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Identification of two marine fungi and evaluation of their antivirus and antitumor activities

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Abstract: [Objective] To identify two marine fungi and evaluate the inhibitory effects of their crude extracts on Tobacco mosaic virus and two tumor cell lines. [Methods] Crude extracts was obtained by extracting with MeOH and evaporated in vacuo. The extracts was water-soluble fraction which was dissolved in water, and the other fraction was water insoluble. The fungi were identified by morphology and Internal Transcribed Spcer (ITS) rDNA molecular methods. The inhibitory effect on Tobacco mosaic virus was evaluated by indirect enzyme linked immuno- sorbent assay, and the anti-tumor activity was tested by methyl thiazolyl tetrazolium method. [Results] The fungi were identified as *Penicillium oxalicum* and *Neosartorya fischeri* There crude extracts inhibited Tobacco Mosaic Virus and two tumor cell lines. The active fraction named $0312F_1$ inhibited Tobacco Mosaic Virus and tumor cell lines and was water-soluble. The fraction named $1008F_1$ inhibited Tobacco Mosaic Virus and was insoluble in water, whereas the fraction inhibited tumor cell lines was water-soluble. [Conclusion] The active fraction named $0312F_1$ inhibited Tobacco Mosaic Virus was different from that named $1008F_1$ inhibited Tobacco Mosaic Virus. The active fraction named $0312F_1$ inhibited tumor cell lines was the same as that named $1008F_1$. Furthermore, the inhibitory activity of water-soluble fraction named $0312F_1$ against BEL-7404 cell line was much higher than that against SGC-7901 cell lines, whereas the inhibitory activity of active fraction named $1008F_1$ against SGC-7901 cell line was much higher.

Keywords: marine fungi; inhibitory activity; Tobacco mosaic virus (TMV); anti-tumor; identification
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The oceans are unique resources that provide a diverse array of natural products[1-2]. The marine environment has more than a million described species[3]. Microorganisms are increasingly exploited as a source of new pharmaceuticals[4]. Due to their huge numbers, their activities have a global impact on Earth. This is particularly relevant when considering marine microorganisms[5]. The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of new drugs[6]. Over the past few years, about 3000 new compounds from various marine sources have been reported and some have entered clinical trials[7-8].

In this paper, we describe the isolation, biological activities of 50 strains isolated from marine organisms. Finally, we identified 2 marine fungi with both higher inhibitory activities against cancer cells proliferation and TMV replication under both morphology and molecular methods.

1 MATERIALS AND METHODS

1.1 Meterials

1.1.1 Culture media: PDA solid and liquid media, Gause's medium.

1.1.2 Cancer cell lines: SGC-7901 and BEL-7404

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tumor cell lines were provided by Hepatobiliary Surgery Institute of Fujian Province, Union Hospital affiliated with Fujian Medical University.

1.1.3 PCR reaction primers: ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'[9], ITS4: 5'-TCCTCCGCTTATTGATATGC-3'[10].

1.1.4 Reagents: All PCR reactions related reagents were bought from TaKaRa company. DNA mag-extracted kit was bought from TOYOBO company. Other reagents were all analytic reagents.

1.2 Sample collection, isolation and cultivation of fungi and actinomycetes

In September, 2006, 14 samples were collected at Changle beach in Fujian, Fuzhou, China. The samples included sea sediments, sea weeds, crabs, sea anemones, several types of snails, medusa and mussels, et al.

The strains were isolated by the following procedures[11-12], then, the plates were incubated at 28℃ for 3 to 20 days. After that, the isolates were grown on PDA solid and liquid media. After different periods of incubation, the broths were harvested[13].

1.3 Cancer cell lines culture conditions

Cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 IU/ml of penicillin-streptomycin in an atmosphere of 5% CO_2 at 37 ℃.

1.4 Extraction procedure

The fermentation broth was extracted using the methods described in our previous work[14]. The other fraction was water insoluble fraction. The extracts were dissolved in DMSO, and diluted to different concentrations.

