

Article

Diversity of *Trichoderma* spp. in Marine Environments and Their Biological Potential for Sustainable Industrial Applications

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Abstract: Microorganisms are regarded as a sustainable source of biologically active molecules. Among them, *Trichoderma* spp. have been an attractive source of biological compounds. However, the study of marine-derived *Trichoderma* has developed slowly because of the difficulty in isolating the fungi. In our study, 30 strains of marine-derived *Trichoderma* were identified through the translation elongation factor 1-alpha (EF1 α) sequences, and their biological activities, such as antioxidant activity by ABTS and DPPH assays, antifungal activity against *Asteromyces cruciatus* and *Lindra thalassiae*, and tyrosinase inhibition activity, were investigated. As a result, the 30 marine *Trichoderma* species were classified into 21 taxa, including three new species candidates. Three strains of *T. asperellum* showed the highest ABTS radical scavenging activity and antifungal activity. *T. bissettii* SFC20170821-M05 and *T. guizhouense* SFC20180619-M23 showed notable DPPH radical scavenging activity and tyrosinase inhibition activity, respectively. This study showed the potential of marine-derived *Trichoderma* as a source of bioactive compounds.

Keywords: antagonistic activity; biological control; phylogenetic analysis; radical scavenging; skin whitening agents

1. Introduction

In a specific environment, biodiversity is the most essential information for sustainable development and is important in discovering biological resources [1–6]. In particular, microorganisms have been considered as a sustainable source of various bioactive compounds as well as useful enzymes [7–14]. Traditional sources of natural products have been terrestrial plants, fungi, and bacteria. The idea of natural products from the ocean has recently been of interest, but the need for additional efforts, such as scuba diving or instruments for collecting or culture, have made the development of marine natural compounds slow in comparison with its terrestrial counterpart [15,16]. Nevertheless, the structural uniqueness and profound effect of marine-derived compounds have been of interest to the pharmaceutical and cosmetic industry [17,18]. A total of 200 million microorganisms in the ocean covering 71% of the surface of the earth have been presumed, although the exact number of microorganisms is still open to debate [19,20]. For this reason, marine organisms, especially microorganisms, have attracted the attention of the pharmaceutical and cosmetic industries as a new reservoir of novel natural compounds [17,20].

The genus *Trichoderma* is well known for its ability to produce antibiotic compounds and parasitize other fungi [21–23]. The actions of antifungal secondary metabolites (i.e., 6-pentyl- α -pyrone and trichodermaiketones) and cell wall hydrolytic enzymes (i.e., β -1,3-glucanases and β -1,6-glucanases) secreted by *Trichoderma* spp. can cause the death of prey [21,24–26]. Several *Trichoderma* provide a beneficial effect to host plants by activating plant defense mechanisms, preventing pathogen attacks and promoting plant growth [21,27]. To date, more than 250 species of the genus *Trichoderma* have been reported [28]. Approximately 78 metabolites have been described from marine-derived *Trichoderma* so far, and most of them showed a variety of industrially useful biological activities, such as antifungal, antibacterial, and antioxidant activity [29,30]. Reliable phylogenetic information is important to discover the diversity of secondary metabolites of microorganisms, and it is difficult to distinguish *Trichoderma* spp. by morphology alone, because they share many morphological features [31–33]. Thus, a molecular biological analysis is essential for the accurate identification of *Trichoderma* [34]. The internal transcribed spacer (ITS) is the most universal fungal molecular barcode [35,36]. However, the ITS has low species resolution in the genus *Trichoderma* [32]. Instead of ITS, translation elongation factor 1-alpha (EF1 α) sequences were recommended for phylogenetic analysis of this genus [32].

The aim of this study was to investigate the diversity of *Trichoderma* spp. in marine environment in South Korea using phylogenetic analysis and evaluate the marine *Trichoderma* spp. as a source of bioactive secondary metabolites by investigating the antifungal, antioxidant, and tyrosinase activities of the fungal extracts.

2. Materials and Methods

2.1. Preparation of *Trichoderma* Cultures

A total of 30 marine *Trichoderma* cultures were obtained from the Marine Fungal Resource Bank (<http://mfrb.snu.ac.kr>; Seoul, Korea). A list of the fungal species with their general information is shown in Table 1. Their sampling sites are indicated in Figure 1. They were subcultured on potato dextrose agar (PDA) media in room temperature: 20–25°C.

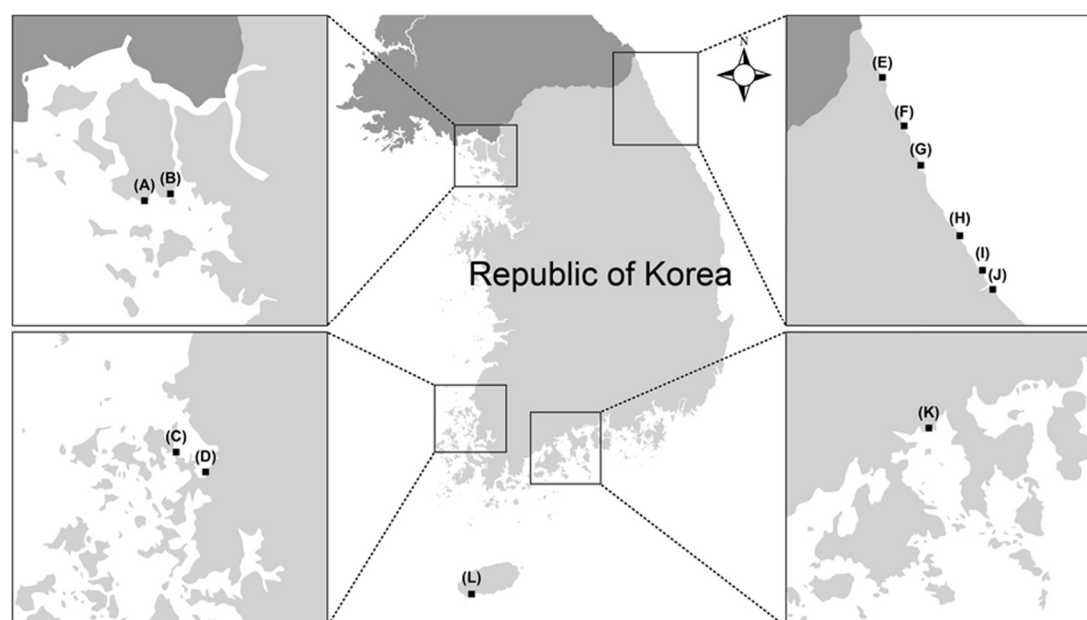


Figure 1. The map of sampling sites: (A) Hwado-myeon, and (B) Gilsang-myeon, Ganghwa-gun, Incheon; (C) seaside, and (D) mudflat in Hyeonnye-myeon, Muan-gun, Jeollanam-do; (E) Hyeonnye-myeon, and (F) Jugwang-myeon Goseong-gun; (G) Sokcho Beach in Sokcho-si; (H) Hyeonnam-myeon, Yangyang-gun; and (I) Jumunjin-eup, and (J) Yeongok-myeon, Gangneung-si, Gangwon-do; (K) Byeollyang-myeon, Suncheon-si, Jeollanam-do; (L) Andeok-myeon, Seogwipo-si, Jeju-do, Korea.

