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Isolation and Characterization of Medicinally Important Marine *Penicillium* Isolates

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Authors' Contributions
IA performed experimental work, analyzed the data and wrote the article. SQ helped in experimental work and analysis of data. GLL performed molecular studies. ZMC and MR helped in data analysis. SA, KSH and ZMC helped in preparation of manuscript.

Key words
Yellow sea, *Penicillium* species, ITS, Internal transcribed spacers.

ABSTRACT

The Yellow Sea is one of the mega diversity centers of the world and contains vast amount of flora and fauna. The *Penicillium* species belongs to one of the most common and economically significant group of marine micro-fungi family. The study comprised isolation, microscopic observations and ribotyping, allowed discrimination of the marine fungal isolates as *Penicillium viticola* F1, *Penicillium restrictum* F2, *Penicillium rubens* F8, *Penicillium implicatum* F10, *Penicillium piceum* F11, *Penicillium oxalicum* F12, *Penicillium sumatrense* F15, and *Penicillium vinaceum* F16. This study demonstrated that Yellow Sea can be considered as a valuable natural source of *Penicillium* species, which can be used for the production of medicinally important different secondary metabolites and organic acids.

INTRODUCTION

The Yellow Sea refers to golden sand particles from Gobi Desert that turn the surface of the water golden yellow during sand storm. Yellow Sea is a marginal sea of the Pacific Ocean that is located between mainland China and the Korean Peninsula. The marine environment harbors plenty of microbes including bacteria, yeasts and fungi which are not macroscopic. These microbes contribute up to 90% hidden majority of ocean biomass (Dolan, 2014).

The marine environment has been increasingly investigated in the search for new microorganism with capability to produce commercially essential products. Besides, marine-derived fungi represent a large, but still little explored potential source of new biological active metabolites, due to the high level of biodiversity found in the various marine ecosystems, and their capability to adjust their metabolism to environmental conditions (Grovel *et al*., 2008). The *Penicillium* spp. belongs to the most common fungal group, which can be isolated easily due to their spore's distribution all over the place (Myung *et al*., 2014; Samson *et al*., 2010). The *Penicillium* genus has capability to produce a wide range of compounds that are useful or harmful to humans, including: organic acids, insecticides, antioxidants, herbicides, antibiotics, mycotoxins and anticancer compounds which make it a genus of interest. The marine environment harbors extreme physiological conditions such as temperature, pressure and salinity which make the marine-derived *Penicillium* species more important and sole sources of unique bioactive compounds under stressed conditions (Myung *et al*., 2014).

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The taxonomy of different *Penicillium* spp. has been considerably studied (Pitt, 1979). Identification of *Penicillium* is mainly performed by phenotypic criteria, including macro and micro-morphology which mainly depends on the growth and sporulation of the respective fungus. Though many biochemical and physiological techniques have provided information useful for the classification, the nucleotide sequence and the organization of DNA are the most likely to give a clear and sensitive distinction between the organisms and indicate clearly their evolutionary and phylogenetic relationship (Cardoso *et al*., 2007). Thus, the comparative nucleotide sequence analysis of the conserved ribosomal RNA (rRNA) genes provide a different and unique way of analysis over a wide range of taxonomic levels by demonstrating the phylogenetic relationships. The small subunit rRNA gene has been used broadly for phylogenetic studies and for the classification purpose at genus or species level in prokaryotes and eukaryotes. The rRNA operon has ITS region which is located between the 18S and 28S rRNA genes and includes the two highly conserved overriding regions that is, ITS1 and ITS2 which are mainly used in phylogenetic analysis (Rasime *et al*., 2013).

This work was aimed to isolate *Penicillium* strains from marine environment of Yellow Sea and to identify these *Penicillium* spp. by using the micro and macro-morphological parameters, as well as molecular base identification, analysing the nucleotide sequences of internal transcribed spacer (ITS).