1.5 Bioactivity assays

The inhibitory activity against TMV replication was performed using the methods described in our previous work[15].

The inhibitory activity against cancer cells proliferation was performed using MTT assay[16]. 5'-Fluoruoracial at a concentration of 50μg/mL was as the positive control. Experiments were conducted in triplicate.

1.6 IC_{50} measurement

IC_{50} values were determined by the inhibitory activities against cancer cell proliferation and TMV replication. The 50% inhibitory concentration for each extract was calculated from concentration-effect-curves after linear regression analysis.

1.7 Morphology identification of fungi with bioactivity

Fungi were identified to the genus level based on microscopic morphology. The results were according to Handbook of fungal identification[17].

1.8 Molecular identification of fungi with bioactivity

The fungi genomic DNA were extracted using DNA mag-extracted kit. The sequences of the ITS regions were amplified by PCR reactions using primers of ITS1 and ITS4. PCR reactions were carried out in a final volume of 25 μL (1 μL genomic DNA template, 12.75 μL ddH_2O, 5 μL Takara PCR buffer, 5 μL dNTPs (2.5 mol/L), 0.5 μL of each primer (10 μmol/L), and 0.25 μL Qiagen HotStarTaq enzyme). After an initial denaturation for 10 min at 95℃, the reaction was run for 36 cycles with the following parameters: denaturation for 1 min at 94℃, annealing for 1 min at 50℃ and extension for 1 min at 72℃. Final extension followed for 10 min at 72℃. Subsequently, the PCR products were sequenced and compared with the sequence reported in GeneBank. Sequence homology trees were constructed by DNAMAN 6.0.40, and phylogenetic trees were constructed under maximum likelihood (ML) method.

1.9 Statistical analysis

Statistical analysis was performed using SPSS 13.0. One-way ANOVA was used to analyze statistical comparisons between groups. Differences with *P*-values less than 0.05 were considered to be statistically significant.

2 RESULTS

2.1 Bioactivities of the fermentation broths of all isolates

The extracts from the fungi $0312F_1$ and $1008F_1$ showed both anti-TMV replication and anti-tumor activities (higher than 50%) (Table 1). To isolate the active fractions of these two fungi, the fermention broth was extracted with MeOH.

Table 1 The taxa with anti-TMV replication, anti-tumor activity

Strains No.	Inhibition rate[a](%)	Inhibition rate[b](%)	Inhibition rate[c](%)	Strains No.	Inhibition rate[a](%)	Inhibition rate[b](%)	Inhibition rate[c](%)
$0101F_2$	30.27	36.92	12.79	$0702F_2$	—	48.25	46.73
$0102F_1$	34.02	—	1.07	$0701F_3$	10.16	45.67	36.36
$0103F_1$	—	56.00	65.17	$0702F_3$	46.70	35.13	20.73
$0301F_1$	—	20.85	35.22	$0801F_1$	18.95	41.22	41.69
$0302F_1$	21.88	3.38	41.88	$1001F_1$	66.56	21.82	26.35
$0303F_1$	—	54.24	13.09	$1002F_1$	60.96	15.18	68.97
$0304F_1$	—	11.03	1.81	$1003F_1$	44.69	21.90	42.49
$0305F_1$	19.23	32.38	58.72	$1004F_1$	32.57	47.98	41.57
$0306F_1$	—	17.07	4.53	$1005F_1$	47.02	20.54	10.65
$0307F_1$	27.23	16.84	14.76	$1006F_1$	—	18.88	7.36
$0308F_1$	39.20	21.39	1.76	$1007F_1$	57.96	29.22	36.36
$0309F_1$	42.14	—	59.29	$1008F_1$	65.89	63.60	56.84
$0311F_1$	—	32.05	20.39	$1009F_1$	40.14	13.52	60.25
$0312F_1$	65.55	52.24	84.61	$1010F_1$	59.63	35.88	19.89
$0313F_1$	58.12	33.93	23.77	$1011F_1$	—	26.81	6.23
$0317F_1$	31.69	63.77	49.94	$1001F_2$	41.25	1.53	31.41
$0318F_1$	—	25.76	13.36	$1002F_2$	20.97	58.75	63.58
$0301F_2$	64.46	34.96	21.89	$1101F_1$	51.95	34.59	1.83
$0302F_2$	47.98	46.62	16.14	$1102F_1$	30.05	19.67	13.27
$0303F_2$	10.71	73.85	68.56	$1104F_1$	—	30.39	12.07
$0301F_3$	23.58	31.12	45.53	$1105F_1$	25.65	35.70	21.49
$0302F_3$	66.49	35.10	56.03	$1301F_2$	13.66	—	31.2
$0402F_1$	26.96	2.40	52.99	$1302F_2$	42.12	35.19	—
$0403F_1$	27.68	1.97	16.88	$1401F_3$	19.02	43.79	21.17
$0502F_1$	46.98	31.56	38.59	$1001B_1$	18.77	18.41	30.17
$0701F_1$	55.07	38.81	—	$1101B_1$	28.98	6.20	13.25

