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研究报告

Diversity of culturable extracellular proteases producing marine fungi isolated from the intertidal zone of Naozhou Island in South China Sea

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Abstract: [Objective] To investigate the diversity of marine fungi producing extracellular protease isolated from the intertidal zone of Naozhou Island in the South China Sea. **[Methods]** The samples of both seawater and sediment were collected from the intertidal zone of Naozhou Island in South China Sea, and used to investigate the diversity of the fungi producing extracellular proteases by culture-dependent method, protease producer plate assay, and a phylogenetic analysis based on the sequences containing internal transcribed spacer 1, 5.8S rRNA gene and internal transcribed spacer 2 (ITS1-5.8S-ITS2). **[Results]** A total of 198 isolates of fungi were isolated, purified and collected from the samples on the plates of Potato dextrose agar (PDA) medium prepared with 50% seawater. Amongst these isolates, 178 strains were identified successfully by PCR amplification, sequencing, BLAST and phylogenetic analysis of the ITS1-5.8S-ITS2 sequences. All these sequences showed ⩾98% identity with the existing relative ITS1-5.8S-ITS2 sequences in the GenBank database, except 10 strains showed the identity <97% with their closest match suggesting their possibility of being novel species. These 178 strains represented 66 species, belonging to 45 genera of 27 families in 16 orders, 6 classes, spreading in two phyla, Ascomycota and Basidiomycota. The predominant genus was *Penicillium*, which takes a great proportion of 28.70%, followed by *Aspergillus*, which takes a proportion of 11.24%. There were 83 identified strains showed clear zone around their colonies on the PDA plates supplemented with skim milk indicating they could produce extracellular proteases. **[Conclusion]** A total of 178 strains of fungi were isolated and identified from the intertidal zone of Naozhou Island in the South China Sea. Amongst these strains, 10 strains would be novel specie Candidates, and 83 strains could produce extracellular proteases.

Keywords: Extracellular protease, Culturable marine fungi, Intertidal zone, Naozhou Island, ITS1-5.8S-ITS2 sequence, Protease producer plate assay

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中国南海硇洲岛潮间带产胞外蛋白酶的可培养海洋真菌多样性

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摘 要: 【目的】研究中国南海硇洲岛潮间带产胞外蛋白酶的可培养海洋真菌多样性。【方法】从中国南海硇洲岛潮间带采集海水和沉积物样品，采用分离培养、蛋白酶生产菌平板检测法和基于内转录间隔区 1-5.8S rRNA 基因内转录间隔区 2 (ITS1-5.8S-ITS2)序列的系统进化分析法，研究产胞外蛋白酶真菌的多样性。【结果】采用 50%海水配制的马铃薯葡萄糖琼脂(PDA)固体培养板从所采集的样品中分离、纯化并收集了 198 株真菌分离株，并采用 ITS1-5.8S-ITS2 序列 PCR 扩增、测序、BLAST 和系统进化分析的方法成功鉴定了其中的 178 株。其中，有 10 株的 ITS1-5.8S-ITS2 序列与其在 NCBI 数据库中最匹配的 ITS1-5.8S-ITS2 序列的一致性<97，表明它们有可能是新的物种；其余 168 株的 ITS1-5.8S-ITS2 序列与 NCBI 数据库中已存在的相关 ITS1-5.8S-ITS2 序列的一致性均≥98%。这 178 株真菌归属为 66 个种，分布在子囊菌门和担子菌门的 6 纲，16 个目，27 个科的 45 个属中。其中的主要属为青霉菌属，占 28.70%；其次为曲霉属，占 11.24%。有 83 株真菌在加在脱脂奶粉的 PDA 固体培养板上的菌落周围有一透明圈，表明其可产生分泌胞外蛋白酶。【结论】从中国南海硇洲岛潮间带共分离、鉴定和收集了 178 株真菌，其中 10 株可能是新的物种，83 株为胞外蛋白酶生产菌。

关键词: 胞外蛋白酶，可培养海洋真菌，潮间带，硇洲岛，ITS1-5.8S-ITS2 序列，蛋白酶生产菌平板检测法

1 Introduction

Enzymes industry is one of the major industries in the world, and there exists a great market for further improvement in this field. The global market for industrial enzymes is estimated to be 3.3 billion dollars in 2010, and is expected to reach more than 4.4 billion dollars by 2015[1-2]. The most important industrial enzymes should be proteases, which account for about 60% of the total worldwide enzyme sales[2-3]. Proteases catalyzing the conversion of proteins to amino acids and peptides are widely used in several industrial applications such as detergent additives, leather processing, textile industry, food processing and pharmaceutical industry[4]. Proteases can be obtained from plants, animals and microorganisms, though the microorganisms show a great potential for protease production due to their broad diversity, renewable property, and susceptibility to genetic manipulation[4-5]. Many microorganisms, secreting enzymes including proteases to the external environment to degrade proteins and using the hydrolysis products as carbon and nitrogen sources for cell growth, are great potential sources of proteases[6].

The industrial demand of proteolytic enzymes, with appropriate specificity to substrates and stability to pH, temperature and chemical agents, continues to motivate the search for new sources[7]. Finding suitable enzyme candidates mainly depends on efficient and sensitive screening strategies and greater diverse candidate organisms. As for screening strategies, the traditional method of obtaining novel enzymes from microorganisms by cultivation and subsequent screening of pure strains of organisms is still a standard, powerful and feasible approach[8]. Amongst the microorganism sources of proteases, the bacterium has been studied extensively by many researchers, especially the genus *Bacillus*. Several *Bacillus* strains such as *B. pumilus*, *B. horikoshii*, *Geobacillus caldoproteolyticus*, *B. clausii*, *B. subtilis*, *B. sphaericus*, *B. pseudofirmus*, *B. aquimaris*, *B. circulans*, *B. licheniformis*, etc. are known to be used to produce industrially important proteases[9]. The proteases added as key ingredients in detergents account for approximately 25% of the total worldwide enzyme sales,

and nearly all detergent proteases currently used in the market are serine proteases produced using *Bacillus* sp.[10]. Recently, the fungi producing protease, such as *Conidiobolus coronatus*[11], *Penicillium* sp.[12-13], *Aspergillus* sp.[5,14-15] and *Engyodontium album*[10] were also studied for application in industry, whereas, marine fungi which have immense potential as the source of exoenzymes, including extracellular proteases, are yet to be used as source of proteases for commercial application.

