ISOLATION AND IDENTIFICATION OF INTERNAL MYCOFLORA OF MARINE ALGAE

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Abstract

In the contemporary medicinal field, various bioactive molecules have been found through experimental research on natural extracts from marine species. Mutualistic symbionts help to improve growth and resist disease or environmental stress. The eleven endophytic fungus isolates found in this study were all found in marine algae samples obtained from the Mandapam rocky shores, Rameswaram, and Ramanathapuram districts of Tamil Nadu. The endophytic fungi were recognized based on the morphology of conidial attachments and the fruit body structure. The evolutionary analysis was conducted in MEGA11. The DNA of fungi was isolated by polymerase chain reaction using a thermal cycler. A Blast search of the sequence showed a maximum similarity of 100% with *Curvularia geniculate* submitted in the gene bank with the accession code OP321580.

Keywords:

Endophytes, Mutualistic symbionts, Drug discovery, Bioprospecting.

Introduction

One definition of an endophyte refers to a bacteria or fungus which has formed a symbiotic relationship with its host plant like alga. Mutualistic symbionts help to improve growth and resist disease or environmental stress of the host. Johann Heinrich Friedrich Link, in 1809, was the first to describe endophyte. Due to their species richness and variety, Many countries have conducted research on marine substances (Calisto et al., 2019).

In general, marine organisms can withstand harsh conditions (Kamat et al., 2020). Owing to their symbiotic association with endophytes, algae from the oceans may survive in harsh environments (Jensen et al., 1994) caused by factors such as salt, and acidity. pollution from chemicals, as well as climate change. Because of their chemical variety and potential to create numerous unique secondary metabolites that may be used for fuel, medicine, restoration, and agriculture, they play an important role in research. Biofuel, environmental remediation, drug discovery, host defense.

Endophytes are not host-specific, according to recent research (Eluvakkal et al., 2019). According to several studies (Tejesvi et al., 2008; Weber et al., 2008; Sappan et al., 2004; Huang et al. 2008; Rao, 1987),

endophytic fungal organisms from medicinal herbs are a potentially abundant source of bioactive metabolites.

Novel secondary metabolites have been discovered in endophytes from angiosperms and gymnosperms. The previous studies focus on the assessment of endophytes in lower plants including bryophytes, algae, pteridophytes, as well as lichens (Zhang et al., 2006; Suryanarayana et al., 2005; Li et al., 2007).

Seaweed-Algae

Seaweed is a species of multicellular, macroscopic, marine algae. They are classified as either brown algae (Phaeophyta), green algae (Chlorophyta), or red algae (Rhodophyta). They are high in Industrial exploitation as they have a source of polysaccharides, proteins, and phenols. (Eluvakkal et al., 2010). Seaweed is used in multiple applications like food, fertilizers, industry, pharmaceutical, and animal feed.

Rhodophyta is the dynamic producer of halogenated compounds such as peptides, polyketides, indoles, phenols, and terpenes that are used as anti-inflammatory, anti-microbial, antiproliferative, cytotoxic, anti-cancer, and insecticidal. Phaeophyta occurs primarily in marine environments and is prized for creating important metabolites such as volatile chemicals, fucoidans, phlorotannins, and fucoxanthins. And are employed as antiviral agents against HIV, CMV, and HSV, as well as in the drug development process. In stacked thylakoids, Chlorophyta contains chlorophyll a & b in their chloroplasts, together with beta carotene as well as xanthophylls, which give them their bright green color. Isoprenoid is an extraordinary derivative discovered in green algae that has anti-cancer properties.

Proposed Marine algae identified for the study and their Medicinal values

Table 1. Marine Algae and their Medicinal Values

S.No	Phylum	Family	Genus	Species	Medicinal Values
1.	Rhodophyta	*Rhodomelaceae*	*Laurancia*	*Laurancia dendroidea*	Cytotoxic against human breast cancer cell line Antibacterial against UTI Anti-ulcer and hepatoprotective activity.
2.	Ochrophyta	*Sargassaceae*	*Sargassum*	*Sargassum polycystum*	Anti-oxidant, anti-inflammatory and anti-cancer
3.	Ochrophyta	*Dictyotaceae*	*Padina*	*Padina Pavonica*	Antioxidant, antifungal and anti-cancer activities