MATERIALS AND METHODS

Cultivation media

Fungal isolation, colony and cell morphology for fungal strains identification were done by using potato dextrose

agar (PDA), malt extract agar (MEA) and Czapek yeast extract agar (CYA) media (purchased from Zhengzhou Sino Chemical Co., Ltd, China), supplemented with 0.05% chloramphenicol (Qayyum *et al*., 2016; Khan *et al*., 2014; Nonaka *et al*., 2011; Kurtzman and Fell, 2000).

Isolation of fungal strains

The seawater (2ml), sea sediment (2g), marine algae and marine fish collected from different places at Yellow Sea were immediately suspended in 50 ml of sterile PDA medium supplemented with 0.5 g/l chloramphenicol in 250-ml shaking flasks after the sampling and cultivated at 28°C for 5 days. After suitable dilution of the cell cultures, the cells were plated on the potato dextrose agar (PDA) medium with 0.5 g/l chloramphenicol, and the plates were incubated at 28°C for 5 days. Subsequently, the different mold colonies were isolated, purified, and then the pure strains were preserved on the PDA plants and stored at -80°C. Finally, eight fungal strains (F1, F2, F8, F10, F12, F15 and F16) were obtained in this study.

Identification of fungal isolates

Microscopic identification

All the fungal strains isolated above were inoculated into PDA for colony characterization and microscopic analysis. The plates containing fungal isolates were incubated in the dark at 28°C for 6 days. Microscopic observations were done by using Olympus U-LH100HG fluorescent microscope with 40x objective. Images were recorded using the cell Sens Standard software as described by Wang *et al*. (2013).

Molecular identification

Fungal mycelium for each of eight fungal strains (F1, F2, F8, F10, F12, F15 and F16) isolated as mentioned above were prepared from its pure cultures grown in 50 ml of YPG broth. Mycelia of each strain from 50 ml broth were harvested separately by centrifugation at 12,000 rpm for 10 min and the cell pellets were used for the fungal genomic DNA extraction by following the modified method of Al-Samarrai and Schmid (2000).

To estimate phylogenetic associations among all the fungal isolates and the typical strains reported at the National Center for Biotechnology Information (NCBI), amplification and sequencing of internal transcribed spacer (ITS) from the fungal strains were performed using the primers IT5: 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ (Pederson *et al*., 1997). For amplification purpose PCR cycler named GeneAmp PCR System 2400 made by Perkin–Elmer was used. The reaction mixture system (25 µl) was composed of 10 × buffer 2.5 µl, ITS-F or ITS-R 0.5 mM, dNTP 0.8 mM, DNA template 1.0 µl, Taq DNA polymerase 1.25 U and H_2O 16.6 µl. The PCR amplification was done by using following conditions: preliminary denaturation at 94°C for 10 min, former denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, finishing extension at 72°C for 10 min. This PCR was processed for 32 cycles. Finally, the 1% agarose gel electrophoresis was used for PCR products separation (Khan *et al*., 2014b) and gel band containing desired product were recovered by using TIANgel Midi Purification kit (Tiagen Biotech, Beijing, China). The transformationof the recovered PCR product into competent cells of *E. coli* DH5α was done by ligation it to pMD19-T simple vector and transformation. The transformant *E. coli* selection was performed on plates supplemented with ampicillin. The plasmids extraction of the transformant *E. coli* cells was done by using method as illustrated by Sambrook *et al*. (1989). The ITS fragments inserted on the vector were sequenced by Genescript Company, Nanjing, China.

Phylogenetic analysis

The sequences obtained as mentioned above were aligned using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences which showed over 98% similarities with currently available sequences were regard as the same species. Furthermore, multiple alignments were performed using Clustal X 1.83 and MEGA 4.0 was used for the construction of the phylogenetic tree (Tamura *et al*., 2007).