The ocean covers approximately 71% or 361 million square kilometers of earth surface with a variable thermal range from the below freezing temperatures in Antarctic waters to about 350 °C in deep hydrothermal vents, pressure range from 1 to 1 000 atm, nutrient range from oligotrophic to eutrophic, and photic range from extensive photic to non-photic zones, which facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals, including fungi[16]. It was estimated that the number of marine fungi might be in excess of 10 000 species or phylotypes, including the described 537 obligate fungi, 100 facultative fungi, 1 500 marine yeasts, 500 misidentified fungi, 1 500 marine derived and sediment fungi, 300 deep-sea fungi, 500 planktonic fungi, and approximately 6 000 endophyte fungi of seaweeds, marine plants, and marine animals[17]. In our knowledge, the diverse marine fungi remain largely unexplored but have a strong potential for the discovery of proteases with unique properties for varied industrial applications. In this work, we attempted to investigate the diversity of marine fungi producing extracellular protease isolated from the intertidal zone of Naozhou Island (20.862 9°–20.946 8°N, 110.552 9°–110.644 9°E) in the South China Sea, which would provide more diverse candidates of marine fungi for discovering of novel protease.

2 Materials and Methods

2.1 Chemicals and samples

Lyticase, casein and most of chemicals for preparation of the media, including potato dextrose agar (PDA, g/L)(potato 200, dextrose 20, agar 16, and 50% sterile nature seawater 1 L), yeast extract peptone dextrose (YPD, g/L)(yeast extract 10, polypeptone 20, glucose 20 and 50% sterile nature seawater 1 L) and skim milk potato dextrose agar (SM-PDA, g/L) (skim milk powder 10, yeast extract 10, polypeptone 20, glucose 20 and 50% sterile nature seawater 1 L), were purchased from Sigma-Aldrich. Fungal Genomic DNA Extraction Kits were purchased from Bruker BioSpin. 2×*Taq* Master Mix were purchased from Vazyme Biotech, fungal PCR general primers were synthesized and the PCR products were sequenced by Shanghai sangon biological engineering co., ltd. All other chemicals used here were of analytical grade.

Different samples of both the seawater at about 20 cm depths from the surface of seawater and the sediment at about 10 cm depths from the surface of the seafloor were collected from four different sampling sites (20.866 8°N, 110.614 0°E; 20.887 4°N, 110.621 3°E; 20.918 3°N, 110.640 8°E, and 20.930 4°N, 110.644 3°E) in the intertidal zone along eastern coast of Naozhou Island (20.862 9°–20.946 8°N, 110.552 9°–110.645 2°E) in South China Sea, carried back to laboratory in an ice box, and stored at 4 °C for further experiments. The samples of seawater and sediment from sampling site (20.866 8°N, 110.614 0°E) are marked with AW and AS, that from sampling site (20.887 4°N, 110.621 3°E) are marked with BW and BS, that from (20.918 3°N, 110.640 8°E) are marked with CW and CS, and from (20.930 4°N, 110.644 3°E) are marked with DW and DS, respectively.

2.2 Isolation, purification and collection of marine fungi

2 g of the sediment samples were suspended in 20 mL of sterile nature seawater, and the supernatants and seawater samples were diluted suitably with sterile nature seawater according to the cell counts in the samples. 50 μL of the diluted samples were spread on the plates with seawater PDA supplemented with 0.1 g streptomycin and 10 000 units of penicillin/100 mL to inhibit bacterial growth. The plates were then incubated at 28 °C for some days, and the morphologically different colonies appeared on the plates were selected and further purified by repeatedly streaking on the same medium plates. The purified isolates were collected and stored both on seawater PDA slants at 4 °C till further analysis, and suspending in 20% glycerol, at −80 °C, and frozen in liquid nitrogen for storage.

2.3 Molecular identification of the fungal isolates

The fungal isolates maintained on seawater PDA slants were sub-cultured and grown at 28 °C for 14 days. Conidial inoculum for fermentation was prepared by dispersing the spores into sterile 0.1% Tween 80. The number of spores per milliliter in the spore suspension was determined with a cell counting chamber. The spore suspension was adjusted to the desired concentration

(about 2×10^8 cells/mL) after counting in a counting chamber.

The spore suspension were inoculated into a 3 mL of seawater YPD broth, incubated for 48 h at 28 °C. The fermentation broth was centrifuged at 1 000×*g* for 3 min, and the mycelia was collected and transferred into a eppendorf tube, suspended with 500 μL PBS (pH 7.0), added 100 U of lyticase, mixed and incubated at 37 °C for 1 h. The fungal genomic DNA was isolated with Fungal Genomic DNA Extraction Kits purchased from Bruker BioSpin according to the methods described in the manual.

The fragment of internal transcribed spacer 1 (ITS1), 5.8S rRNA gene and internal transcribed spacer 2 (ITS2) (ITS1-5.8S-ITS2) in fungal rDNA of each isolated strain was PCR amplified using fungal specifically universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al[5,18-20]. Amplification was carried out in 50 μL reaction mixture containing 20 μL 2×*Taq* Master Mix, 18 μL PCR water, 1 μL of each primer (0.5 μmol/L), and 1 μL (200 ng) DNA template, and conditioned as 5 min initial denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 52 °C and 1 min elongation at 72 °C; and a 7 min final elongation at 72 °C. The PCR products ITS1-5.8S-ITS2 sequences were separated with 1.5% agarose gel electrophoresis and sent to sequencing performed commercially by Sangon Biotech (Shanghai, China). The fungal ITS1-5.8S-ITS2 sequences obtained were aligned with known ITS1-5.8S-ITS2 sequences in the GenBank database using the basic local alignment search tool BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify fungal isolates.