Table 1. General information of 30 marine-derived *Trichoderma* spp.

Identity	ID	Sampling Date	Sampling SITE *	Isolation Source
<i>Trichoderma afroharzianum</i>	SFC20160907-M20	January 2015	Hyeonnae-myeon, Goseong-gun, Gangwon-do ^E	Eggs of <i>Arctoscopus japonicus</i>
	SFC20180619-M25	January 2017	Gilsang-myeon, Ganghwa-gun, Incheon ^B	Mudflat
	SFC20190312-M15	July 2016	Gilsang-myeon, Ganghwa-gun, Incheon ^B	Mudflat
<i>T. asperelloides</i>	SFC20180619-M20	January 2015	Hyeonnae-myeon, Goseong-gun, Gangwon-do ^E	Eggs of <i>Arctoscopus japonicus</i>
<i>T. asperellum</i>	SFC20160907-M21	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
	SFC20180619-M22	October 2016	Hyeongyeong-myeon, Muan-gun, Jeollanam-do ^D	Mudflat
	SFC20180619-M24	October 2016	Byeollyang-myeon, Suncheon-si, Jeollanam-do ^K	Sea sand
<i>T. atroviride</i>	SFC20190312-M11	January 2015	Hyeonnae-myeon, Goseong-gun, Gangwon-do ^E	Eggs of <i>Arctoscopus japonicus</i>
<i>T. atroviride</i>	SFC20161110-M05	December 2015	Jugwang-myeon, Goseong-gun, Gangwon-do ^F	<i>Agarum clathratum</i>
<i>T. bissettii</i>	SFC20170821-M05	October 2016	Byeollyang-myeon, Suncheon-si, Jeollanam-do ^K	Mudflat
<i>T. capillare</i>	SFC20180619-M19	January 2015	Sokcho-si, Gangwon-do ^G	Eggs of <i>Arctoscopus japonicus</i>
<i>T. citrinoviride</i>	SFC20180510-M16	July 2016	Hwado-myeon, Ganghwa-gun, Incheon ^A	Sea sand
<i>T. gamsii</i>	SFC20160907-M22	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
<i>T. guizhouense</i>	SFC20160907-M23	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
	SFC20180619-M21	August 2015	Jumunjin-eup, Gangneung-si, Gangwon-do ^I	<i>Agarum clathratum</i>
	SFC20180619-M23	October 2016	Hyeongyeong-myeon, Muan-gun, Jeollanam-do ^D	Mudflat
	SFC20190312-M12	January 2017	Andeok-myeon, Seogwipo-si, Jeju-do ^L	Sea sand
	SFC20180510-M09	July 2016	Gilsang-myeon, Ganghwa-gun, Incheon ^B	Mudflat
<i>T. hamatum</i>	SFC20171019-M03	July 2016	Gilsang-myeon, Ganghwa-gun, Incheon ^B	Mudflat
<i>T. longibrachiatum</i>	SFC20170718-M02	January 2017	Byeollyang-myeon, Suncheon-si, Jeollanam-do ^K	Mudflat
<i>T. orientalis</i>	SFC20170718-M02	January 2017	Byeollyang-myeon, Suncheon-si, Jeollanam-do ^K	Mudflat
<i>T. paraviridescens</i>	SFC20160907-M24	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
<i>T. pyramidale</i>	SFC20160907-M25	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
<i>T. songyi</i>	SFC20171120-M04	October 2016	Hyeongyeong-myeon, Muan-gun, Jeollanam-do ^C	Sea sand
<i>T. subviride</i>	SFC20170919-M07	July 2016	Andeok-myeon, Seogwipo-si, Jeju-do ^L	Sea sand
<i>T. virens</i>	SFC20180817-M24	October 2016	Hyeongyeong-myeon, Muan-gun, Jeollanam-do ^D	Mudflat
<i>Trichoderma</i> sp. 1	SFC20190312-M13	January 2015	Yeongok-myeon, Gangneung-si, Gangwon-do ^J	Eggs of <i>Arctoscopus japonicus</i>
	SFC20190312-M14	July 2016	Gilsang-myeon, Ganghwa-gun, Incheon ^B	Mudflat
	SFC20190312-M17	November 2015	Hyeonnam-myeon, Yangyang-gun, Gangwon-do ^H	<i>Agarum clathratum</i>
<i>Trichoderma</i> sp. 2	SFC20190312-M16	October 2015	Hyeonnam-myeon, Yangyang-gun, Gangwon-do ^H	<i>Agarum clathratum</i>
<i>Trichoderma</i> sp. 3	SFC20161110-M06	September 2015	Jumunjin-eup, Gangneung-si, Gangwon-do ^I	<i>Agarum clathratum</i>

* The sampling sites in Figure 1 were indicated by A–L.

2.2. Phylogenetic Analysis

EF1 α sequences of marine *Trichoderma* were obtained from Marine Fungal Resource Bank. The closely related sequences for references were downloaded from GenBank using nucleotide BLAST. Type specimens were chosen for reference sequences except for *Trichoderma virens*. EF1 α sequence data of the CBS 249.59, type specimen of *T. virens* has only 200 bp in GenBank. *Protocrea illinoensis* GJS 94-54 (EU703904) was downloaded as outgroup of the genus *Trichoderma*. The obtained sequences were aligned using MAFFT 7.388 [37]. The aligned dataset was proofread and modified manually using MacClade 4.08 [38]. The aligned dataset contained 113 taxa and 752 characters. A neighbor joining tree was constructed with PAUP 4.0b10 [39]. The Kimura 2-parameter model was applied [40]. To indicate branch stability, 1000 replications of bootstrap analysis were carried out.