4.	Ochrophyta	*Sargassaceae*	*Sargassum*	*Sargassum wightii*	Anti-cholinesterase, anti-inflammatory, and antioxidant
5.	Rhodophyta	*Gracilariaceae*	*Gracilaria*	*Gracilaria corticata*	Antioxidant and anti-microbial activity.
6.	Rhodophyta	*Gracilariaceae*	*Gracilaria*	*Gracilaria crassa*	Antioxidant and gastro protective effect.
7.	Ochrophyta	*Dictyotaceae*	*Padina*	*Padina Pavonica*	Antioxidant, antifungal and anti-cancer activities
8.	Rhodophyta	*Gracilariaceae*	*Gracilaria*	*Gracilaria edulis*	Antitumor
9.	Rhodophyta	*Solieriaceae*	*Kappaphycus*	*Kappaphycus alvarezii*	Anti-oxidant
10.	Chlorophyta	*Ulvaceae*	*Ulva*	*Ulva fasciata*	Anti-hypercholesterolemic effect production of bioethanol and Cytotoxic against human colon cancer cell line.
11.	Ochrophyta	*Sargassaceae*	*Turbinaria*	*Turbinaria conoides*	anti-cancer

Sample Collections

Algal specimens were obtained discretely from Mandapam rocky coastlines (Lat9˚27’70N, Long79˚12’52E) in Rameswaram, Ramanathapuram district, to the geographical separation of endophytic fungi, Tamil Nadu. From December 2020 - February 2021 (15 months), algal samples were collected at three-month intervals. Soil sediments were collected at 30cm depth using a plastic borer and transferred to sterile plastic zip lock covers and were transported immediately to the laboratory and stored for further study.

A taxonomical study of Seaweed

The collected seaweeds were investigated for taxonomical traits such as substratum nature, seaweed behavior, and species abundance. Endophytic fungus identification and isolation with relation to Iswarya et al., 2019. Bioprospecting and bioactive chemical profiling of seaweed-associated endophytic fungus and marine actinomycetes from Chennai shores.

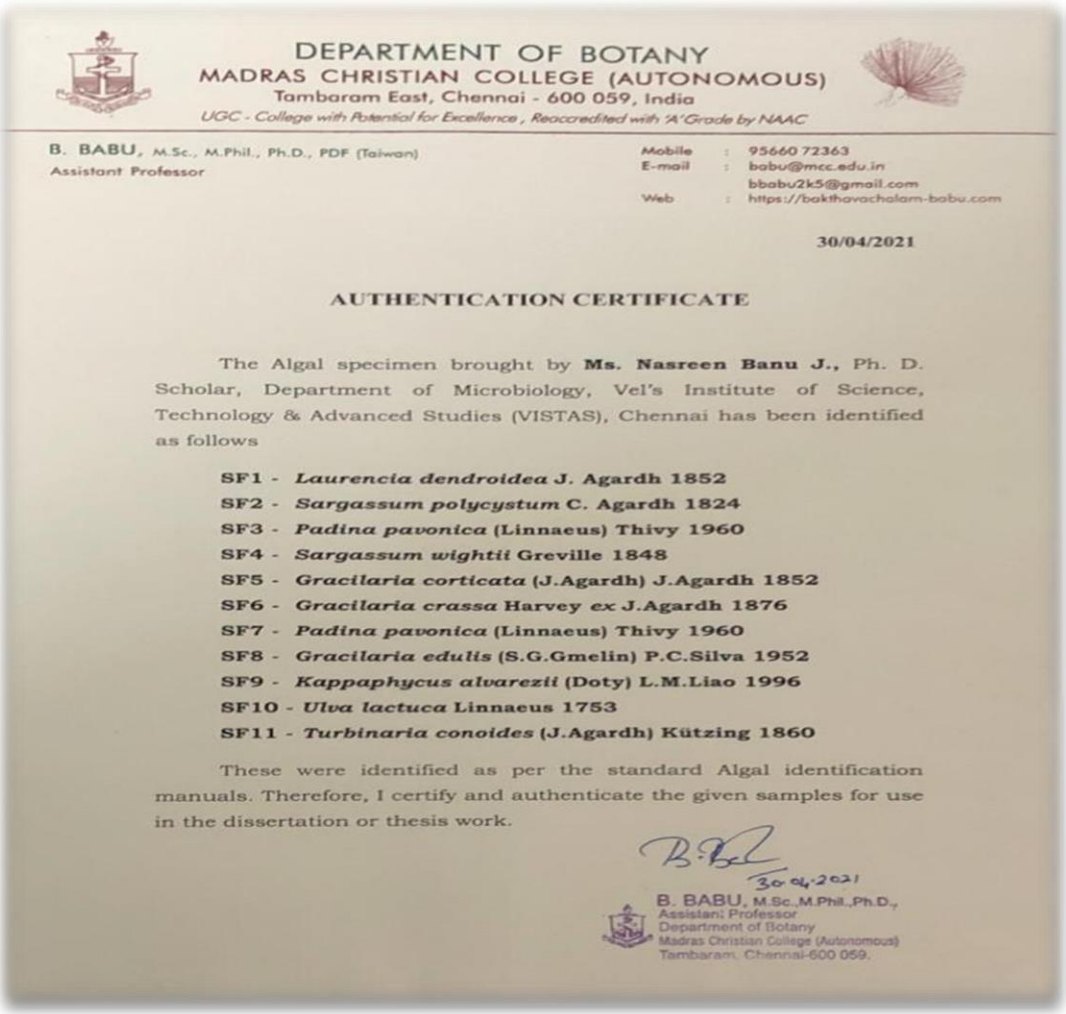
Nomenclature are adopted (Appeltans et al., 2012) and taxa were allocated from pieces of literature Umamaheswara Rao et al., 1987; 1990, 1998; Krishnamurthy et al., 1999).