2.4 Screening for fungi producing extracellular proteases

The isolates used to screening for fungi producing extracellular proteases were inoculated on the plates with seawater SM-PDA, and incubated at 28 °C for 24–72 h, and checked for each day the colonies showing around clearance zone produced by casein hydrolysis, which were considered as a positive fungi producing protease. The diameter of the around hydrolysis zone each positive fungal colonies was measured after incubating for 24, 48 and 72 h. Then, for each strain, the diameter of its colony and the diameter of the hydrolytic zone it produced were measured, and a ratio of the hydrolytic zone diameter to the colony diameter (hydrolytic zone/colony, H/C) was calculated[21].

2.5 Phylogenetic analysis of the fungal strains

Multiple sequence alignments of both obtained fungal ITS1-5.8S-ITS2 sequences and the reference sequences showing sequence homology from GenBank were performed using ClustalW. Phylogenetic trees were constructed with Phylip version 3.695 using a Neighbor-Joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1 000 replicates was performed.

2.6 Nucleotide sequence accession numbers

All the ITS1-5.8S-ITS2 sequences resulting from 178 fungal strains in this study have been deposited in the GenBank database under the accession numbers from KM277955 to KM278132.

3 Results

3.1 Isolation and identification of the marine fungi

The samples of both seawater and sediment at the same points were collected from four different sampling sites in the intertidal zone along eastern coast of Naozhou Island, in face of South China Sea. A total of 198 isolates of fungi were isolated, 110 isolates from seawater samples and 78 from sediment samples, and further purified by repeatedly streaking, on the plates of seawater PDA from these samples.

The genomic DNA of all fungal isolates was isolated and the ITS1-5.8S-ITS2 sequences of these fungal isolates were amplified by PCR technique. The amplified PCR products were separated by 1.5% agarose gel electrophoresis and viewed under the UV transilluminator (Figure 1), and sent to sequencing by Sangon Biotech. The PCR product, amplified using fungal universal primers ITS1 and ITS4, was a fragment of approximately 550 bp. This size corresponded to the expected size as compared to other fungi. Out of the total 198 fungal ITS1-5.8S-ITS2 sequences of collected isolates, 178 were sequencing successfully. These fungal ITS1-5.8S-ITS2 sequences were aligned respectively with known ITS1-5.8S-ITS2 sequences in the GenBank database using the basic local alignment search tool BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and percent homology scores were generated to identify fungal isolates (Table 1).

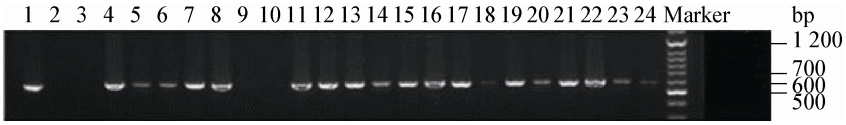

Figure 1 Agarose gel electrophoresis of PCR amplification products of ITS1-5.8S-ITS2 fragments of isolated strains
图 1 分离菌株的 ITS1-5.8S-ITS2 片段的 PCR 产物琼脂糖凝胶电泳图

Note: ITS1-5.8S-ITS2 fragments in rRNA gene of isolated strains was PCR amplified using fungal specifically universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and separated with 1.5% agarose gel electrophoresis.

注:分离菌株的 ITS1-5.8S-ITS2 片段是由引物 ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′)和 ITS4 (5′-TCCTCCGCTTATTGATATG C-3′)扩增的，并用 1.5%琼脂糖凝胶分离.

Table 1 The BLAST alignment analysis of the ITS1-5.8S-ITS2 sequences and the activity detection of extracellular protease of the marine fungi isolated from the intertidal zone of Naozhou Island in South China Sea
表 1 中国南海硇洲岛潮间带产胞外蛋白酶可培养海洋真菌 ITS1-5.8S-ITS2 序列的 BLAST 比对分析及其产胞外蛋白酶菌株的酶活性检测

Isolated strain number 分离菌株号	The closest identified ITS1-5.8S-ITS2 in GenBank GenBank 中一致性最高的 ITS1-5.8S-ITS2 序列			Isolated strains from 分离菌株来源		
	Scientific name 物种名称	Accession number 序列登录号	Identity 一致性 (%)	Water (sampling site) 水样(采样点)	Soil (sampling site) 土样(采样点)	Protease activity 蛋白酶活性
mf179	*Ascomycete* sp. RM2-5	DQ993625.1	99		CS	++
mf008	*Aspergillus aculeatinus*	JX406504.1	99	DW		+
mf003	*Aspergillus aculeatus*	HF545315.1	99	CW		−
mf013	*Aspergillus aculeatus*	JQ670921.1	99	AW		−
mf021	*Aspergillus aculeatus*	KC621081.1	99	CW		−
mf039	*Aspergillus aculeatus*	GU595032.1	100	BW		+
mf048	*Aspergillus aculeatus*	GU134884.1	99	BW		−
mf073	*Aspergillus aculeatus*	JX501384.1	100	CW		−
mf113	*Aspergillus aculeatus*	GU134884.1	99	BW		−
mf022	*Aspergillus aculeatus*	JX501375.1	99		DS	−
mf153	*Aspergillus sydowii*	JN850979.1	99		DS	++
mf172	*Aspergillus sydowii*	JN851001.1	99		CS	++++
mf184	*Aspergillus versicolor*	HQ285619.1	99		CS	+++
mf081	*Aspergillus versicolor*	KF986551.1	99		DS	++++
mf118	*Aspergillus versicolor*	FJ878625.1	99	AW		++
mf119	*Aspergillus versicolor*	KF954909.1	99	CW		++
mf126	*Aspergillus versicolor*	EU709775.1	99		DS	−
mf168	*Aspergillus versicolor*	EU833210.1	99	DW		++
mf133	*Aspergillus versicolor*	HQ285619.1	99		BS	−
mf166	*Aspergillus versicolor*	JN368459.1	99	CW		+++

