differed from the available type strain data, and may be new species or known, but unsequenced *Penicillium* species.

This study increases our knowledge of marine-derived *Penicillium* diversity and Korean *Penicillium* species in general. The published literature on marine-derived *Penicillium* indicates that approximately 30 species have been isolated from marine environments (Sonjak et al. 2006; Burtseva et al. 2010; Paz et al. 2010; Ding et al. 2011; Singh et al. 2012; Zhang et al. 2012). Only six species are shared between our work and these previous studies; our study identified 30 additional marine-derived *Penicillium* species, approximately doubling the known marine-derived *Penicillium* diversity. With respect to *Penicillium* diversity in Korea, five species (*P. nordicum*, *P. rubifaciens*, *P. rubens*, *P. virgatum*, and *P. yarmokense*) are new distribution records. These records increase the known *Penicillium* diversity in Korea to approximately 104 species. This discovery of several new distribution records in Korea and potential new *Penicillium* species lead us to believe that marine-derived fungal diversity is largely underestimated and is in need of further investigation.

The diversity of species and strains is unequal across sites within Korea. Sites such as Jeju and Wando have relatively high diversity, while sites such as Namhae, Dadaepo, and Uljin have extremely low diversity (Fig. 1). Our results do not show any clear pattern of *Penicillium* diversity in relation to geography. However, our data do reveal that the number of strains and species isolated from soil and macroorganisms were much greater than from seawater (Table 1). Furthermore, we did not observe clear substrate specificity for any particular species. In general, the rare species (1–2 strains) were isolated from a single substrate, whereas the more common species tended to be found on multiple substrate types. Whether the rare species are truly exhibiting substrate specificity or this pattern is the result of incomplete sampling will need to be tested with a larger dataset that compares multiple substrates.

Biological activity of the strains

Members of the genus *Penicillium* are known for producing numerous bioactive compounds (Frisvad et al. 2004). Therefore, we tested the extracellular enzyme and antifungal activity of the strains isolated in this study. Extracellular enzymes produced by *Penicillium* species can aid in the degradation of various compounds (Burtseva et al. 2010; Dubrovskaya et al. 2012). In the present study, we screened for alginase, β-glucosidase, and endoglucanase. Alginase is important for the degradation of alginate, a compound commonly found in seaweed, and the activity of this enzyme has the potential to be useful in the production of bioenergy and biofunctional oligosaccharides (Kim et al. 2010). Alginases are produced by a variety of sources including marine and soil-derived bacteria (Doubet and Quatrano 1982) and marine-derived fungi such as *Corollospora intermedia*, *A. cruciatus*, and *Dendryphiella salina* (Schaumann and Weide 1990). Presently, there are only two *Penicillium* species reported to exhibit alginase activity: *P. canescens* (Dubrovskaya et al. 2012) and *P. cyaneum* (Burtseva et al. 2010). In our study, 22 strains of 11 species demonstrated alginase activity (Tables 2 and 3), with *P. chrysogenum* (strain