2.3. Preparation of Fungal Extracts

All of the fungal species were cultivated on 50 mL of PDA at 25 °C for seven days in darkness. After 7 days of cultivation, the solid media were extracted with 200 mL of methanol for 24 h. the methanol solution was filtrated with Whatman No. 1 filter paper. The filtrated solutions were evaporated at 37 °C under a vacuum. The condensed residues were dissolved in 20 mL of ethyl acetate and 20 mL of distilled water. After 6 h, the supernatant, the partitioned ethyl acetate fraction, was evaporated. The extracts were stored at 4 °C. The fungal cultures were incubated in triplicate, and all the subsequent biological assays were performed in triplicate.

2.4. Measurement of Antioxidant Activity by ABTS Scavenging Ability

The 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, Inc., St. Louis, MO) solution dissolved in PBS (7 mM) was oxidized with potassium persulfate (2.45 mM) for 24 h in darkness at room temperature. The ABTS^{•+} solution was diluted with PBS to an absorbance of 0.70 (\pm 0.02) at a wavelength of 734 nm. Then, 990 μ L of the ABTS^{•+} solution and 10 μ L of each fungal extract sample (10 mg/mL in DMSO) were mixed in the cuvette and measured at 734 nm after 6 min.

2.5. Measurement of Antioxidant Activity by DPPH Radical Scavenging Ability

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Inc., St. Louis, MO, USA) was dissolved in methanol (80%) at 150 μ M. An aliquot (22 μ L) of the fungal extracts (10 mg/mL) were each mixed with 200 μ L of DPPH solution in the 96-well plate. The mixture was stored at room temperature for 30 min and measured at 520 nm.

2.6. Measurement of Antifungal Activities

Asteromyces cruciatus SFC20161110-M19 was obtained from Marine Fungal Resource Bank (MFRB) at Seoul National University as a marine bioresource bank of Korea by the Ministry of Oceans and Fisheries, and *Lindra thalassiae* NBRC106646 was purchased from Biological Resource Center under National Institute of Technology and Evaluation. *Asteromyces cruciatus* SFC20161110-M19 and *Lindra thalassiae* NBRC106646 were tested as the target fungi and antifungal activity was determined in a 96-well plate. The 25 μ L of spore suspensions (4×10^5 conidia/mL) of the target fungi was added to each well containing 49 μ L of distilled water and 25 μ L of potato dextrose broth. The 2 μ L of *Trichoderma* extracts solubilized in dimethyl sulfoxide (DMSO) to a final concentration of 100 μ g/mL was added to each well at the last. The 96-well plates were incubated at 25 °C for 3 days and the fungal growth was detected by measuring the absorbance at the wavelength of 595 nm [41]. The minimum inhibitory concentration (MIC) values were determined in the concentration range of 6.25–50 μ g/mL.

2.7. Tyrosinase Inhibition Activity

The assay was modified from the method described by Lai et al. [42]. An amount of 40 μ L of each fungal extract samples dissolved in 50% DMSO at the same concentration (2.5 mg/mL), 70 μ L of

a 0.1 M potassium phosphate buffer (pH 6.8), and 30 µL of 0.02 mg/mL of tyrosinase were mixed in 96-well plate. The mixture was heated to 30 °C for 5 min and mixed with 100 µL of 2.5 mM L-dihydroxyphenylalanine (L-DOPA). After 30 min, to terminate the reaction put the plate in the ice and the absorbance was measured at 492 nm. Kojic acid was used as a positive control and all mixtures without L-DOPA was used as a blank.

3. Results and Discussion

3.1. Identification and Phylogeny

The marine *Trichoderma* were identified by phylogeny based on the EF1α sequences (Figure 2). A tree containing 86 taxa of *Trichoderma*, including 83 known species and three new species candidates, was constructed. Among them, 25 of the sequences obtained were identified as eighteen previously reported species. Eleven of the sequences were grouped in the Harzianum clade: SFC20190312-M14, SFC20190312-M15, SFC20180619-M23, SFC20190312-M12, SFC20180619-M25, SFC20190312-M13, SFC20160907-M23, SFC20160907-M25, SFC20160907-M20, SFC20180619-M21, and SFC20190312-M17 [32,43]. The morphology of the tree was similar to that in other studies [32,43]. SFC20190312-M15, SFC20180619-M25, and SFC20160907-M20 were identified as *T. afroharzianum* with a high bootstrap value (100%). SFC20180619-M23, SFC20190312-M12, SFC20160907-M23, and SFC20180619-M21 were grouped with *T. guizhouense*, but the bootstrap value was not very high (62.9%). SFC20190312-M14, SFC20190312-M13, and SFC20190312-M17 had their own group without a close reference and were regarded as new species candidates. In this study, these cultures were named *Trichoderma* sp. 1. Their closest sister group contains *T. afarasin*, *T. harzianum*, and *T. camerunense*.

SFC20180817-M24 was placed in the Virens clade [43] and identified as *T. virens* (bootstrap value: 97.7%). Its closely related species were *T. crassum* [43]. SFC20180510-M16, SFC20171019-M03, SFC20170821-M05, SFC20170718-M02, and SFC20160907-M2190 were placed in the Longibrachiatum clade [44,45]. SFC20171019-M03 was identified as *T. longibrachiatum* with high support (bootstrap value: 98.3%). SFC20170718-M02 was grouped with *T. orientale* with a bootstrap value of 100%. SFC20180510-M16 was identified as *T. citrinoviride* (bootstrap value: 100%). SFC20160907-M2190 was grouped with *T. capillare* (bootstrap value: 100%). Though SFC20170821-M05 was placed in a paraphyletic group with *T. bissettii*, it showed 100% similarity with the type specimens of *T. bissettii* based on a BLAST search. Thus, it was regarded as *T. bissettii*.

Eleven sequences were placed in the Viride clade: SFC20180510-M09, SFC20170919-M07, SFC20180619-M22, SFC20171120-M04, SFC20180619-M24, SFC20160907-M21, SFC20160907-M22, SFC20180619-M20, SFC20190312-M11, SFC20161110-M06, and SFC20161110-M05 [46–48]. Among them, SFC20160907-M22 was grouped with *T. gamsii* (bootstrap value: 100%). SFC20190312-M11 was identified as *T. paratroviride* with a bootstrap value of 100%. SFC20161110-M05 made a monophyletic group with *T. atroviride* (bootstrap value: 99.9%). SFC20170919-M07 was placed in a monophyletic clade with high support (bootstrap value: 100%). SFC20171120-M04 was grouped with *T. songyi* with a bootstrap value of 100%. This species is known to be associated with *Tricholoma matsutake* [48]. SFC20180619-M22, SFC20180619-M24, and SFC20160907-M21 were in a monophyletic clade with *T. asperellum* (bootstrap value: 99.9%). SFC20180619-M20 was identified as *T. asperelloides* (bootstrap value: 100%). SFC20180510-M09, and SFC20161110-M06 were not assigned to a clade through close references; therefore, we regarded them as new species candidates. These cultures were named *Trichoderma* sp. 2 and 3, respectively.