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mf188	*Aspergillus versicolor*	JN368459.1	100		CS	+
mf092	*Beauveria bassiana*	HQ222971.1	100		CS	−
mf198	*Cladosporium sphaerospermum*	EU759978.1	99	CW		++++
mf043	*Cladosporium sphaerospermum*	KF938401.1	99	CW		+++
mf047	*Cladosporium sphaerospermum*	KC113297.1	99	CW		++
mf069	*Cladosporium sphaerospermum*	EU759978.1	98	BW		+++
mf070	*Cladosporium sphaerospermum*	KC113297.1	99	BW		−
mf125	*Cladosporium sphaerospermum*	JN084018.1	99	DW		++
mf146	*Cladosporium sphaerospermum*	KC113297.1	99	BW		++++
mf169	*Colletotrichum pisi*	GU934514.1	99	BW		++
mf007	*Corynespora cassiicola*	JN541214.1	99	BW		−
mf187	*Curvularia hawaiiensis*	FR717533.1	100	BW		−
mf052	*Curvularia lunata*	KF031026.1	100	DW		−
mf056	*Curvularia lunata*	KF031026.1	100	DW		+
mf045	*Daldinia eschscholtzii*	GU222391.1	99	CW		−
mf120	*Daldinia eschscholtzii*	KC895542.1	99	CW		−
mf062	*Diaporthe phaseolorum*	GU066628.1	98		DS	−
mf170	*Emericellopsis terricola*	FJ713093.1	99		DS	++
mf164	*Eutypella scoparia*	HM052827.1	99		AS	+++
mf156	*Flavodon flavus*	JQ638521.1	99	DW		−
mf159	*Flavodon flavus*	JQ638521.1	99	DW		+
mf020	*Fusarium chlamydosporum*	EU214561.1	100	AW		−
mf112	*Fusarium chlamydosporum*	EU214561.1	99	AW		−
mf090	*Fusarium decemcellulare*	HM775326.1	99	BW		−
mf167	*Fusarium decemcellulare*	AB587017.1	99	CW		+
mf030	***Fusarium decemcellulare***	**HM775326.1**	**93**		BS	++
mf066	*Fusarium decemcellulare*	HM775326.1	99		BS	++
mf186	*Fusarium solani*	KF751072.1	99	BW		++++
mf009	*Gibberella moniliformis*	GU723435.1	99	AW		−
mf192	*Gliomastix polychroma*	AB540548.1	99	CW		+++
mf028	***Gliomastix* sp. HSAUP063167**	**FJ946481.1**	**86**		BS	−
mf191	*Humicola fuscoatra*	GU183113.1	99		AS	−
mf180	*Hypocreales* sp. KH00321	GU017526.1	98	BW		−
mf094	*Hypoxylon investiens*	JN979428.1	99	DW		+
mf005	*Lasiodiplodia theobromae*	KJ381073.1	99	AW		−
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mf017	*Lasiodiplodia theobromae*	JX275790.1	99	AW		−
mf023	*Lasiodiplodia theobromae*	JX945583.1	99	AW		−
mf027	*Lasiodiplodia theobromae*	JX945583.1	100	AW		−
mf034	*Lasiodiplodia theobromae*	JX868613.1	99	AW		−
mf105	*Lasiodiplodia theobromae*	JX868613.1	99	AW		−
mf111	*Lasiodiplodia theobromae*	JX945583.1	100	AW		−
mf002	*Lasiodiplodia theobromae*	JQ658976.1	99	AW		−
mf165	*Leaf litter ascomycete* strain its342	AF502849.1	98		AS	−
mf117	*Lentinus squarrosulus*	GU001951.1	99		AS	+
mf010	*Microsphaeropsis arundinis*	HM049173.1	100	BW		++
mf177	*Microsphaeropsis arundinis*	JN851034.1	99	BW		+
mf091	*Microsphaeropsis arundinis*	AB775571.1	100	BW		++
mf171	*Microsphaeropsis arundinis*	JQ344344.1	99	CW		−
mf173	*Microsphaeropsis arundinis*	EF094551.1	98		DS	++
mf044	*Musicillium theobromae*	AJ292422.1	99	DW		−
mf157	*Myrmecridium schulzeri*	JN851038.1	99		BS	+
mf123	*Myrmecridium schulzeri*	KF986544.1	99	BW		+++
mf095	*Neofusicoccum parvum*	JX096632.1	99	BW		−
mf054	*Nigrospora oryzae*	KF192823.1	98	DW		−
mf185	*Paecilomyces formosus*	HQ444388.1	99	BW		−
mf175	*Paecilomyces formosus*	FJ389929.1	99		BS	−
mf114	***Peniophora lycii***	**JX046435.1**	**93**	AW		+++
mf176	*Paecilomyces formosus*	FJ389929.1	99		BS	−
mf182	*Paraconiothyrium cyclothyrioides*	KC215138.1	99	CW		+
mf195	*Paraconiothyrium cyclothyrioides*	KC215138.1	100		DS	++
mf181	*Paraphaeosphaeria* sp. HKA26	DQ092509.1	100		CS	++
mf100	*Paraphaeosphaeria* sp. HKA26	DQ092509.1	99		BS	++
mf194	*Paraphaeosphaeria* sp. HKA26	DQ092509.1	99		DS	++
mf025	*Paraphaeosphaeria* sp. HKA26	DQ092509.1	99		CS	+
mf064	*Paraphaeosphaeria* sp. HKA26	DQ092509.1	99		CS	++
mf193	***Pleosporales* sp. E9321b**	**JN545763.1**	**93**		DS	−
mf042	*Paraphaeosphaeria* sp. HLS214	FJ770071.1	98	CW		++
mf143	*Paraphaeosphaeria* sp. QTYC11	KJ469553.1	99		BS	−
mf174	*Paraphoma radicina*	JN851036.1	99	CW		++
mf011	*Penicillium chermesinum*	AY742693.1	97		BS	−
mf050	*Penicillium chermesinum*	AY742693.1	98	AW		+