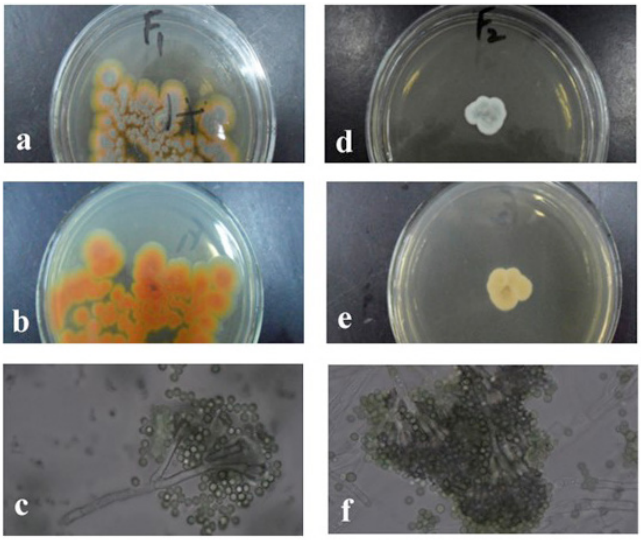

Fig. 1. The colony morphology of the strain F1 (*Penicillium viticola*) F2 (*Penicillium restrictum*) (a, front; b, back; c, mycelium and the chains of its conidia).

RESULTS

Fungal isolates

The colonies of the strain F1 grown on PDA medium were of characteristic of *Penicillium viticola* (Nonaka *et al*., 2011). The front colonies of F1 strain on the PDA plate were 34–35 mm in diameter after 7 days at 25°C (Fig. 1a), radically sulcate and velutinous. Its mycelia were at the pink margins and the colonies were covered with olive gray conidia in their center. Their margins were entire and soluble pigment was not produced. Colonies from back side

were red (Fig. 1b). The mycelium of the strain F1 grown on PDA usually consisted of an extremely branched complex network of septate, colorless and multinucleate hyphae and its fruiting body that was conidiophores consisted of the chains of conidia that resembled a broom which was an essential feature of *Penicillium* species (Fig. 1c).

Macroscopic features of F2 strain when grown on potato dextrose agar (PDA) medium for 7 days at 25°C, showed colony diameter 18–27 mm, plane or umbonate, radially and concentrically sulcate or wrinkled, low to moderately deep, mycelium white, texture velutinous to slightly floccose, exudate clear to yellow-brown, red-brown soluble pigment occasionally produced (Fig. 1d), back side was typically pale to pale grey, less commonly light yellow or red brown (Fig. 1e). Furthermore, their sporulation was light to moderate with broom like conidial spores (Fig. 1f), indicating that all the characteristics of F2 strain resembled those of *Penicillium restrictum* (Andrade *et al*., 2014).

Furthermore, the colonies of the strain F8 grown on PDA plate showed characteristics of *Penicillium ruben* (Houbraken *et al*., 2011). The characteristics of the colony were documented after 7 days of incubation at 25°C. The F8 strain typically consisted of pale grey colony with an extremely branched systematic network of multinucleate mycelium, colorless and septate hyphae, with pale yellow back side (Fig. 2a, b) and also possessed the chains like arrangements of conidia which resembled a broom indicating basic characteristics of *Penicillium* species (Fig. 2c).