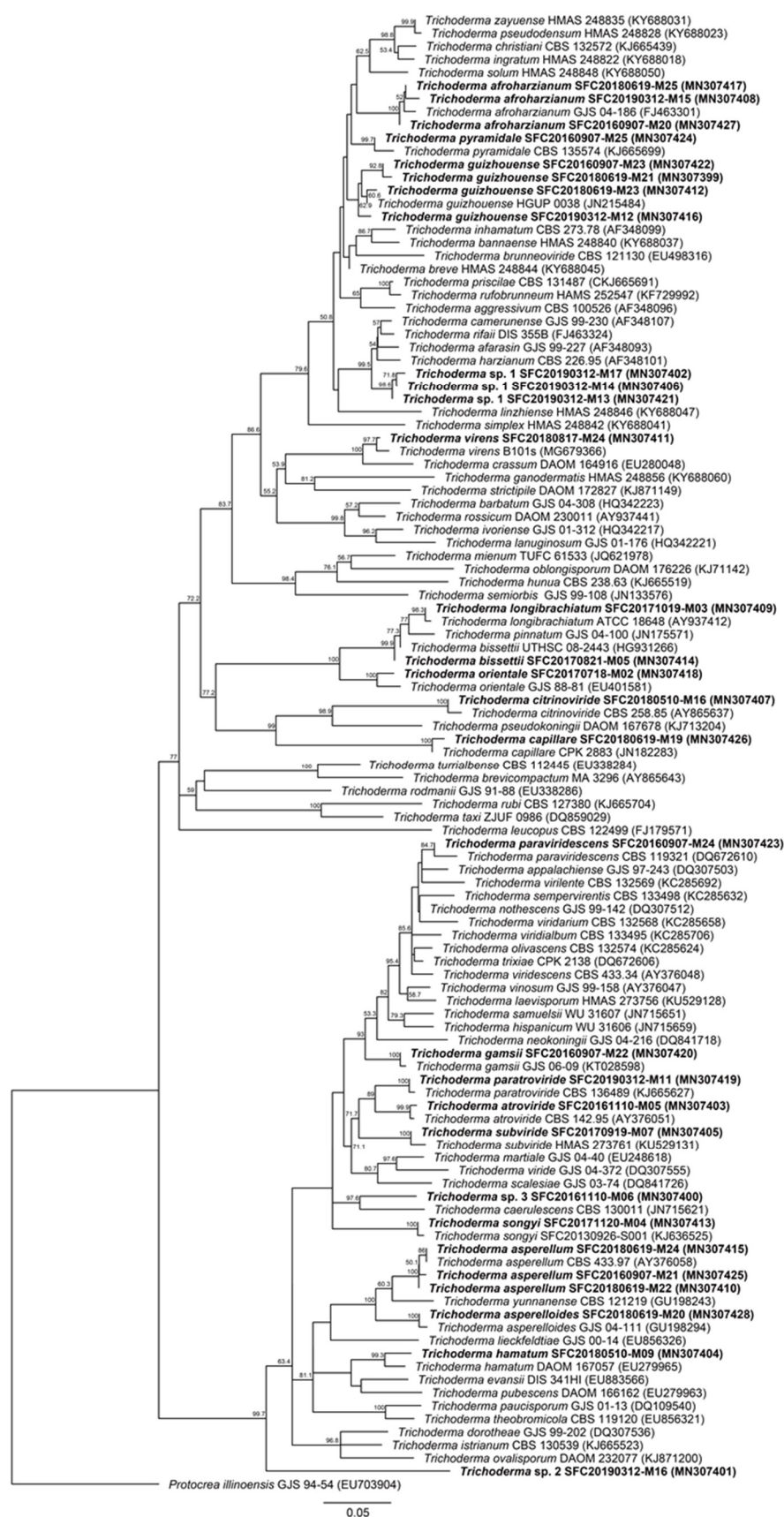


Figure 2. The neighbor joining tree of the EF-1 α sequences dataset. Numbers above branches indicate bootstrap values. Bootstrap values less than 50 were not shown. Fungal cultures collected in this study are in bold and colored. GenBank accession numbers are in parentheses.

3.2. Antioxidant Activity

ABTS and DPPH radical scavenging assays were used to evaluate the antioxidant activity of 30 methanol crude fungal extracts of marine *Trichoderma*, and many of the marine *Trichoderma* extracts contained antioxidative compounds. *Trichoderma asperellum* SFC20160907-M21, SFC20180619-M22, and SFC20180619-M24 showed over 70% ABTS radical scavenging activity, and *T. bissettii* SFC20170821-M05 and *T. longibrachiatum* SFC20171019-M03 exhibited over 70% DPPH radical scavenging activity (Table 2). Other extracts showed lower inhibition rates from 2.3% to 69%. Only four strains, *T. bissettii* SFC20170821-M05, *Trichoderma* sp. 1 SFC20190312-M13, *T. longibrachiatum* SFC20171019-M03, and *T. subviride* SFC20170919-M07, exhibited over 50% antioxidant activities in both the ABTS and DPPH radical scavenging tests. Numerous studies have shown that various marine-derived natural compounds have radical scavenging properties that could be used as raw materials in the cosmetic and pharmaceutical industries [18,49,50]. For example, the major antioxidant in the food supplement Seanol is phlorotannins extracted from *Ecklonia Cava* [51]. Several natural compounds that have antioxidant ability, such as polyphenols and vitamins, also have other biological activities, such as antiaging and skin-whitening [52,53]. Some antioxidants have the ability to inhibit tyrosinase by eliminating reactive quinone products [52]. In this study, the extracts of *T. atroviride* SFC20161110-M05, *T. gamsii* SFC20160907-M22, *T. guizhouense* SFC20180619-M23, and *T. songyi* SFC20171120-M04, which showed a remarkable ability to inhibit tyrosinase ($IC_{50} < 100 \mu\text{g/mL}$), exhibited low radical scavenging activity (<50%), indicating that they have other mechanisms for inhibiting tyrosinase rather than scavenging reactive quinone products. For examples, they may be the competitive inhibitors such as copper chelators that inhibit this metalloenzyme or suicide inhibitors that inactivate tyrosinase by changing the tertiary and quaternary structures of the enzyme [54].