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mf084	*Penicillium chermesinum*	AY742693.1	99	DW		−
mf162	*Penicillium chermesinum*	AY742693.1	99	DW		−
mf130	*Penicillium citreonigrum*	GU934550.1	99		BS	−
mf141	*Penicillium citreonigrum*	GU934550.1	99	CW		+
mf103	*Penicillium citreonigrum*	GU934550.1	99		BS	−
mf160	*Penicillium citreonigrum*	EU497959.1	99	CW		++
mf178	*Penicillium citrinum*	KJ173539.1	99		DS	++++
mf024	*Penicillium citrinum*	KJ173539.1	99		BS	++++
mf031	*Penicillium citrinum*	EU645682.1	99	BW		+++
mf057	*Penicillium citrinum*	JQ647899.1	99	CW		++
mf078	*Penicillium citrinum*	JN851040.1	99	AW		−
mf080	*Penicillium citrinum*	EU645682.1	99		AS	++++
mf082	*Penicillium citrinum*	JQ647899.1	99		DS	−
mf099	*Penicillium citrinum*	KJ173539.1	99		BS	++
mf110	*Penicillium citrinum*	HQ889713.1	99	DW		++++
mf121	*Penicillium citrinum*	JX192960.1	99	CW		++++
mf128	*Penicillium citrinum*	JX192960.1	99	DW		+++
mf139	*Penicillium citrinum*	EU821333.1	99	DW		−
mf147	*Penicillium citrinum*	KF986420.1	99		DS	++++
mf149	*Penicillium citrinum*	HQ608026.1	99	CW		−
mf151	*Penicillium citrinum*	JQ082503.1	99	DW		+++
mf161	*Penicillium citrinum*	KF999813.1	99	DW		−
mf040	*Penicillium citrinum*	JQ082503.1	100		AS	+++
mf059	*Penicillium citrinum*	KF999813.1	99	BW		+++
mf068	*Penicillium citrinum*	JQ082503.1	99	CW		−
mf124	*Penicillium citrinum*	JN851046.1	99	BW		+++
mf197	*Penicillium citrinum*	JF793520.1	99		AS	++++
mf032	*Penicillium citrinum*	HQ671192.1	99		CS	+
mf108	*Penicillium citrinum*	HQ671192.1	99	BW		++++
mf037	*Penicillium corylophilum*	JN585947.1	100	CW		−
mf006	*Penicillium gerundense*	EU427291.1	98	AW		−
mf041	*Penicillium gerundense*	EU427291.1	98		BS	−

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(续表)

mf148	*Penicillium gerundense*	EU427291.1	97	CW		−
mf051	*Penicillium griseofulvum*	KF811439.1	99		BS	+++
mf152	*Penicillium griseofulvum*	KF572453.1	99		AS	++++
mf154	*Penicillium indicum*	AY742699.1	97		CS	−
mf104	*Penicillium janthinellum*	GU565146.1	99		AS	+
mf014	*Penicillium oxalicum*	JN676116.1	99		DS	−
mf029	*Penicillium toxicarium*	FJ557247.1	99		AS	−
mf065	*Penicillium toxicarium*	FJ557247.1	99	BW		+
mf087	*Penicillium toxicarium*	KJ173540.1	99	CW		−
mf098	*Penicillium toxicarium*	KJ173540.1	99	AW		+
mf106	*Penicillium toxicarium*	KJ173540.1	99	BW		++
mf131	*Penicillium toxicarium*	KJ173540.1	99	DW		−
mf134	*Penicillium toxicarium*	KJ173540.1	99	DW		−
mf140	*Penicillium toxicarium*	JX140942.1	99		BS	−
mf144	*Penicillium toxicarium*	KJ173540.1	100	BW		++
mf026	*Penicillium toxicarium*	KJ173540.1	99		AS	−
mf129	***Penicillium toxicarium***	**KJ173540.1**	**93**	BW		−
mf038	*Penicillium verruculosum*	JQ717338.1	99	DW		+
mf036	*Pestalotiopsis vismiae*	JX305708.1	99	DW		+
mf196	*Phaeosphaeriopsis musae*	JX156375.1	99	CW		+++
mf122	*Phaeosphaeriopsis musae*	JX156375.1	99		AS	+++
mf158	*Phanerochaete chrysosporium*	AF475147.1	99	DW		−
mf135	***Phanerochaete chrysosporium***	**AF475147.1**	**95**		BS	−
mf189	*Plectosphaerella cucumerina*	EU594566.1	98	BW		−
mf132	***Pseudolagarobasidium belizense***	**NR_120036.1**	**95**	CW		−
mf089	*Pyrenochaetopsis decipiens*	KC427074.1	99	DW		+++
mf190	*Rigidoporus vinctus*	HQ400710.1	99	CW		−
mf088	*Subulicystidium longisporum*	JQ905612.1	99		DS	+
mf096	***Stachybotrys chartarum***	**KF999001.1**	**95**		DS	−
mf150	***Setophoma terrestris***	**KF512828.1**	**87**		AS	−

(待续)

						(续表)
mf115	*Subulicystidium longisporum*	JQ905612.1	97		DS	++++
mf085	*Talaromyces marneffei*	KF990134.1	99		CS	−
mf102	*Talaromyces verruculosus*	JF682635.1	99	DW		−
mf071	*Trametes versicolor*	JQ886402.1	99	DW		−
mf018	*Trametes versicolor*	JQ886402.1	99		DS	−
mf116	*Trichoderma harzianum*	KF053675.1	98	DW		−
mf015	*Trichoderma harzianum*	JX173851.1	99	CW		−
mf016	*Trichoderma harzianum*	FJ412026.1	99	DW		−
mf049	*Trichoderma harzianum*	KC847190.1	99		DS	−
mf058	*Trichoderma harzianum*	FJ517550.1	99		DS	−
mf060	*Trichoderma harzianum*	KC847190.1	99	CW		−
mf061	*Trichoderma harzianum*	KF986660.1	98	CW		−
mf063	*Trichoderma harzianum*	FJ517550.1	99		DS	−
mf072	*Trichoderma harzianum*	JX173851.1	99		DS	−
mf074	***Trichoderma harzianum***	**JX173834.1**	**96**	BW		+++
mf075	*Trichoderma harzianum*	JX173851.1	99		DS	−
mf076	*Trichoderma harzianum*	JX069199.1	98	DW		−
mf079	*Trichoderma harzianum*	JN716380.1	99	DW		−
mf097	*Trichoderma harzianum*	JX232597.1	98	BW		−
mf101	*Trichoderma harzianum*	JX173851.1	99	DW		−
mf107	*Trichoderma harzianum*	FJ517550.1	99		DS	−
mf109	*Trichoderma harzianum*	KC576701.1	99	DW		−
mf163	*Trichoderma harzianum*	JX173847.1	100	DW		−
mf183	*Verticillium theobromae*	AJ292422.1	98		CS	+
mf053	*Westerdykella purpurea*	FJ624258.1	98		CS	++
Number of strains				110	68	83

Note: The protease activity levels were designated on the basis of the *D* value (the difference between the transparent circle diameter and the diameter of the colony) of the transparent circle produced by casein hydrolysis as: ++++: *D*=8−10 mm; +++: *D*=5−7 mm; ++: *D*=2−4 mm; +: *D*=0−1 mm; −: No transparent circle. Sequences showing <97% identity values are represented in bold. The samples of seawater and sediment from (20.866 8°N, 110.614 0°E) are marked with AW and AS, that from (20.887 4°N, 110.621 3°E) are marked with BW and BS, from (20.918 3°N, 110.640 8°E) are marked with CW and CS, and from (20.930 4°N, 110.644 3°E) are marked with DW and DS, respectively.