Fungi fermentation broth diluted in 10^{-1}.

[a]: anti-TMV replication rates of fermentation broth of strains. [b]: anti-tumor activity rates of fermentation broth of strains against SGC-7901 cell line. [c]: anti-tumor activity rates of fermentation broth of strains against BEL-7404 cell line.

2.2 Bioactivities of the MeOH extracts of two fungi

The anti-TMV replication and anti-tumor fractions of stain $0312F_1$ were both water soluble, the anti-TMV replication fraction of stain $1008F_1$ was insoluble in water, whereas the anti-tumor fraction was also water soluble (Table 2).

Table 1 The IC_{50} values of two fungi with bioactivity ($*$ $P < 0.05$)

No.	IC_{50}[a] (mg/mL ± SD)	IC_{50}[b] (mg/mL ± SD)	IC_{50}[c] (mg/mL ± SD)
A1	0.554* ± 0.23	2.921* ± 0.24	0.107* ± 0.08
C1	1.807 ± 0.17	244.119 ± 12.36	1.604 ± 0.54
A2	2.136 ± 0.92	0.020* ± 0.01	0.198* ± 0.06
C2	0.871* ± 0.42	1.130 ± 0.35	0.908 ± 0.10

Extracts concentration diluted in 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL.

A1: water-soluble fraction of MeOH extracts of strain $0312F_1$. A2: water-soluble fraction of MeOH extracts of strain $1008F_1$. C1: water insoluble fraction of MeOH extracts of strain $0312F_1$. C2: water insoluble fraction of MeOH extracts strain $1008F_1$.

[a]: IC_{50} values of extracts of two fungi of anti-TMV replication rates, [b]: IC_{50} values of extracts of two fungi of anti-tumor activity rates against SGC-7901 cell line, [c]: IC_{50} values of extracts of two fungi of anti-tumor activity rates against BEL-7404 cell line.

One-way ANOVA was used to analyze statistical comparisons between groups A1 and C1, A2 and C2, separately.

2.3 Morphology identification of two active fungi

The morphologic character of strain $0312F_1$ was similar to *Penicillium sp*. based on microscopic morphology after growing on the solid medium for about 7 days at 28℃ (Fig. 1).

The morphologic character of strain $1008F_1$ was similar to *Aspergillus sp*. based on microscopic morphology after growing on the solid medium for about 7 days at 28℃ (Fig. 2).