TABLE 2. LIST OF COLLECTED SEAWEEDS

Sample No.	Fungal Strains	Sea weeds	Morphological structure
1	SF1	*Laurancia dendroidea*	
2	SF2	*Sargassum polycystum*	
3	SF3	*Padina Pavonica*	
4	SF4	*Sargassum wightii*	

5	SF5	*Gracilaria* ***corticata***	
6	SF6	*Gracilaria* ***Crassa***	
7	SF7	*Padina Pavonica*	
8	SF8	*Gracilaria edulis*	
9	SF9	*Kappaphycus alvarezii*	
10	SF10	*Ulva fasciata*	
11	SF11	*Turbinaria conoides*	

Fig. 1 Authentication Certificate of collected Marine Algae



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30/04/2021

AUTHENTICATION CERTIFICATE

The Algal specimen brought by **Ms. Nasreen Banu J.,** Ph. D. Scholar, Department of Microbiology, Vel's Institute of Science, Technology & Advanced Studies (VISTAS), Chennai has been identified as follows

SF1 - *Laurencia dendroidea* J. Agardh 1852
SF2 - *Sargassum polycystum* C. Agardh 1824
SF3 - *Padina pavonica* (Linnaeus) Thivy 1960
SF4 - *Sargassum wightii* Greville 1848
SF5 - *Gracilaria corticata* (J.Agardh) J.Agardh 1852
SF6 - *Gracilaria crassa* Harvey *ex* J.Agardh 1876
SF7 - *Padina pavonica* (Linnaeus) Thivy 1960
SF8 - *Gracilaria edulis* (S.G.Gmelin) P.C.Silva 1952
SF9 - *Kappaphycus alvarezii* (Doty) L.M.Liao 1996
SF10 - *Ulva lactuca* Linnaeus 1753
SF11 - *Turbinaria conoides* (J.Agardh) Kützing 1860

These were identified as per the standard Algal identification manuals. Therefore, I certify and authenticate the given samples for use in the dissertation or thesis work.