注: 蛋白酶的活性水平根据酪蛋白水解产生的透明圈 *D* 值(透明圈直径与菌落直径的差值)分别定义为：++++：*D*=8−10 mm；+++：*D*=5−7 mm；++：*D*=2−4 mm；+：*D*=0−1 mm；−：没有透明圈. 序列最相似的百分比<97%以粗体表示. 采样点(20.866 8°N，110.614 0°E)来源的海水样品和沉积物样品分别标记为 AW 和 AS，采样点(20.887 4°N，110.621 3°E)来源的分别标记为 BW 和 BS，采样点(20.918 3°N，110.640 8°E)来源的分别标记为 CW 和 CS，采样点(20.930 4°N，110.644 3°E)来源的分别标记为 DW 和 DS.

3.2 Diversity of culturable marine fungi

These 178 strains represented 66 species, belonging to 45 genera, in more than 27 families, 16 orders, 6 classes, spreading in two phyla, Ascomycota and Basidiomycota. The predominant genus is *Penicillium* which takes a great proportion of 28.7%, followed by *Aspergillus* which takes a proportion of 11.2%, and then *Trichoderma* which takes a proportion of 10.1% (Table 2). All the ITS1-5.8S-ITS2 sequences from these178 strains showed ≥97% similarity with the existing fungal ITS1-5.8S-ITS2 sequences in the GenBank database, except 10 strains including mf028, mf030, mf043, mf074, mf096, mf114, mf129, mf132, mf135 and mf150, showed a similarity <97% with their closest match (Table 3).

Table 2 Groups distribution of 178 isolated fungal strains and the strains with extracellular protease activities
表 2 178 株分离菌株及产胞外蛋白酶菌株的类群分布

Distribution groups 类群分布					Number of strains 菌株数			Strains with protease activity 蛋白酶菌株数		
Phylum 门	Class 纲	Order 目	Family 科	Genus 属	From water 水	From soil 泥沙	Total 总共	From water 水	From soil 泥沙	Total 总共
Ascomycota	Ascomycete	?	?	?		2	2		1	1
	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	*Lasiodiplodia*	8		8			
				Neofusicoccum	1		1			
		Capnodiales	Cladosporiaceae	*Cladosporium*	7		7	6		6
		Pleosporales	Corynesporascaceae	*Corynespora*	1		1			
			Cucurbitariaceae	*Pyrenochaetopsis*	1		1	1		1
			Sporormiaceae	*Westerdykella*		1	1		1	1
			Montagnulaceae	*Paraphaeosphaeria*	1	6	7	1	5	6
				Paraconiothyrium	1	1	2	1	1	2
			Phaeosphaeriaceae	*Paraphoma*	1		1	1		1
				Phaeosphaeriopsis	1	1	2	1	1	2
				Setophoma		1	1			
			Pleosporaceae	*Curvularia*	3		3	1		1
			?	?		1	1			
	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	12	8	20	6	5	11
				Penicillium	30	21	51	16	11	27
			Thermoascaceae	*Paecilomyces*	1	2	3			
			Trichocomaceae	*Talaromyces*	2	1	3	1		1
	Sordariomycetes	Diaporthales	Diaporthaceae	*Diaporthe*		1	1			
		Glomerellales	Glomerellaceae	*Colletotrichum*	1		1	1		1
			Plectosphaerellaceae	*Musicillium*	1	1	2		1	1
				Plectosphaerella	1		1			
		Hypocreales	Bionectriaceae	*Gliomastix*	1	1	2	1		1
			Cordycipitaceae	*Beauveria*		1	1			
			Hypocreaceae	*Trichoderma*	12	6	18	1		1
			Nectriaceae	*Fusarium*	6	2	8	2	2	4
			?	?	1		1			

(待续)

(续表)

			?	*Emericellopsis*		1	1		1	1
			?	*Stachybotrys*		1	1			
		Sordariales	Chaetomiaceae	*Humicola*		1	1			
		Trichosphaeriales	?	*Nigrospora*	1		1			
		Xylariales	Amphisphaeriaceae	*Pestalotiopsis*	1		1	1		1
			Diatrypaceae	*Eutypella*		1	1		1	1
			Xylariaceae	*Daldinia*	2		2			
				Hypoxylon	1		1	1		1
		?	?	*Myrmecridium*	1	1	2	1	1	2
	?	?	?	*Microsphaeropsis*	4	1	5	3	1	4
Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	*Phanerochaete*	1	1	2			
				Subulicystidium		2	2		2	2
		Polyporales	Coriolaceae	*Rigidoporus*	1		1			
				Trametes	1	1	2			
			Lentinaceae	*Lentinus*		1	1		1	1
			?	*Flavodon*	2		2	1		1
			Phanerochaetaceae	*Pseudolagarobasidium*	1		1			
		Russulales	Peniophoraceae	*Peniophora*	1		1	1		1
Number of strains					110	68	178	48	35	83

Note: The closest identified ITS1-5.8S-ITS2 in GenBank has no corresponding Class or Order or Family or Genus are represented in?.
注：分离菌株的 ITS1-5.8S-ITS2 序列在 GenBank 中同一性最高的物种没有相应纲、或目、或科、或属以?表示.