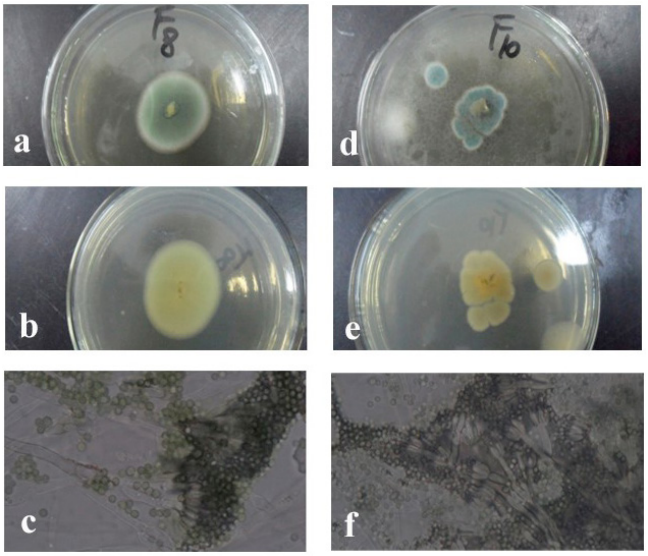

Fig. 2. The morphologies of the colony of the strain F8 (*Penicillium ruben*) (a, front; b, back; c, mycelium and the chains of its conidia and the strain) F10 (*Penicillium implicatum*) (d, front; e, back; f, mycelium and the chains of its conidia).

F10 strain colonies were 20–25 mm diameter with surface velutinous, to some extent floccose in the center, margins arachnoid, plane, centrally raised; mycelium white, sporadically prominent in the centers; intense colonies with abundant sporulation, bluish grey; lacking exudates and produced dull reddish-brown or yellow soluble pigment (Fig. 2d), back side of colonies were yellowish to reddish brown (Fig. 2e), having conidiophore with broom like structure (Fig. 2f) when grown on PDA, medium at 25°C for 7 days which resembled with the characteristics of *Penicillium implicatum* (Khokhar *et al*., 2013).

The morphological analyses of F11 strain on potato dextrose agar (PDA) medium at 25°C for 7 days resembled with that of *Penicillium piceum* (Sidrim *et al*., 2010), having following characteristics; colonies growing moderately, rapidly on PDA, pale to bright yellow, velutinous to floccose, biverticillate penicillin cells (Fig. 3a) and colonies reverse dark brown to orange-brown (Fig. 3b), with conidia, smooth-walled and olivaceous green conidia present on Conidiophore with broom like appearance (Fig. 3c).

The colonies of the strain F12 grown on PDA at 25°C for 7 days showed the characteristics which were identical with those of *Penicillium oxalicum* (Tiwari *et al*., 2011), that was, with rapid growth, colony was dark green color, powdery, compact and the back side of colony was yellowish cream in color on PDA media with colourless mycelium (Fig. 3d, e) and the conidia spores arrangements resembled a broom (Fig. 3f).

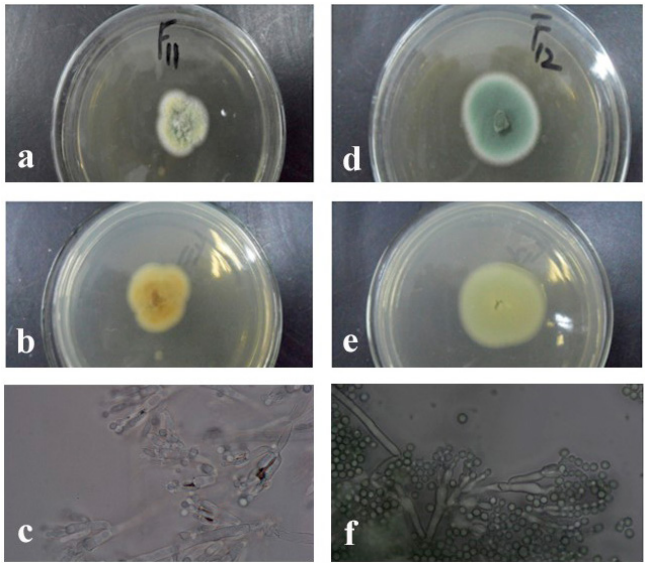

Fig. 3. The morphologies of the colony of the strain F11 (*Penicillium piceum)* (a, front b, back and c, mycelium and the chains of its conidia; the strain F12 (*Penicillium oxalicum)* (d, front; e, back; f, mycelium and the chains of its conidia).