SFC20140101-M828
P. glabrum CBS 125543T
P. glabrum NRRL35684
P. asperosporum CBS 324.83
P. spinulosum CBS 374.48T
P. subericola CBS 125096T
P. palmense CBS 336.79T
P. patens CBS 260.87T
P. thomii CBS 347.59
P. crocicola CBS 745.70T
SFC20140101-M756
P. corylophilum CBS 330.79
P. rubefaciens CBS 145.83T
SFC20140101-M799
P. decumbens CBS 230.81T
Eu. meridianum CBS 314.67T
SFC20140101-M762
P. cinereoatrum CBS 222.66T
P. heteromorphum CBS 226.89T
P. restrictum CBS 367.48T
P. abidjanum CBS 246.67T
SFC20140101-M660
SFC20140101-M639
SFC20140101-M839
SFC20140101-M477
P. janthinellum CBS 340.48T
P. sumatrense NRRL779T
SFC20140101-M747
P. gorlenkoanum CBS 408.69T
SFC20140101-M662
P. heteringtonii CBS 122392T
P. sizovae CBS 413.69T
P. tropicoides CBS 122410T
P. tropicum CBS 112584T
P. steckii CBS 260.55T
P. coralligerum CBS 123.65T
P. novae-zeelandiae CBS 137.41T
SFC20140101-M746
P. antarcticum CBS 100492T
SFC20140101-M744
P. janczewskii CBS 221.28T
P. yarmokense CBS 410.69T
SFC20140101-M833
P. murcianum CBS 161.81T
P. canescens CBS 300.48T
P. jensenii CBS 216.28T
P. virgatum CBS 114838T
SFC20140101-M659
P. soppii CBS 226.28T
P. jamesonlandense CBS 102888T
P. swieckii CBS 119391T
SFC20140101-M830
P. fennelliae CBS 711.68T
P. bialowiezense CBS 227.28T
SFC20140101-M712
P. neocrassum CBS 122428T
P. brevicompactum CBS 257.29T
SFC20140101-M641
P. ribeum CBS 127809T
P. kojigenum CBS 345.61T
P. atramentosum CBS 291.48T
SFC20140101-M769
P. nalgiovense CBS 352.48T
P. aethiopicum CBS 484.84T
SFC20140101-M675
P. confertum CBS 171.87T
P. flavigenum CBS 419.89T
SFC20140101-M647
P. rubens CBS 205.57T
SFC20140101-M682
SFC20140101-M734
P. brevistipitatum DTO105I7T
P. coprophilum CBS 110760
P. sclerotigenum CBS 101033T
SFC20140101-M717
SFC20140101-M724
P. italicum CBS 339.48T
SFC20140101-M737
P. digitatum CBS 112082
SFC20140101-M667
SFC20140101-M777
SFC20140101-M836
SFC20140101-M802
SFC20140101-M742
SFC20140101-M697
P. hirsutum CBS 135.41T
SFC20140101-M780
SFC20140101-M781
SFC20140101-M767
P. commune NRRL35686
P. aurantiogriseum CBS 324.89
SFC20140101-M733
P. polonicum CBS 222.28T
SFC20140101-M815
0.07
Aspergillus niger NRRL326T

◀ **Fig. 4** Phylogenetic tree for *Penicillium* and related species based on ML analysis of RNA polymerase subunit II (RPB2). Bootstrap scores 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

SFC20140101-M797) showing especially strong activity (Table 3).

β-Glucosidase and endoglucanase are important enzymes that assist in the degradation of cellulose (Pointing 1999). The importance of cellulase activity has been recognized as a result of its potential ability to convert plant biomass to fuels and chemicals (Lynd et al. 1991; Himmel et al. 1999). More than 70 % of the strains examined in this study (133 strains of 27 species) showed β-glucosidase activity. The strains showing the strongest β-glucosidase activity were *P. oxalicum* (strain SFC20140101-M839) and *Penicillium* sp. 5 (strains SFC20140101-M820, SFC20140101-M847, and SFC20140101-M756; Table 3). Yoon et al. (2007) reported that 103 of 106 *Penicillium* species from terrestrial environments produced strong β-glucosidase activity. Of the 14 species common to the Yoon et al. study and our present investigation, 11 showed β-glucosidase activity in both studies. For the remaining three species, Yoon et al. found β-glucosidase activity while we did not, thus highlighting a potential difference between terrestrial and marine-derived strains. Our study also identified nine species with β-glucosidase activity that were not studied in (Yoon et al. 2007). Although several *Penicillium* species have been known to produce endoglucanase to degrade cellulose (Wood et al. 1980; Steiner et al. 1994; Jørgensen et al. 2003; Adsul et al. 2004; Dutta et al. 2008), we did not detect endoglucanase activity in our study, despite testing all of the isolated strains.

Penicillium species are known as potential biological control agents owing to their antifungal activity (Frisvad et al. 2004). In our study, more than half the strains (50.5 %) showed antifungal activity against at least one of the plant pathogens tested, and 38 strains showed antifungal activity against both plant pathogens. Of the species tested in our study, *P. citrinum* showed relatively high antifungal activity against the two plant pathogens, with strain SFC20140101-M662 exhibiting the strongest activity (Table 3). *P. citrinum* is commonly isolated from marine environments (Paz et al.