Table 3 BLAST alignment analysis of the ITS1-5.8S-ITS2 sequences of 10 potential novel species
表 3 10 个潜在新物种 ITS1-5.8S-ITS2 序列的 BLAST 比对分析

Strains of novel candidates 潜在新物种菌株	ITS1-5.8S-ITS2 sequences of the closest identified species in GenBank GenBank 中最相似物种的 ITS1-5.8S-ITS2 序列		
	Scientific name 物种名称	Accession number 登录号	Identity 一致性(%)
mf028	*Gliomastix* sp. HSAUP063167	FJ946481.1	86
mf030	*Fusarium decemcellulare*	HM775326.1	93
mf043	*Ascomycete* sp. RM2-5	DQ993625.1	93
mf074	*Trichoderma harzianum*	JX173834.1	96
mf096	*Stachybotrys chartarum*	KF999001.1	95
mf114	*Peniophora lycii*	JX046435.1	93
mf129	*Penicillium toxicarium*	KJ173540.1	93
mf132	*Pseudolagarobasidium belizense*	NR_120036.1	95
mf135	*Phanerochaete chrysosporium*	AF475147.1	95
mf150	*Setophoma terrestris*	KF512828.1	87

3.3 The fungi producing extracellular proteases

All 178 strains, identified by ITS1-5.8S-ITS2 rRNA gene sequence analysis, were inoculated on the plates with seawater SM-PDA and incubated at 28 °C. Amongst the 178 strains, 83 strains showed clear zone around their colonies on the plates indicating they could produce extracellular proteases (Figure 2, Table 2). These 83 strains of extracellular proteases producing marine fungi belonged to 41 species, distributed in 27 genera, 16 families, 13 orders, 6 classes, spreading in Ascomycota and Basidiomycota. The predominant genus is *Penicillium* which contained 27 strains taking a great proportion of 32.5%, followed by *Aspergillus* which contained 11 strains taking takes a proportion of 13.3%.

3.4 Phylogenetic analysis of the fungi producing extracellular proteases

The ITS1-5.8S-ITS2 sequences from the fungi producing extracellular proteases were aligned with the reference sequences showing sequence homology from GenBank using ClustalW. Phylogenetic trees were constructed with Phylip version 3.695 using a Neighbor-Joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1 000 replicates was performed (Figure 3).

4 Discussion

Marine fungi are an ecological rather than a taxonomic group and comprise two distinct groups. The obligate marine fungi grow and sporulate exclusively in marine aquatic habitat, and their spores are capable of germinating in sea water. On the other hand, facultative marine fungi are those originate from fresh water or terrestrial environment and adapt to marine conditions that allow them to grow and possibly also sporulate in the marine environment[22-23]. Most of the strains isolated here are facultative marine fungi. The predominant genus of the strains is *Penicillium* which takes a great proportion of 28.7%, followed by *Aspergillus* which takes a proportion of 11.2% (Table 2), both of which are the common genera of facultative marine fungi isolated from diverse terrestrial and marine habitats[23-24]. *Penicillium* and *Aspergillus* were found to be notably sodium chloride tolerance with the majority of their species able to grow in the presence of more than 20% or more sodium chloride[25-26], and have been isolated from several marine habitats including marine sediments, and deep-sea hydrothermal vents[23,26-28].

The genomic DNA of all fungal isolates was isolated and the ITS1-5.8S-ITS2 sequences of these fungal isolates were amplified by PCR technique using fungal universal primers ITS1 and ITS4[5,18-20]. The amplified PCR products, separated by 1.5% agarose gel electrophoresis and viewed under the UV transilluminator, was a fragment of approximately 550 bp (Figure 1). Although, The primer pair of ITS1 and ITS4, which amplify the entire of ITS1-5.8S-ITS2 region, were the most commonly used primers published early in the 1990[20]. An *in silico* approach using EcoPCR revealed the potential PCR biases of the primers including

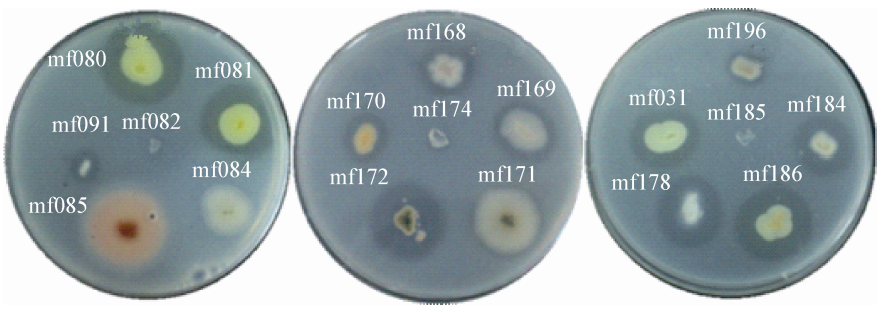

Figure 2 Screening fungi producing extracellular proteases on the potato dextrose agar plates supplemented with skimmed milk

图 2 在含有脱脂奶的 PDA 平板上产胞外蛋白酶菌株的筛选

Note: The isolated strains were inoculated on the plates of potato dextrose agar supplemented with skimmed milk, incubated at 28 °C for 24–72 h, and checked each day for the colonies showing around clearance zone produced by casein hydrolysis, which were considered as a positive fungi extracellular proteases.

注：被分离的菌株接种在含有脱脂奶粉的 PDA 平板上，28 °C 培养 24−72 h，每天检查菌落周围由于菌株分泌扩散出的蛋白酶使酪蛋白水解而出现溶解圈的阳性菌株.