In case of F15 strain when grown on PDA at 25°C for 7 days showed moderate or good sporulation, mycelium not immediately obvious, smooth, conidia dull-green, exudate present as small or totally absent, occasionally clear or light brown, colorless margin entire (Fig. 4a), reverse in shades of yellow or beige-brown (Fig. 4b). Furthermore, conidiophores predominantly biverticillate with broom like structure having conidiabroadly ellipsoidal or subglobose, sporadically smooth, delicately roughened (Fig. 4c),

which were the characteristics of *Penicillium sumatrense* (Houbraken *et al*., 2011).

Moreover, the F16 strain morphology characteristics were noticed on PDA at 25°C after 7 days which were: colony diameters 13-16 mm, center low, deep, radially sulcate, dense, floccose, margin narrow, entire; mycelium white, exudate absent (Fig. 4d), reverse light brown (Fig. 4e) and broom like conidiophores on PDA borne from aerial mycelium; stipes septate, apices nonvesiculate, smooth, thin walled, strictly monoverticillate (Fig. 4f), which resembled with the features of *Penicillium vinaceum* (Zheng *et al*., 2012).

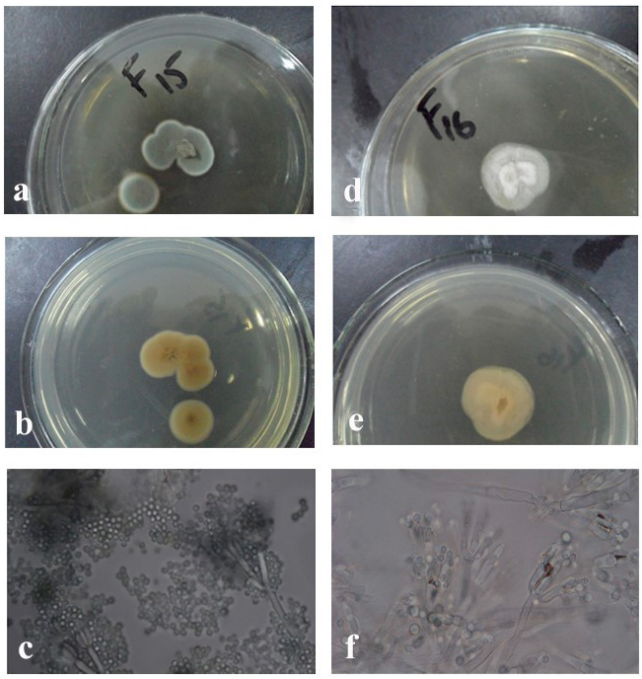

Fig. 4. The morphologies of the colony of the strain F15 (*Penicillium sumatrense)* (a, front; b, back; c, mycelium and the chains of its conidia; the srain F16 (*Penicillium vinaceum)* (d, front; e, back; f, mycelium and the chains of its conidia).

Molecular identification and Phylogenetic analysis

Total genomic DNA from all the fungal isolates were extracted (Fig. 5) and a PCR product of 541 bp band was obtained by using gDNA as template (Fig. 6).

ITS sequences of all the fungal isolates were aligned and a phylogenetic tree was created. The investigation for resemblance between ITS of the isolates and those in the NCBI database showed that many phylogenetically associated *Penicillium* spp. were analogous to the fungal isolates obtained in this study (Fig. 7). The topology of the phylograms confirmed that the fungal isolates used in this study were assigned to F1 (*Penicillium viticola*), F2 (*Penicillium restrictum*), F8 (*Penicillium rubens*), F10 (*Penicillium implicatum*), F11 *(Penicillium piceum*), F12 (*Penicillium oxalicum*), F15 (*Penicillium sumatrense*), and F16 (*Penicillium vinaceum*). Furthermore, *Aspergillus flavus* CBS 260.73 was used as out group.