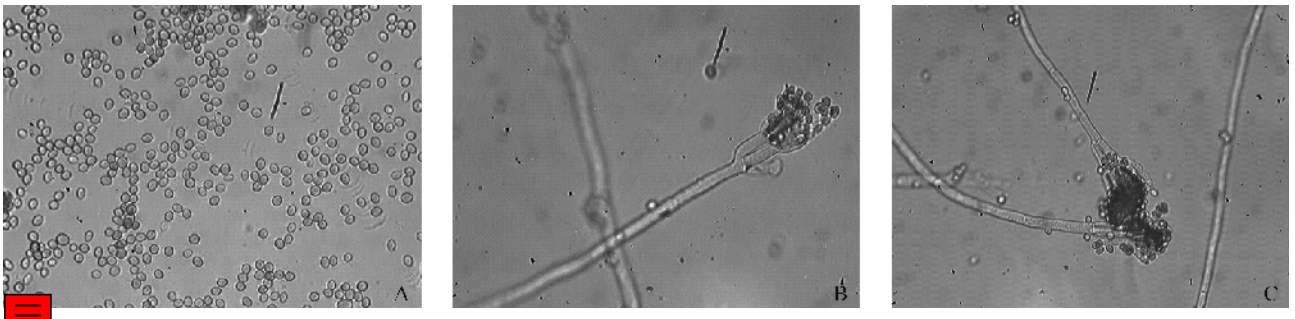

Fig.1 Morphology of strain $0312F_1$. A: Conidia of strain $0312F_1$ (400×); (B) Conidiophores of strain $0312F_1$ (400×). (C) Conidia and conidiophores of $0312F_1$ (400×).

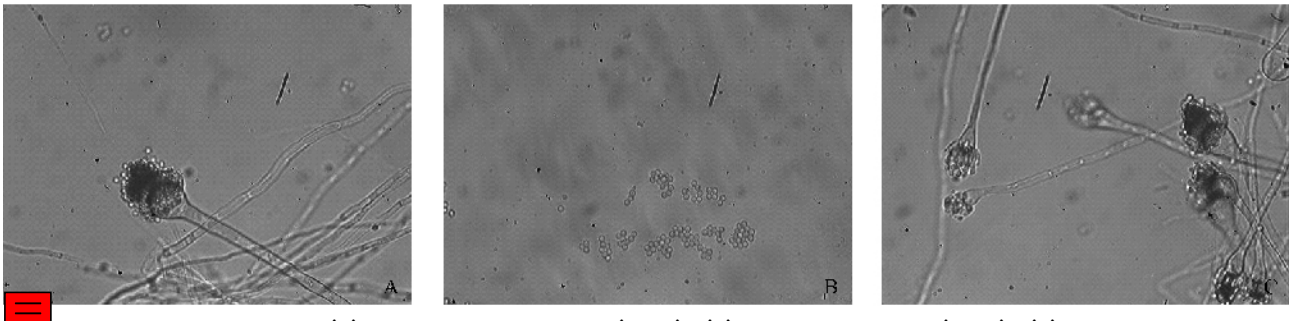

Fig.2 Morphology of strain $1008F_1$. (A) Conidiophores of strain $1008F_1$ (400×); (B) Conidia of strain $1008F_1$ (400×); (C) Conidia and conidiophores of $1008F_1$ (400×).

2.4 Molecular identification of two active fungi

Comparison study supported a strong relationship between strain $0312F_1$ (GenBank accession number: EU926977) and members of *Penicillium sp.*, and particularly revealed the highest homology (100%) with EF103455.1 (*Penicillium oxalicum*). Homology relationship of closely related microorganisms is shown (Fig. 3).

Comparison study supported a strong relationship between strain $1008F_1$ (GenBank accession number: EU926976) and members of *Neosartorya sp.* (sexual phase of *Aspergillus sp.*) and particularly revealed the highest homology (100%) with AF176661.1 (*Neosartorya fischeri*). Homology relationship of closely related microorganisms is shown (Fig. 4).