Table 2 The number of *Penicillium* strains showing extracellular enzyme activity and/or in vitro antifungal activity

Species	No. of strain(s)	No. of positive strains			
		AA	GA	AC	AF
P. aethiopicum	2	0	0	1	0
P. allii	2	0	2	0	0
P. antarcticum	10	0	9	6	0
P. atramentosum	4	1	0	3	1
P. bialowiezense	2	0	1	1	1
P. brevicompactum	2	0	1	1	1
P. chrysogenum	21	4	20	5	0
P. citrinum	12	3	1	11	11
P. crustosum	20	0	4	12	2
P. daleae	1	0	0	1	1
P. digitatum	3	0	3	1	1
P. expansum	2	1	2	2	1
P. freii	2	0	2	2	2
P. glabrum	11	1	11	6	0
P. italicum	2	0	2	0	0
P. nordicum	3	2	2	3	2
P. oxalicum	1	0	1	0	0
P. polonicum	36	4	36	12	7
P. radicicola	1	0	1	1	1
P. raperi	1	1	1	1	0
P. rubefaciens	2	0	2	1	1
P. rubens	21	2	20	2	0
P. solitum	2	0	0	1	0
P. sumatrense	4	0	1	3	0
P. ulaiense	1	0	1	0	0
P. virgatum	1	0	0	1	1
P. yarmokense	1	0	1	1	1
Penicillium sp. 1	1	1	0	1	1
Penicillium sp. 2	1	0	1	1	0
Penicillium sp. 3	1	0	1	1	1
Penicillium sp. 4	3	0	2	2	2
Penicillium sp. 5	3	2	3	3	1
Penicillium sp. 6	1	0	0	1	1
Penicillium sp. 7	1	0	1	1	0
Penicillium sp. 8	2	0	0	1	1
Penicillium sp. 9	1	0	0	1	0
Total	184	22	132	90	41

AA alginase activity, *GA* β-glucosidase activity, *AC* antifungal activity against *C. acutatum* (SFC20130816-01), *AF* antifungal activity against *F. oxysporum* (SFC20130816-02)

Table 3 Select isolates showing relatively high extracellular enzyme activity and/or in vitro antifungal activity

Species	Strain	Clear zone (mm)		Inhibition zone (mm)	
		AA	GA	AC	AF
P. antarcticum	SFC20140101-M835	–	10.0	1.0	–
	SFC20140101-M745	–	11.5	1.5	–
	SFC20140101-M747	–	11	–	–
	SFC20140101-M748	–	9.5	–	–
P. allii	SFC20140101-M742	–	9.0	–	–
P. bialowiezense	SFC20140101-M642	–	–	5.0	3.7
P. brevicompactum	SFC20140101-M640	–	–	3.0	3.3
P. chrysogenum	SFC20140101-M797	5.0	7.0	–	–
	SFC20140101-M647	2.5	–	1.0	–
	SFC20140101-M711	2	7.0	–	–
	SFC20140101-M831	2.8	6.0	–	–
P. citrinum	SFC20140101-M656	–	–	4.5	3.0
	SFC20140101-M481	–	–	4.5	2.3
	SFC20140101-M492	–	–	5.0	2.7
	SFC20140101-M493	–	–	4.5	2.7
	SFC20140101-M483	2.5	–	4.0	3.0
	SFC20140101-M490	–	–	5.5	2.0
	SFC20140101-M662	–	–	7.0	4.0
P. freii	SFC20140101-M754	–	2.5	4.5	4.0
	SFC20140101-M815	–	8.0	4.5	3.0
P. nordicum	SFC20140101-M683	2.0	4.0	3.0	–
P. oxalicum	SFC20140101-M839	–	20.0	–	–
P. polonicum	SFC20140101-M816	–	9.5	1.0	–
P. rubens	SFC20140101-M682	3.0	6.0	–	–
P. virgatum	SFC20140101-M659	–	–	4.0	3.7
P. yarmokense	SFC20140101-M833	–	6.0	3.0	3.7
Penicillium sp. 1	SFC20140101-M830	1.0	–	5.0	3.0
Penicillium sp. 3	SFC20140101-M836	–	8.0	4.0	3.7
Penicillium sp. 4	SFC20140101-M680	–	6.0	6.5	1.7
	SFC20140101-M810	–	–	5.5	1.0
Penicillium sp. 5	SFC20140101-M820	–	16.0	1.8	–
	SFC20140101-M847	1.0	20.0	1.7	–
	SFC20140101-M756	1.5	19.5	2.3	–
Penicillium sp. 6	SFC20140101-M744	–	–	3.5	3.0