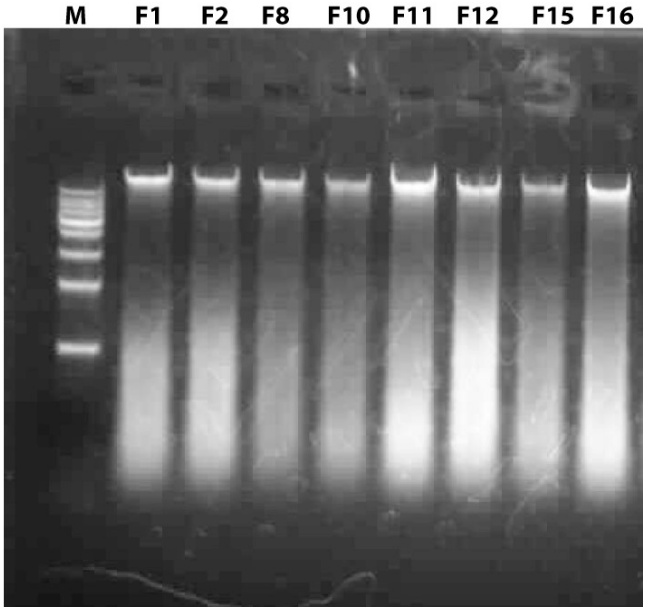

Fig. 5. Genomic DNAs of the fungal isolates; lane M: 1 kb DNA ladder, Lane F1, F2 F8, F10, F11, F12, F15, and F16 are the genomic DNAs of them.

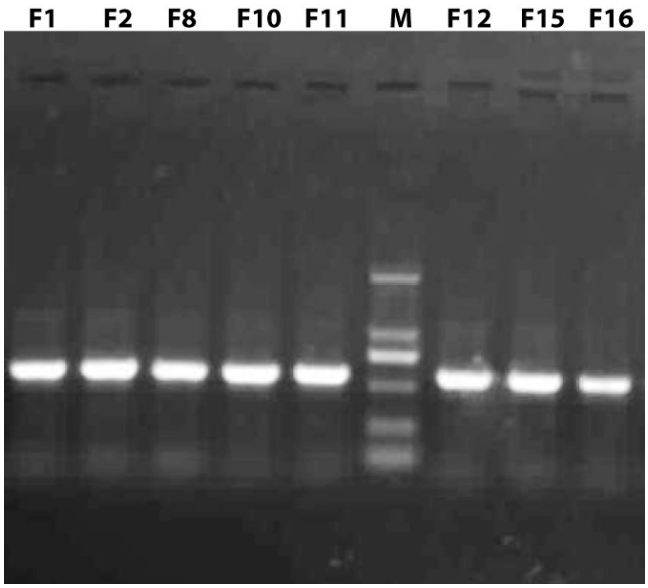

Fig. 6. Resolving pattern of PCR amplified ITS sequences on 1% Agarose gel; Lanes F1, F2, F8, F10, F11, F12, F15, and F16 showed PCR products of the fungal isolates and Line M: DNA ladder, fragment size from top to bottom is 2000bp, 1000bp, 750bp, 500bp, 250bp and100bp.

DISCUSSION

The fungi especially *Penicillium* species gained immense importance during recent years due to capacity of producing plenty of bioactive compounds (*e.g*. vinblastine, vincristine, taxol, camptothecine and its analogs, etoposide and its analogs, resveratrol, *etc.*), as well as efficient antimicrobial agents with diverse mode of actions (Joseph and Priya, 2011).