Table 2. Biological activities of the marine *Trichoderma* spp. extracts.

Fungal Name	ID	Radical-Scavenging Activity (%)		Antifungal Activity (MIC ³ , µg/mL)		Tyrosinase Inhibition (IC ₅₀ ⁴ , µg/mL)
		ABTS ¹	DPPH ²	<i>L. thalassiae</i>	<i>A. cruciatus</i>	
<i>T. afroharzianum</i>	SFC20160907-M20	27.16	24.47	N.D.	100	N.D. ⁵
	SFC20190312-M15	69.92	25.29	N.D.	>100	N.D.
	SFC20180619-M25	66.70	33.77	>100	>100	N.D.
<i>T. asperelloides</i>	SFC20180619-M20	47.70	41.06	50	>100	N.D.
<i>T. asperellum</i>	SFC20160907-M21	73.38	30.62	6.25	50	>417
	SFC20180619-M22	74.70	37.83	>100	25	>417
	SFC20180619-M24	72.63	32.34	6.25	25	104.23
<i>T. atroviride</i>	SFC20161110-M05	32.18	38.03	N.D.	N.D.	15.30
	SFC20190312-M11	48.48	34.80	N.D.	N.D.	>417
<i>T. bissettii</i>	SFC20170821-M05	57.17	79.60	N.D.	>100	>417
<i>T. capillare</i>	SFC20180619-M19	29.37	49.62	>100	>100	N.D.
<i>T. citrinoviride</i>	SFC20180510-M16	25.52	48.27	>100	>100	N.D.
<i>T. gamsii</i>	SFC20160907-M22	15.82	14.69	N.D.	N.D.	52.08
<i>T. guizhouense</i>	SFC20160907-M23	51.18	31.27	N.D.	N.D.	N.D.
	SFC20180619-M21	14.65	18.62	>100	N.D.	N.D.
	SFC20180619-M23	18.17	23.41	N.D.	N.D.	7.28
	SFC20190312-M12	32.73	36.76	N.D.	N.D.	152.07
<i>T. hamatum</i>	SFC20180510-M09	23.29	35.33	N.D.	N.D.	N.D.
<i>T. longibrachiatum</i>	SFC20171019-M03	66.36	72.72	N.D.	>100	252.26
<i>T. orientalis</i>	SFC20170718-M02	28.62	32.22	N.D.	N.D.	N.D.
<i>T. paraviridescens</i>	SFC20160907-M24	49.59	38.32	N.D.	>100	118.61
<i>T. pyramidale</i>	SFC20160907-M25	25.02	2.36	N.D.	>100	N.D.
<i>T. songyi</i>	SFC20171120-M04	27.14	14.60	N.D.	N.D.	23.77
<i>T. subviride</i>	SFC20170919-M07	61.71	53.43	N.D.	N.D.	N.D.
<i>T. virens</i>	SFC20180817-M24	42.20	45.53	>100	100	N.D.
<i>Trichoderma</i> sp. 1	SFC20190312-M14	42.09	44.05	N.D.	N.D.	N.D.
	SFC20190312-M13	61.57	55.03	>100	N.D.	119.74
	SFC20190312-M17	40.11	55.81	>100	N.D.	148.1
<i>Trichoderma</i> sp. 2	SFC20190312-M16	48.48	34.80	N.D.	N.D.	N.D.
<i>Trichoderma</i> sp. 3	SFC20161110-M06	43.96	33.57	N.D.	N.D.	N.D.

Ascorbic acid *	13.70	6.80	
Kojic acid *			49.32

¹ 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; ² DPPH, 2,2-diphenyl-1-picrylhydrazyl; ³ minimum inhibitory concentration; ⁴ half maximal inhibitory concentration; ⁵ not detected; * positive controls.

3.3. Antifungal Activity

The antifungal activity was determined using the fungal extracts of marine *Trichoderma* on *Asteromyces cruciatus* and *Lindra thalassiae* as target fungi. *A. cruciatus* is a ubiquitous marine fungus and has the ability to degrade alginate, which is the major material of the brown algal construct; therefore, it is regarded as a potentially harmful fungus to brown algae [55]. *Lindra thalassiae* is a well-known pathogen of brown algae and seagrasses and causes raisin disease [56]. In this study, eight strains showed remarkable growth inhibitory ability against both *A. cruciatus* and *L. thalassiae*: *T. afroharzianum* SFC20180619-M25, *T. asperelloides* SFC20180619-M20, *T. asperellum* SFC20160907-M21, SFC20180619-M22, and SFC20180619-M24, *T. capillare* SFC20160907-M2190, *T. citrinoviride* SFC20180510-M16 and *T. virens* SFC20180817-M24. *T. asperellum* SFC20160907-M21, SFC20180619-M22, and SFC20180619-M24 showed a high inhibitory effect against both target fungi (Table 2). In particular, *T. asperellum* SFC20160907-M21 and SFC20180619-M24 showed the highest inhibitory effect, as they could inhibit *L. thalassiae* at 6.25 µg/mL. Since *Trichoderma* spp. are well known producers of antifungal compounds, such as 6-pentyl- α -pyrone and trichoderma ketones, it was expected that *Trichoderma* species could exhibit antagonistic ability against *A. cruciatus* and *L. thalassiae*, which are regarded as pathogenic fungi to algae [25,26]. Similarly, *T. asperellum* has already been investigated for its antagonistic ability against pathogenic fungi in several previous studies [57–59]. In addition, the synergistic effects of antifungal secondary metabolites and enzymes from *Trichoderma* could provide support to the plant rhizosphere attacked by other pathogenic fungi [21,27]. The results of this antifungal activity suggested that the antifungal compounds from *Trichoderma* could also support marine algae defense systems that are related to symbiosis, similar to the action of the plant rhizosphere.