3 DISCUSSIONS

There have been many researches about Isolation of *Penicillium sp.* and *Aspergillus sp.* from marine organisms in china particularly from Mangrove[18, 19]. So strains in *Penicillium sp.* and *Aspergillus sp.* are universal. But most of them are associated with diverse bioactivity, for example, antitumor activity[20], antimicrobial activity[21-25], and activity associated producing related enzymes[26-28]. Strains associated with anti-phytovirus activity are less reported compared to these.

The fermentation broths of most of the strains showed anti-tumor activities against two cell lines (Table 1), but the inhibition rates against SGC-7901 cell line were lower than those against BEL-7404 cell line. It might be a result of tumor cell lines specificity.

Since the 1990s, marine-derived fungi have been recognized as a rich source of novel bioactive metabolites[29]. The IC_{50} values (Table 2) of the crude extracts were in a range of 0.020 mg/mL to 244.119 mg/mL, from which the active fractions were identified. A lot of anti-tumor and anti-microbial compounds of alkaloids have been isolated from strains in *Aspergillus sp.*[30, 31], suggesting that active fractions might consist of alkaloids to a certain extent. Our further research will emphasize isolation of the active compounds from the extracts from fermentation broth of the two fungi.

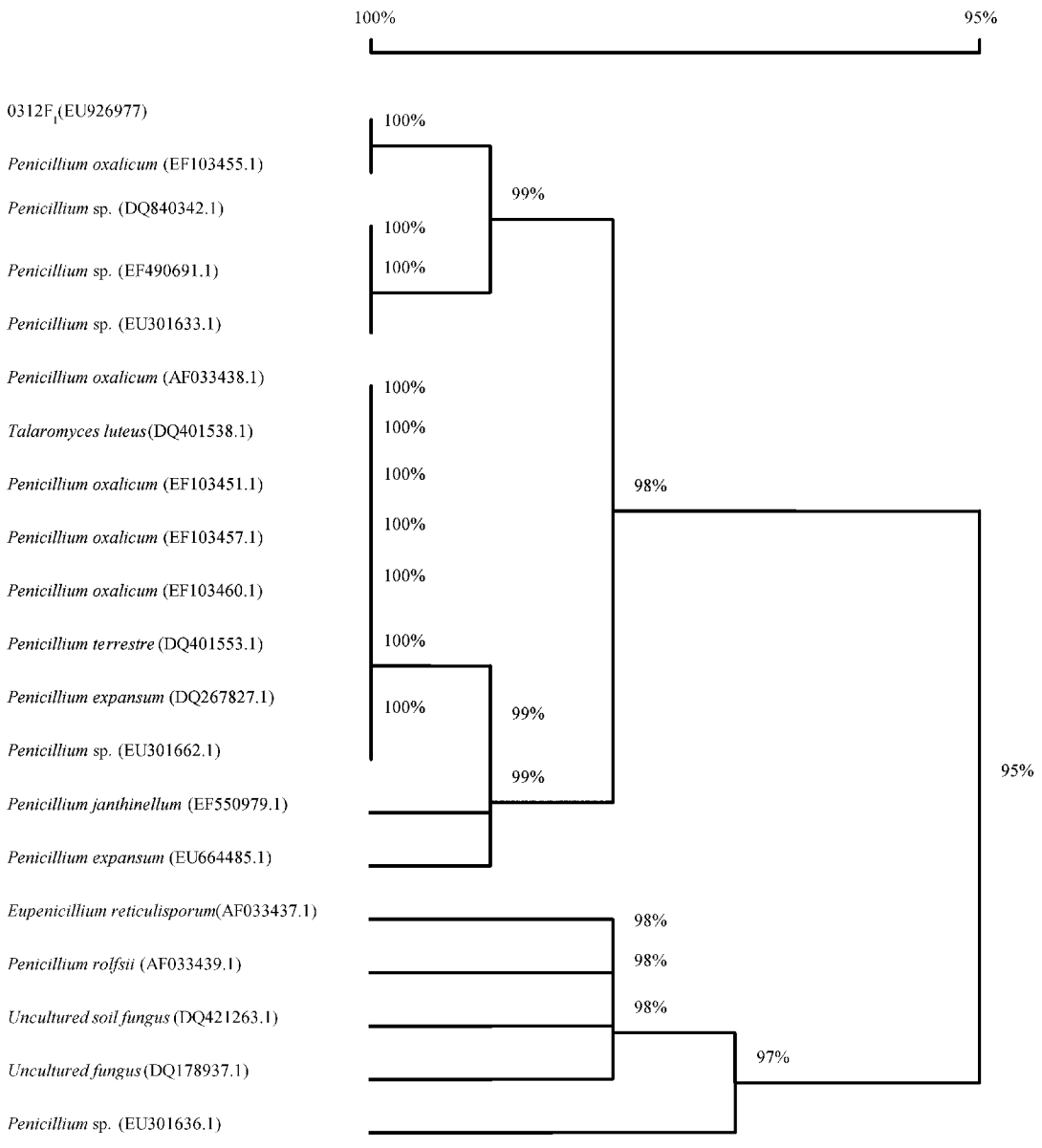

Fig.3 Homology tree of strain $0312F_1$ isolated to other fungi from GeneBank, deduced from sequence of ITS rDNA. Numbers in parentheses represent the sequences accession numbers in Genbank. The bootstrap values (in percent) calculated illustrated homology relationship between strain $0312F_1$ and other strains. Bar, 95% – 100% homology persentage.

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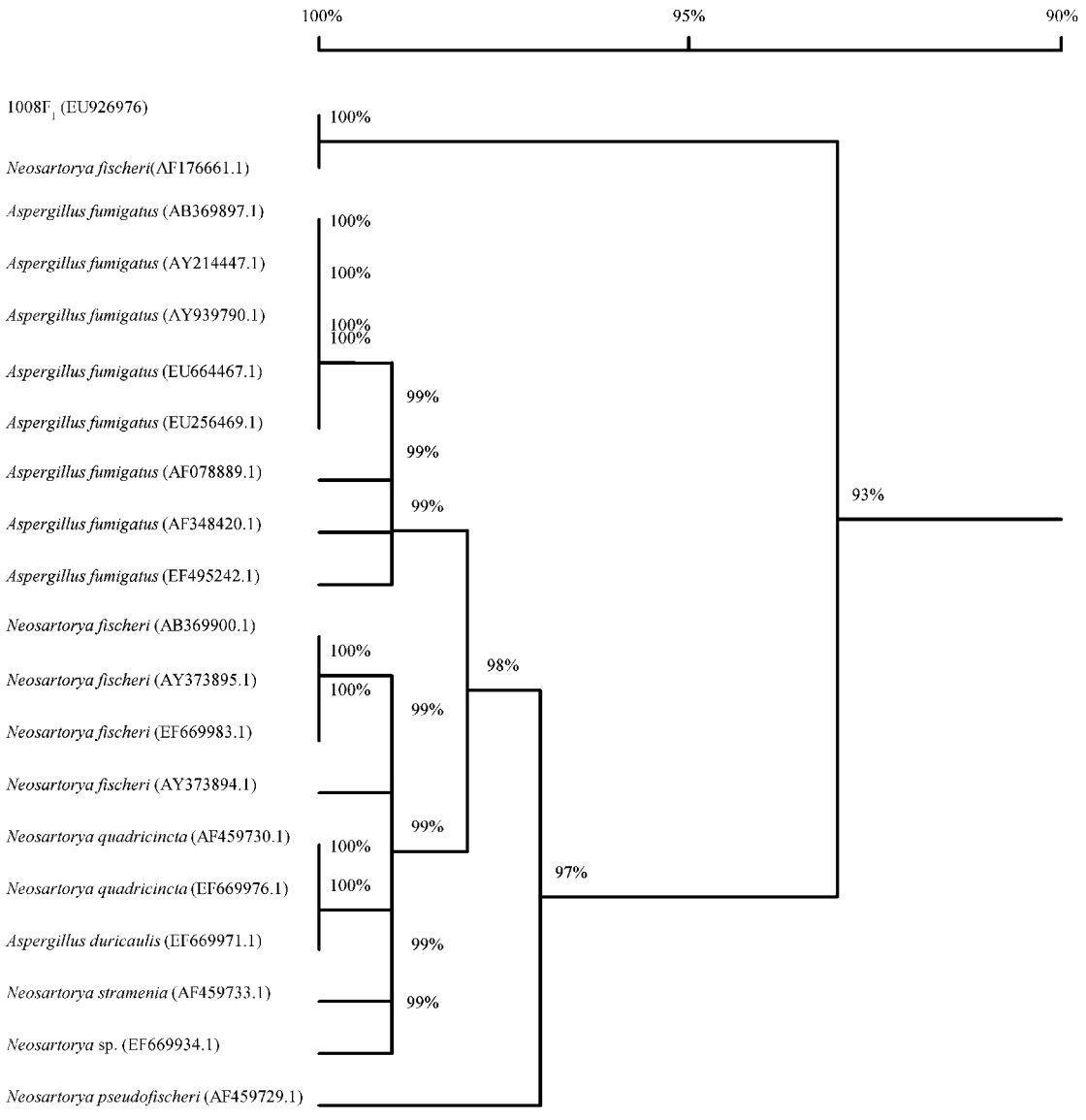