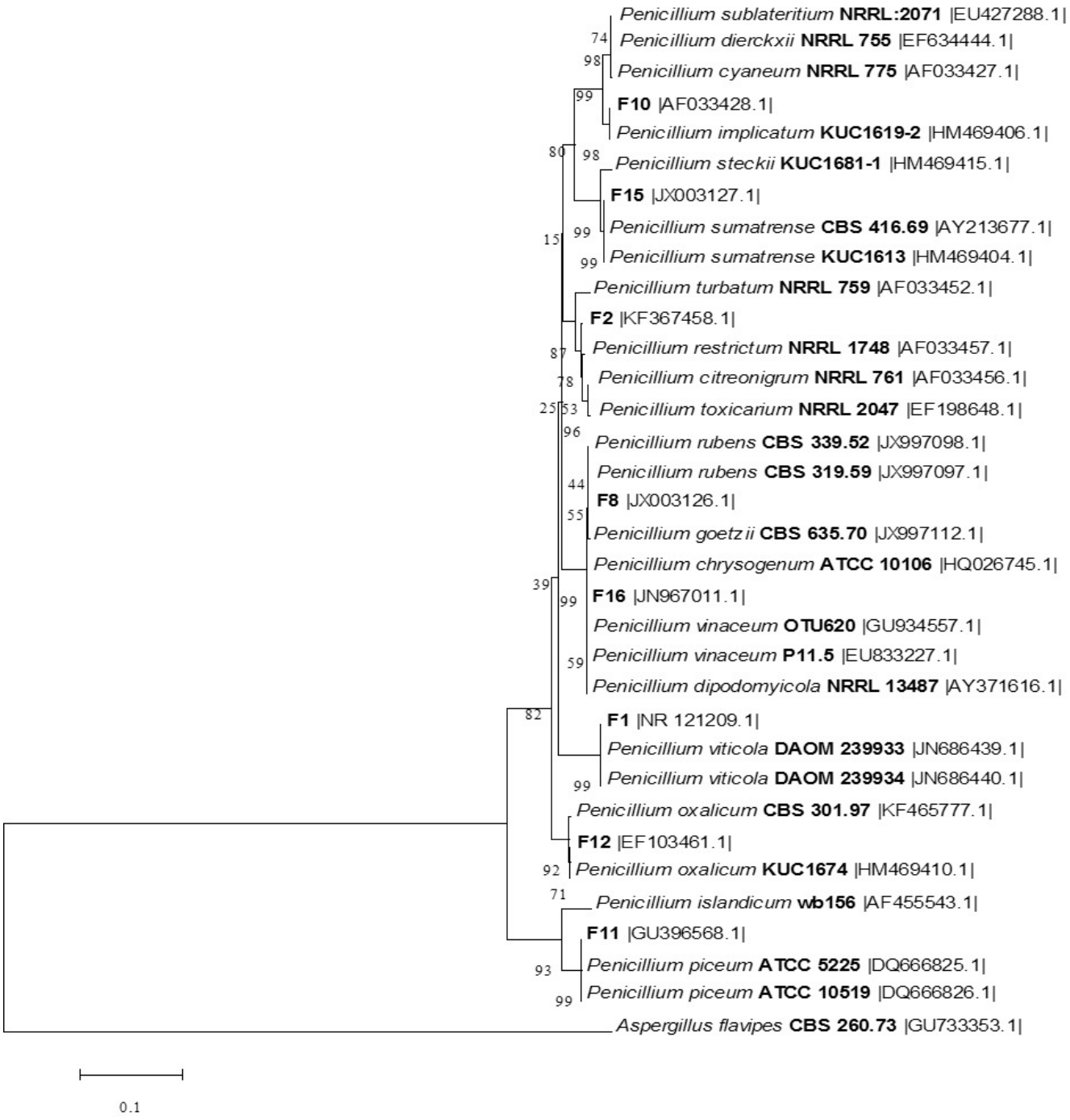

Fig. 7. The phylogenetic tree of the all fungal isolates and other *Penicillium* species relatives based on a neighbor-joining analysis of ITS sequences. *Aspergillus flavus* CBS 260.73 was used as out group.