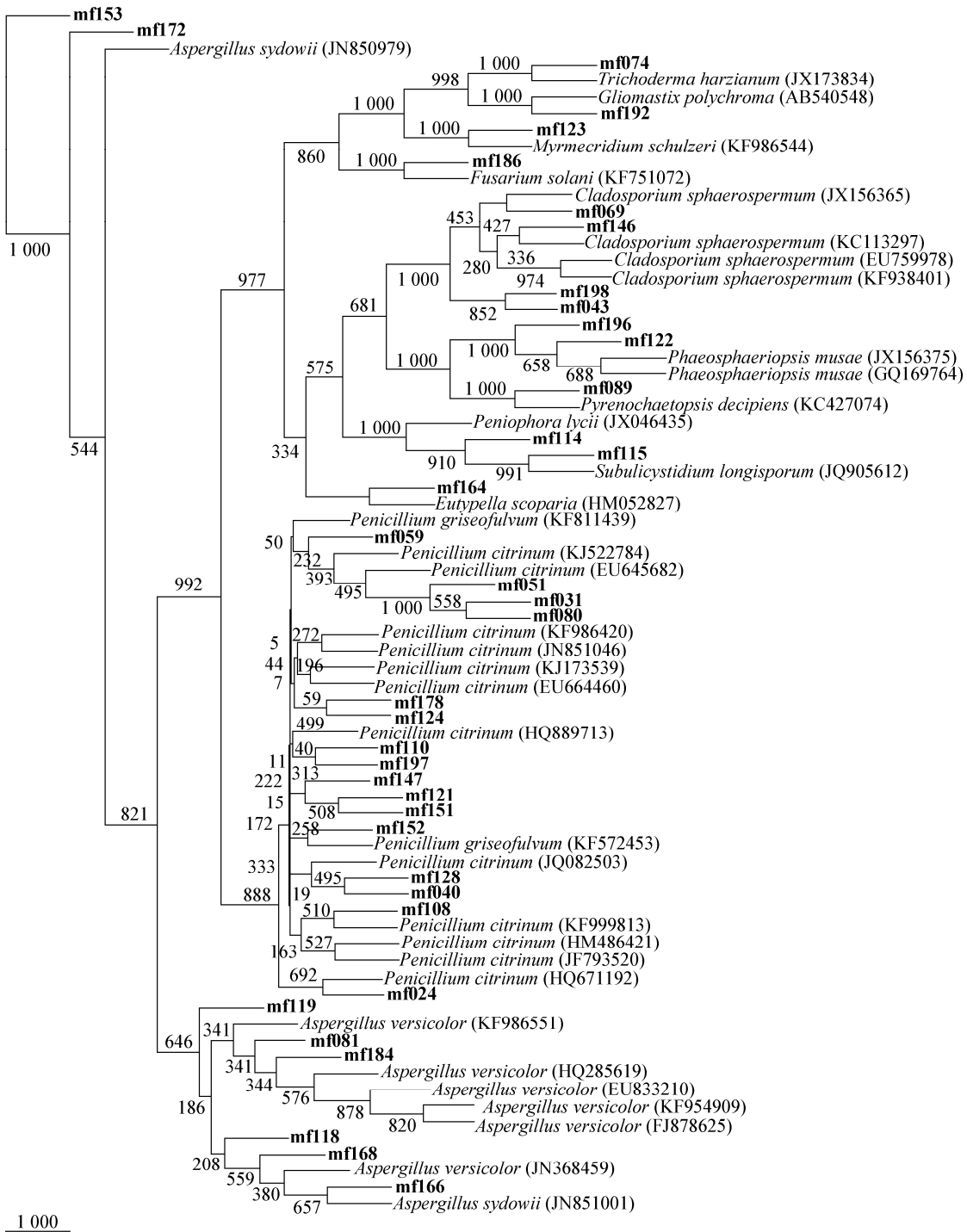

Figure 3 Phylogenetic tree of ITS1-5.8S-ITS2 sequence of the isolated fungal strains with obvious extracellular protease activity (≥++) and appropriate fungal species in GenBank database

图 3 分离蛋白酶活性(≥++)菌株与 GenBank 数据库中相应真菌菌种的 ITS1-5.8S-ITS2 序列系统进化树

Note: The number of fungal strains isolated in this study are indicated in bold. Multiple Sequence alignment was performed using ClustalW. Phylogenetic tree was constructed with Phylip version 3.695 using a Neighbor-Joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1 000 replicates was performed.

注：分离的真菌菌株号用加粗表示，采用 ClustalW 两两比对，用 Phylip 软件按照邻接法聚类(选择 Bootstrap 检验值≥50%，1 000 次重复)进行系统树的构建.

ITS1 and ITS4 which only amplified about 92% of the fungal ITS sequences when allowing three mismatches[29]. Here in this study, out of the total 198 fungal ITS1-5.8S-ITS2 sequences from collected isolates, 178 (89.9%) were sequencing successfully, and 20 (10.1%) could not be amplified by PCR methods using primers of ITS1 and ITS4. It is suggested that different primer pairs targeting different parts of the ITS1-5.8S-ITS2 region should be used to analyze in parallel.

Comparing with terrestrial fungi, marine fungi have been poorly investigated, which means marine environments are habitats hosting previously unexplored fungi[17,30]. Out of the total 178 marine fungal strains isolated here, 10 strains (6%) including mf028, mf030, mf043, mf074, mf096, mf114, mf129, mf132, mf135 and mf150 showed a similarity <97 % (from 86% to 96%) with their closest match *Gliomastix* sp. HSAUP063167, *Fusarium decemcellulare*, *Pleosporales* sp. E9321b, *Trichoderma harzianum*, *Stachybotrys chartarum*, *Peniophora lycii*, *Penicillium toxicarium*, *Pseudolagarobasidium belizense*, *Phanerochaete chrysosporium* and *Setophoma terrestris*, respectively (Table 3), suggesting their possibility of being novel species[31-33]. A multigene analysis combined with detailed morphological and ultra structural studies will aid in deciding their novelty[31].

Method using the agar plates supplemented with deferent concentration of skimmed milk is still the standard method of screening for proteolytic activity[34-35]. The clearance zone appeared around the colonies on the plate indicated proteolytic activity positive. Fungi as enzyme producers have many advantages, since they are generally regarded as safe and the produced enzymes are extracellular which makes its easy recuperation from fermentation broth. Reports available on protease produced by fungi are mainly about the genera *Aspergillus*, *Penicillium* and *Rhizopus*[36]. Among these genera, *Aspergillus* have a long history of use as producers of secreted proteins including glucoamylase, amylase, pectinases and proteases. They are capable of growing on inexpensive media prepared with solid agro-industry by products and secrete bulk quantities of extracellular enzymes[5,37]. About 47% of the marine fungal strains isolated here, including 27 strains belonging to *Penicillium* sp. and 11 strains belonging to *Aspergillus* sp., were found to be able to produce extracellular proteases by this method (Figure 2, Table 2). This result indicated that marine fungi remain largely unexplored but have a strong potential for the discovery of proteases with unique properties for varied industrial applications.

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