Strain numbers are from the Seoul National University Fungal Collection (SFC)

AA alginase activity, *GA* β-glucosidase activity, *AC* antifungal activity against *C. acutatum* (SFC20130816-01), *AF* antifungal activity against *F. oxysporum* (SFC20130816-02)

2010; Zhang et al. 2012; Li and Wang 2009), soil, indoor air, and food, and is also a plant endophyte (Posada et al. 2007). This species is known to produce a variety of active compounds, including the mycotoxins citrinin and citreoviridin (Houbraken et al. 2011), the enzymes cellulase (Dutta et al. 2007) and xylulase (Wakiyama et al. 2008), the plant growth regulators citrinolactones A–C, sclerotinin C, and gibberellin (Kuramata et al. 2007; Khan et al. 2008), and antifungal compounds against *Sclerotsinia minor* (Melouk and Akem 1987). Our study has identified a large pool of *Penicillium* species that could potentially be used as biological control agents—particularly those with strong antifungal activity, such as *P. citrinum*.

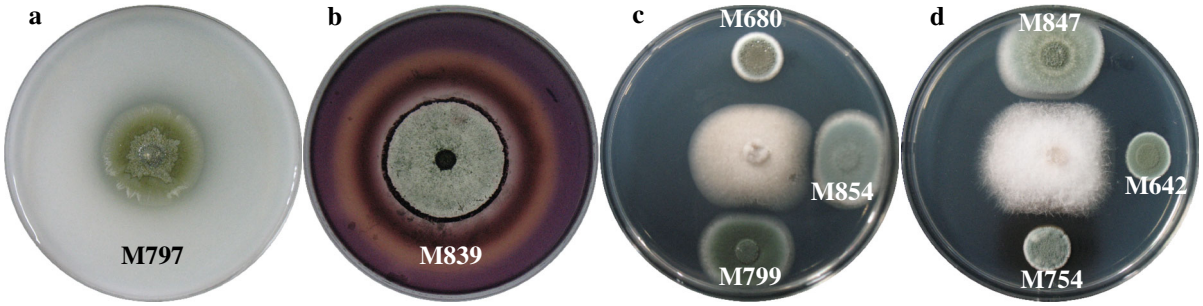


Fig. 5 Examples of cultures exhibiting extracellular enzyme activity **a** alginase, **b** β-glucosidase, and antifungal activity against **c** *C. acutatum* (SFC20130816-01), **d** *F. oxysporum* (SFC20130816-02). Strain numbers in the figure all begin with the prefix "SFC20140101"

Conclusions

The number of natural bioactive products from marine-derived fungi has dramatically increased in recent years (Blunt et al. 2009). However, our knowledge of the diversity and functions of *Penicillium* species isolated from marine habitats is still limited. Since terrestrial and marine environments are different in their biotic and abiotic conditions, we believe it is possible for facultative marine fungi strains to evolve different bioactive compounds compared to their terrestrial counterparts. In this report, we have described 36 marine-derived *Penicillium* species from Korea, a relatively high proportion of which showed enzyme and antifungal activity. These findings bolster the idea that marine-derived fungi, especially species in the genus *Penicillium*, are a valuable resource for discovering natural bioactive compounds. As the marine environment is relatively understudied compared to terrestrial environments, we believe that many novel species and bioactive products await discovery.

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