In the present investigation, total eight fungal strains were isolated in present study which were named as F1, F2, F8, F10, F11, F12, F15, and F16. Furthermore, the morphological characteristic of all the eight fungal isolates were determined, which were totally in accordance with the study of Pitt (1979) as demonstrated in results (Figs. 1, 2, 3, 4). According to Pitt (1979), the colonies of *Penicillium* spp. always are fast growing, smooth and filamentous. The colonies are primarily white and turn into blue green, dark green, olive gray, gray green, pink or yellowish in time. Moreover, various pigments are more or less typical for the *Penicillium* species. The colony reverse is usually pale to yellowish or brownish. Furthermore, Pitt (1979) also mentioned, for *Penicillium* species branched or simple conidiophores, phialides, metulae and conidia are observed. The appearance of the spore head is like that of a brush; and the spore head is called a *penicillus* which is Latin for a brush. Thus, based on microscopic investigations it was proved that examined fungal isolates are characterized by conidiophores and microscopic elements typical for

Penicillium genus. Demirel *et al*. (2013) also isolated nine terverticillate *Penicillium* strains that were (*Eupenicillium egyptiacum, P. chrysogenum, P. expansum, P. viridicatum, P. crustosum, P. aurantiogriseum, P. griseofulfum, P. primulinum, P. puberulum*) from 56 soil samples and also genetically categorized by using PCR method (Demirel *et al*., 2013). The taxonomy of *Penicillium* spp. is complex due to these large number of species which have very few differences. Despite that the classification systems of organisms are based on the observable characteristics, many species classified as *Penicillium* are morphologically similar, and this method of identification remains difficult (Cardoso *et al*., 2007). Thus, the morphological study of *Peniciillium* spp. had some distinguishable characters and the discrimination of these characters was very difficult by microscopic investigation. On the other hand, ITS (internal transcribed spacer) base PCR identification techniques provided highly useful information about the molecular identification and distinction of fungi, especially closely related fungi. Moreover, Hairul *et al*. (2010) also conducted similar study to determine genetic inconsistency and relationship among twenty *Penicillium* isolates by means of morphological and random amplification polymorphic DNA (RAPD) molecular technique from diverse locations in Western part of Borneo Island and these strains were kept in the pure culture collection of University Malaysia Sarawak (Hairul *et al*., 2010).

Therefore, for further verification of morphological identified strains by the rDNA gene, was done by using taxonomic and identification studies. When each of these sequences was investigated with BLAST on NCBI database and different maximum identification values were obtained, with the highest being 99% and the lowest 97% in the GenBank database of NCBI. In this comparison, the F1 strain that was identified as *P. viticola* by the traditional identification methods showed a similarity to *P. viticola* strains with 99% maximum identification, which correlated with study conducted by (Nonaka *et al*., 2011). The F2 strain identified as *P. restrictum*as it showed a similarity with 99% maximum similarities, alike result was also shown by Mario *et al*. (2014). While, ITS blast result of F8 showed its resemblance 99% with *P. rubens* (Jos *et al*., 2011). Furthermore, the fungal isolates F10 and F11, identified as *P. implicatum* and *P. piceum,* respectively with a 97% and 99% similarity, which relates to the finding of Ibatsam *et al*. (2013) and Sidrim *et al*. (2010). Moreover, F12, F15, and F16 fungal strains used in this study showed 98%, 99% and 99% similarities with *P. oxalicum, P. sumatrense* and *P. vinaceum,* respectively, which is in accordance with the prior studies (Tiwari *et al*., 2011; Houbraken *et al*., 2011). Phylogenetic relationship of all the *Penicillium* spp. with other fungi is shown in Figure 7.

CONCLUSIONS

Total eight fungal cultures were isolated from different sources of Yellow Sea and their morphology, microscopy and molecular base analysis revealed that fungal strains isolated in this study belong to *Penicillium* genus. Thus, this study demonstrates that Yellow Sea environment can be considered as a valuable natural source of filamentous fungi isolation specially *Penicillium* genus, which can be used for the production of different bioactive compound or secondary metabolites with diverse application in several industries including medicine. Moreover, our results indicate that this work has aided the development in fungal studies to make it more valuable in the production of organic acids, having considerable attention for their role in natural ecology and their potential industrial applications.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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