3.4. Tyrosinase Inhibition Activity

Among the fungal extracts, 14 extracts showed tyrosinase inhibition activity (Table 2). Three strains of *T. asperellum* (SFC20160907-M21, SFC20180619-M22, and SFC20180619-M24) showed weak inhibitory activity. *T. guizhouense* SFC20180619-M23, *T. atroviride* SFC20161110-M05, and *T. songyi* SFC20171120-M04 exhibited the highest tyrosinase inhibitory effect. Interestingly, the extracts of *T. guizhouense* SFC20180619-M23, *T. atroviride* SFC20161110-M05, and *T. songyi* SFC20171120-M04 exhibited even higher activities than kojic acid, the most widely studied tyrosinase inhibitor from fungi. Kojic acid, which is produced by various species of *Aspergillus* and *Penicillium*, inhibits the formation of melanin and supports skin whitening [60–62]. Considering that the fungal extracts used in the experiments, unlike the single compound kojic acid, were crude extracts, these three *Trichoderma* species are expected to produce highly active tyrosinase inhibitors or produce multiple compounds with synergistic effects. In addition to kojic acid isolated from mushrooms, other tyrosinase inhibitors from fungi have been found recently, including 1 β ,5 α ,6 α ,14-tetraacetoxy-9 α -benzoyloxy-7 β H-eudesman-2 β ,11-diol and 4 α ,5 α -diacetoxy-9 α -benzoyloxy-7 β H-eudesman-1 β ,2 β ,11,14-tetraol [63]. Moreover, the novel tyrosinase inhibitor from marine *Trichoderma* was purified. The structure of the compound is the same as antibiotics from *T. koningii* and *T. harzianum* [64]. Many antibiotics and antioxidants also have potent tyrosinase inhibition activity, although the mechanism of various tyrosinase inhibitors is different [52]. In this study, the strains that exhibited high potent tyrosinase inhibition activity (*T. guizhouense* SFC20180619-M23, *T. atroviride* SFC20161110-M05, and *T. songyi* SFC20171120-M04) did not directly relate to the radical scavenging activities and antifungal activity. However, some strains that exhibited relatively weak tyrosinase inhibition activity showed radical scavenging activity and antifungal effects (*T. asperellum* SFC20160907-M21, SFC20180619-M22, and SFC20180619-M24; *T. bissettii* SFC20170821-M05; *Trichoderma* sp. 1 SFC20190312-M13; *T. longibrachiatum* SFC20171019-M03).

4. Conclusions

We investigated the diversity of marine-derived *Trichoderma* spp. and provided reliable DNA information of 30 marine *Trichoderma* species isolated in South Korea as well as their exploitable

biological activities. Based on the phylogenetic analysis, the 30 marine *Trichoderma* species were classified into 21 taxa, including three new species candidates. Among them, three species—*Trichoderma* sp. 1, *T. asperellum*, and *T. longibrachiatum*—showed remarkable abilities in most of the biological activities investigated in this study. The potent bioactive compounds of these species will be studied in the near future. This study proved that marine-derived *Trichoderma* can be a useful source of bioactive compounds.

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References

1. Freguia, S.; Logrieco, M.E.; Monetti, J.; Ledezma, P.; Viridis, B.; Tsujimura, S. Self-powered bioelectrochemical nutrient recovery for fertilizer generation from human urine. *Sustainability* **2019**, *11*, 5490.
2. Chou, J.-S.; Yu, C.-P.; Truong, D.-N.; Susilo, B.; Hu, A.; Sun, Q. Predicting microbial species in a river based on physicochemical properties by bio-inspired metaheuristic optimized machine learning. *Sustainability* **2019**, *11*, 6889.
3. Vieira, T.A.; Panagopoulos, T. Urban forestry in brazilian amazonia. *Sustainability* **2020**, *12*, 3235.
4. Heo, Y.M.; Lee, H.; Kim, K.; Kwon, S.L.; Park, M.Y.; Kang, J.E.; Kim, G.-H.; Kim, B.S.; Kim, J.-J. Fungal diversity in intertidal mudflats and abandoned solar salterns as a source for biological resources. *Mar. Drugs* **2019**, *17*, 601.
5. Lee, H.; Lee, D.W.; Kwon, S.L.; Heo, Y.M.; Jang, S.; Kwon, B.-O.; Khim, J.S.; Kim, G.-H.; Kim, J.-J. Importance of functional diversity in assessing the recovery of the microbial community after the hebei spirit oil spill in korea. *Environ. Int.* **2019**, *128*, 89–94.
6. Lee, H.; Heo, Y.M.; Kwon, S.L.; Yoo, Y.; Lee, A.H.; Kwon, B.-O.; Kim, G.-H.; Khim, J.S.; Kim, J.-J. Recovery of the benthic bacterial community in coastal abandoned saltern requires over 35 years: A comparative case study in the yellow sea. *Environ. Int.* **2020**, *135*, 105412.
7. Romano, G.; Costantini, M.; Sansone, C.; Lauritano, C.; Ruocco, N.; Ianora, A. Marine microorganisms as a promising and sustainable source of bioactive molecules. *Mar. Environ. Res.* **2017**, *128*, 58–69.
8. Tourova, T.; Sokolova, D.; Nazina, T.; Grouzdev, D.; Kurshev, E.; Laptev, A. Biodiversity of microorganisms colonizing the surface of polystyrene samples exposed to different aqueous environments. *Sustainability* **2020**, *12*, 3624.
9. Ge, S.; Ma, J.; Liu, L.; Yuan, Z. The impact of exogenous aerobic bacteria on sustainable methane production associated with municipal solid waste biodegradation: Revealed by high-throughput sequencing. *Sustainability* **2020**, *12*, 1815.
10. Han, S.-I.; Jeon, M.S.; Heo, Y.M.; Kim, S.; Choi, Y.-E. Effect of *pseudoalteromonas* sp. Mebic 03485 on biomass production and sulfated polysaccharide biosynthesis in *porphyridium cruentum* utex 161. *Bioresour. Technol.* **2020**, *302*, 122791.
11. Ham, J.; Lim, W.; Kim, K.; Heo, Y.M.; Ryu, S.M.; Lee, D.; Kim, J.-J.; Song, G. Gentisyl alcohol inhibits proliferation and induces apoptosis via mitochondrial dysfunction and regulation of mapk and pi3k/akt pathways in epithelial ovarian cancer cells. *Mar. Drugs* **2019**, *17*, 331.
12. Heo, Y.M.; Lee, H.; Lee, C.; Kang, J.; Ahn, J.-W.; Lee, Y.M.; Kang, K.-Y.; Choi, Y.-E.; Kim, J.-J. An integrative process for obtaining lipids and glucose from *chlorella vulgaris* biomass with a single treatment of cell disruption. *Algal Res.-Biomass Biofuels Bioprod.* **2017**, *27*, 286–294.
13. Heo, Y.M.; Kim, K.; Kwon, S.L.; Na, J.; Lee, H.; Jang, S.; Kim, C.H.; Jung, J.; Kim, J.-J. Investigation of filamentous fungi producing safe, functional water-soluble pigments. *Mycobiology* **2018**, *46*, 269–277.