Fig.4 Homology tree of strain $1008F_1$ isolated to other fungi from GeneBank, deduced from sequence of ITS rDNA. Numbers in parentheses represent the sequences accession numbers in Genbank. The bootstrap values (in percent) calculated illustrated homology relationship between strain $1008F_1$ and other strains. Bar, 90% – 100% homology persentage.

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两株海洋真菌的鉴定及其次级代谢产物抑制烟草花叶病毒及抗肿瘤活性

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摘要:【目的】通过对 2 株活性海洋真菌发酵产物提取物抑制烟草花叶病毒和抗肿瘤活性进行研究,为进一步得到活性纯品化合物作为抗病毒及抗肿瘤的先导化合物奠定基础。【方法】菌株发酵产物的粗提物是通过甲醇浸取并在真空条件下蒸干得到的。粗提物中溶于水的部分为水溶性部分,不溶于水的部分为脂溶性部分。通过间接酶联免疫法检测样品抑制烟草花叶病毒的活性,通过四甲基偶氮唑盐微量酶反应比色法(MTT 法)检测样品抗肿瘤活性,通过形态及 ITS rDNA 序列法进行菌株鉴定。【结果】两株海洋真菌抑制烟草花叶病毒活性和抗肿瘤的活性均较高。分子鉴定结果显示,两株真菌分别与 *Penicillium oxalicum* 和 *Neosartorya fischeri* 的同源性极高。菌株 $0312F_1$ 发酵液的水溶性部分具有抗病毒及抗肿瘤活性,菌株 $1008F_1$ 发酵液的脂溶性部分具有抑制烟草花叶病毒活性,而水溶性部分具有抗肿瘤活性。【结论】菌株 $0312F_1$ 和菌株 $1008F_1$ 发酵液的提取物抑制烟草花叶病毒的活性部位不同,而抗肿瘤活性部位相同。菌株 $0312F_1$ 发酵液提取物的水溶性活性部位对肝癌细胞 BEL-7404 的抑制效果比对胃癌细胞 SGC-7901 的抑制效果明显,而菌株 $1008F_1$ 发酵液提取物的水溶性活性部位对胃癌细胞 SGC-7901 的抑制效果比对肝癌细胞 BEL-7404 的抑制效果明显。

关键词:海洋真菌 抑制活性 烟草花叶病毒(Tobacco Mosaic Virus, TMV) 抗肿瘤 鉴定

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