14. Lee, H.; Lee, Y.M.; Heo, Y.M.; Lee, J.; Kim, J.S.; Kang, K.Y.; Kim, J.-J. Utilization of agricultural residues for enhancement of cellulolytic enzyme production and enzymatic saccharification by *trichoderma harzianum* kuc1716. *Ind. Crops Prod.* **2017**, *109*, 185–191.
15. Newman, D.J.; Cragg, G.M. Marine natural products and related compounds in clinical and advanced preclinical trials. *J. Nat. Prod.* **2004**, *67*, 1216–1238.
16. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **2007**, *70*, 461–477.
17. Agrawal, S.; Adholeya, A.; Barrow, C.J.; Deshmukh, S.K. Marine fungi: An untapped bioresource for future cosmeceuticals. *Phytochem. Lett.* **2018**, *23*, 15–20.
18. Molinski, T.F.; Dalisay, D.S.; Lievens, S.L.; Saludes, J.P. Drug development from marine natural products. *Nat. Rev. Drug Discov.* **2009**, *8*, 69.
19. Troussellier, M.; Escalas, A.; Bouvier, T.; Mouillot, D. Sustaining rare marine microorganisms: Macroorganisms as repositories and dispersal agents of microbial diversity. *Front. Microbiol.* **2017**, *8*, 947.
20. Kong, Q. Marine microorganisms as biocontrol agents against fungal phytopathogens and mycotoxins. *Biocontrol Sci. Technol.* **2018**, *28*, 77–93.
21. Druzhinina, I.S.; Seidl-Seiboth, V.; Herrera-Estrella, A.; Horwitz, B.A.; Kenerley, C.M.; Monte, E.; Mukherjee, P.K.; Zeilinger, S.; Grigoriev, I.V.; Kubicek, C.P. Trichoderma: The genomics of opportunistic success. *Nat. Rev. Microbiol.* **2011**, *9*, 749.
22. Sivasithamparan, K.; Ghisalberti, E. Secondary metabolism in trichoderma. *Trichoderma Gliocladium. Vol. 1 Basic Biol. Taxon. Genet.* **2014**, *1*, 139.
23. Lu, Z.; Tombolini, R.; Woo, S.; Zeilinger, S.; Lorito, M.; Jansson, J.K. In vivo study of trichoderma-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* **2004**, *70*, 3073–3081.
24. Reino, J.L.; Guerrero, R.F.; Hernández-Galán, R.; Collado, I.G. Secondary metabolites from species of the biocontrol agent trichoderma. *Phytochem. Rev.* **2008**, *7*, 89–123.
25. Hong, J.-H.; Lee, J.; Min, M.; Ryu, S.-M.; Lee, D.; Kim, G.-H.; Kim, J.-J. 6-pentyl- α -pyrone as an anti-sapstain compound produced by *trichoderma gamsii* kuc1747 inhibits the germination of ophiostomatoid fungi. *Holzforschung* **2014**, *68*, 769–774.
26. Song, F.; Dai, H.; Tong, Y.; Ren, B.; Chen, C.; Sun, N.; Liu, X.; Bian, J.; Liu, M.; Gao, H.; et al. Trichoderma ketones a–d and 7-o-methylkoninginin d from the marine fungus trichoderma koningii. *J. Nat. Prod.* **2010**, *73*, 806–810.
27. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. Trichoderma species—opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43.
28. Bissett, J.; Gams, W.; Jaklitsch, W.; Samuels, G.J. Accepted trichoderma names in the year 2015. *IMA Fungus* **2015**, *6*, 263–295.
29. Su, D.; Ding, L.; He, S. Marine-derived trichoderma species as a promising source of bioactive secondary metabolites. *Mini-Rev. Med. Chem.* **2018**, *18*, 1702–1713.
30. Zhang, J.-C.; Chen, G.-Y.; Li, X.-Z.; Hu, M.; Wang, B.-Y.; Ruan, B.-H.; Zhou, H.; Zhao, L.-X.; Zhou, J.; Ding, Z.-T. Phytotoxic, antibacterial, and antioxidant activities of mycotoxins and other metabolites from trichoderma sp. *Nat. Prod. Res.* **2017**, *31*, 2745–2752.
31. Samson, R.A.; Hoekstra, E.S.; Frisvad, J.C. *Introduction to Food and Airborne Fungi*; Centraalbureau Voor Schimmelcultures (CBS): New York, NY, USA, 2004.
32. Chaverri, P.; Branco-Rocha, F.; Jaklitsch, W.; Gazis, R.; Degenkolb, T.; Samuels, G.J. Systematics of the trichoderma harzianum species complex and the re-identification of commercial biocontrol strains. *Mycologia* **2015**, *107*, 558–590.
33. Lind, A.L.; Wisecaver, J.H.; Lameiras, C.; Wiemann, P.; Palmer, J.M.; Keller, N.P.; Rodrigues, F.; Goldman, G.H.; Rokas, A. Drivers of genetic diversity in secondary metabolic gene clusters within a fungal species. *PLoS Biol.* **2017**, *15*, e2003583.
34. Samuels, G.J.; Dodd, S.L.; Gams, W.; Castlebury, L.A.; Petrini, O. Trichoderma species associated with the green mold epidemic of commercially grown *agaricus bisporus*. *Mycologia* **2002**, *94*, 146–170.
35. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. *PCR Protoc. A Guide Methods Appl.* **1990**, *18*, 315–322.

36. Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W.; Bolchacova, E.; Voigt, K.; Crous, P.W.; et al. Nuclear ribosomal internal transcribed spacer (its) region as a universal DNA barcode marker for fungi. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 6241–6246.
37. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780.
38. Maddison, D.R.; Maddison, W.P. *Macclade 4*; Sinauer: Sunderland, MA, USA, 2000.
39. Swofford, D.L. *Phylogenetic Analysis Using Parsimony*; Illinois Natural History Survey: Champaign, IL, USA, 1993.
40. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *J. Mol. Evol.* **1980**, *16*, 111–120.
41. Hafidh, R.R.; Abdulmir, A.S.; Vern, L.S.; Bakar, F.A.; Abas, F.; Jahanshiri, F.; Sekawi, Z. Inhibition of growth of highly resistant bacterial and fungal pathogens by a natural product. *Open Microbiol. J.* **2011**, *5*, 96.
42. Lai, H.Y.; Lim, Y.Y.; Tan, S.P. Antioxidative, tyrosinase inhibiting and antibacterial activities of leaf extracts from medicinal ferns. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1362–1366.
43. Zhu, Z.; Zhuang, W. Trichoderma (hypocrea) species with green ascospores from china. *Pers. Mol. Phylogeny Evol. Fungi* **2015**, *34*, 113.
44. Samuels, G.J.; Ismaiel, A.; Mulaw, T.B.; Szakacs, G.; Druzhinina, I.S.; Kubicek, C.P.; Jaklitsch, W.M. The longibrachiatum clade of trichoderma: A revision with new species. *Fungal Divers.* **2012**, *55*, 77–108.
45. Sandoval-Denis, M.; Sutton, D.A.; Cano-Lira, J.F.; Gené, J.; Fothergill, A.W.; Wiederhold, N.P.; Guarro, J. Phylogeny of the clinically relevant species of the emerging fungus trichoderma and their antifungal susceptibilities. *J. Clin. Microbiol.* **2014**, *52*, 2112–2125.
46. Jaklitsch, W.; Samuels, G.; Ismaiel, A.; Voglmayr, H. Disentangling the trichoderma viridescens complex. *Pers. Mol. Phylogeny Evol. Fungi* **2013**, *31*, 112.
47. Jaklitsch, W.M. European species of hypocrea part ii: Species with hyaline ascospores. *Fungal Divers.* **2011**, *48*, 1–250.
48. Park, M.S.; Oh, S.-Y.; Cho, H.J.; Fong, J.J.; Cheon, W.-J.; Lim, Y.W. Trichoderma songyi sp. Nov., a new species associated with the pine mushroom (tricholoma matsutake). *Antonie Van Leeuwenhoek* **2014**, *106*, 593–603.
49. Ze-Hong, W.; Dong, L.; Ying, X.; Jian-Liang, C.; Wen-Han, L. Antioxidant xanthenes and anthraquinones isolated from a marine-derived fungus aspergillus versicolor. *Chin. J. Nat. Med.* **2018**, *16*, 219–224.
50. Gogineni, V.; Hamann, M.T. Marine natural product peptides with therapeutic potential: Chemistry, biosynthesis, and pharmacology. *Biochim. Biophys. Acta* **2018**, *1862*, 81–196.
51. Douglas, T.E.; Dokupil, A.; Reczyńska, K.; Brackman, G.; Krok-Borkowicz, M.; Keppler, J.K.; Božič, M.; Van Der Voort, P.; Pietryga, K.; Samal, S.K. Enrichment of enzymatically mineralized gellan gum hydrogels with phlorotannin-rich ecklonia cava extract seanol® to endow antibacterial properties and promote mineralization. *Biomed. Mater.* **2016**, *11*, 045015.
52. Chang, T.-S. An updated review of tyrosinase inhibitors. *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475.
53. Mas-Chamberlin, C.; Mondon, P.; Lamy, F.; Peschard, O.; Lintner, K. Strategies of antiaging actives in sunscreen products. *Cosmet. Toilet.* **2006**, *121*, 45.
54. Zolghadri, S.; Bahrami, A.; Hassan Khan, M.T.; Munoz-Munoz, J.; Garcia-Molina, F.; Garcia-Canovas, F.; Saboury, A.A. A comprehensive review on tyrosinase inhibitors. *J. Enzyme Inhib. Med. Chem.* **2019**, *34*, 279–309.
55. Heo, Y.M.; Kim, K.; Ryu, S.M.; Kwon, S.L.; Park, M.Y.; Kang, J.E.; Hong, J.-H.; Lim, Y.W.; Kim, C.; Kim, B.S.; et al. Diversity and ecology of marine algicolous arthrini species as a source of bioactive natural products. *Mar. Drugs* **2018**, *16*, 508.
56. Porter, D. 13 mycoses of marine organisms: An overview of pathogenic fungi. In *The Biology of Marine Fungi*. Cambridge University Press: Cambridge CB2 1TN, UK, 1986. ISBN-13: 978-0521308991
57. Skaptsov, M.; Smirnov, S.; Kutsev, M.; Uvarova, O.; Sinitsyna, T.; Shmakov, A.; Matsura, A. Antifungal activity of several isolates of trichoderma against cladosporium and botrytis. *Ukr. J. Ecol.* **2018**, *8*, 88–91.
58. Vinodkumar, S.; Indumathi, T.; Nakkeeran, S. Trichoderma asperellum (nvta2) as a potential antagonist for the management of stem rot in carnation under protected cultivation. *Biol. Control* **2017**, *113*, 58–64.

59. Wu, Q.; Sun, R.; Ni, M.; Yu, J.; Li, Y.; Yu, C.; Dou, K.; Ren, J.; Chen, J. Identification of a novel fungus, *trichoderma asperellum* gdfs1009, and comprehensive evaluation of its biocontrol efficacy. *PLoS ONE* **2017**, *12*, e0179957.
60. Chen, J.S.; Wei, C.I.; Marshall, M.R. Inhibition mechanism of kojic acid on polyphenol oxidase. *J. Agric. Food Chem.* **1991**, *39*, 1897–1901.
61. May, O.; Moyer, A.; Wells, P.; Herrick, H. The production of kojic acid by *aspergillus flavus*. *J. Am. Chem. Soc.* **1931**, *53*, 774–782.
62. Parrish, F.; Wiley, B.; Simmons, E.; Long Jr, L. Production of aflatoxins and kojic acid by species of *aspergillus* and *penicillium*. *Appl. Microbiol.* **1966**, *14*, 139.
63. Wu, B.; Wu, X.; Sun, M.; Li, M. Two novel tyrosinase inhibitory sesquiterpenes induced by *cuc12* from a marine-derived fungus *pestalotiopsis* sp. Z233. *Mar. Drugs* **2013**, *11*, 2713–2721.
64. Tsuchiya, T.; Yamada, K.; Minoura, K.; Miyamoto, K.; Usami, Y.; Kobayashi, T.; Hamada-Sato, N.; Imada, C.; Tsujibo, H. Purification and determination of the chemical structure of the tyrosinase inhibitor produced by *trichoderma viride* strain h1-7 from a marine environment. *Biol. Pharm. Bull.* **2008**, *31*, 1618–1620.



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