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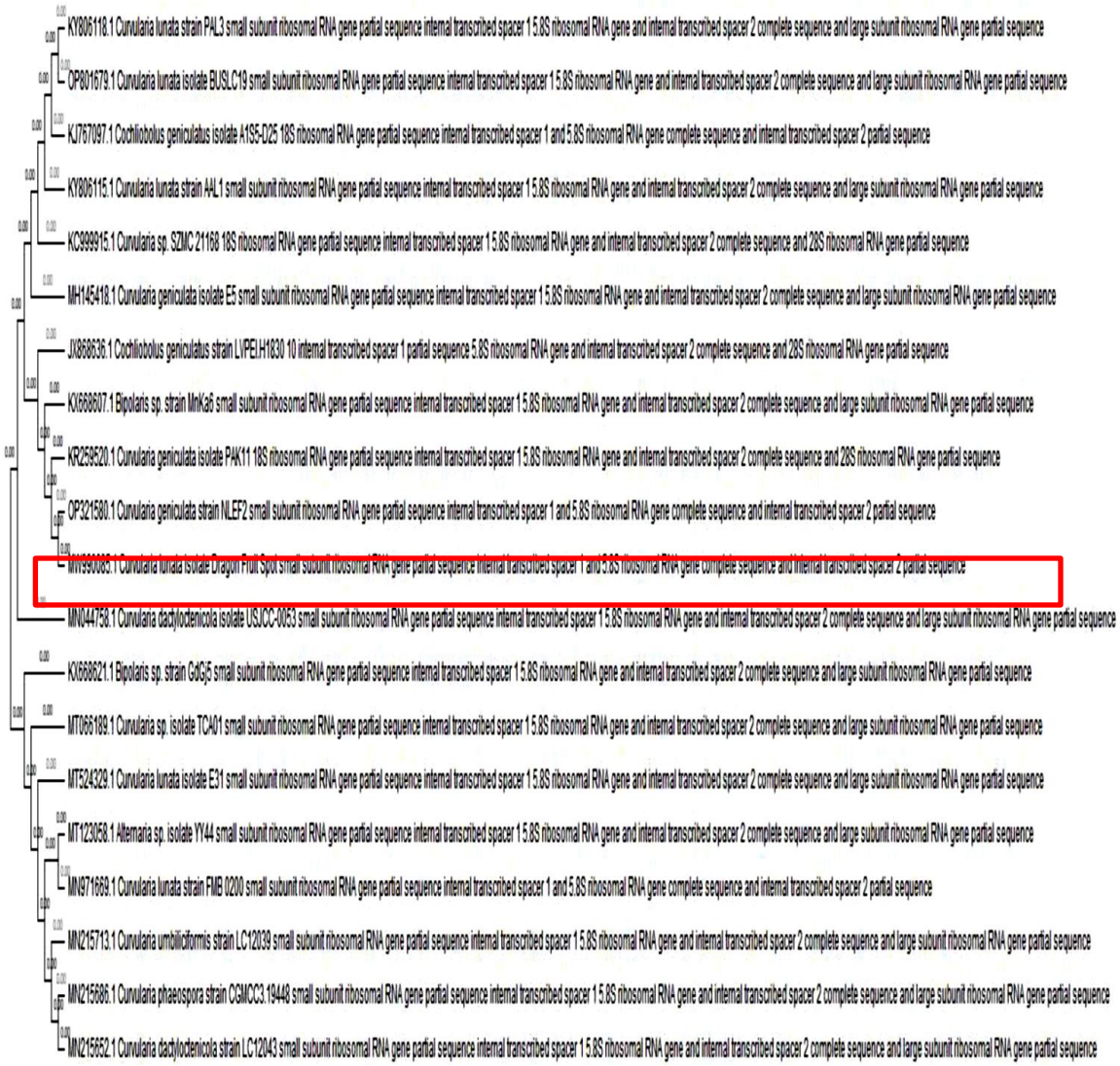
Fig. 2 Phylogenetic Tree



Evolutionary Relationship of Taxa

Saitou et al. (1987) proposed the use of a neighbor-joining method for making such inferences about the evolution of life. The most beautiful tree (near the trunk) is shown. Maximum composite likelihood (Tamura et al., 2004) was used to determine the biological distances, which are expressed as the number of substituting bases per site. Twenty distinct nucleotide sequences were employed in this investigation. All possible points of ambiguity have been removed (using the pairwise deletion method) from every combination set. The datasets produced a total of 1763 locations. Tamura et al. (2021) utilized MEGA11 to analyze evolutionary data.

Maintenance of Isolated fungal cultures

Derived endophytic fungal strains had been further grown in a PDA medium until purity was achieved. We inundated pure mother cultures containing 20% glycerol and then preserved them at -20 degrees Celsius.

Morphological Characterization

The isolated endophytic fungus was identified based on colony and conidiophore morphology, as described in Ellis et al., 2007.

Coverslip Culture Technique

The mycelium and its spore characteristics were studied by the coverslip culture technique by Harris et al., 1986.

A loop of endophytic fungal isolate spore suspension was distributed at the intersection of the casein starch agar medium on the glass slide, which was then covered with the coverslip.

For 4-8 days, these plates were refrigerated at 28 degrees Celsius. The coverslips were removed at 2-4 day intervals and examined under a light microscope. Aerial mycelium morphology, sporogenous hyphae organization, and spore structures were recorded

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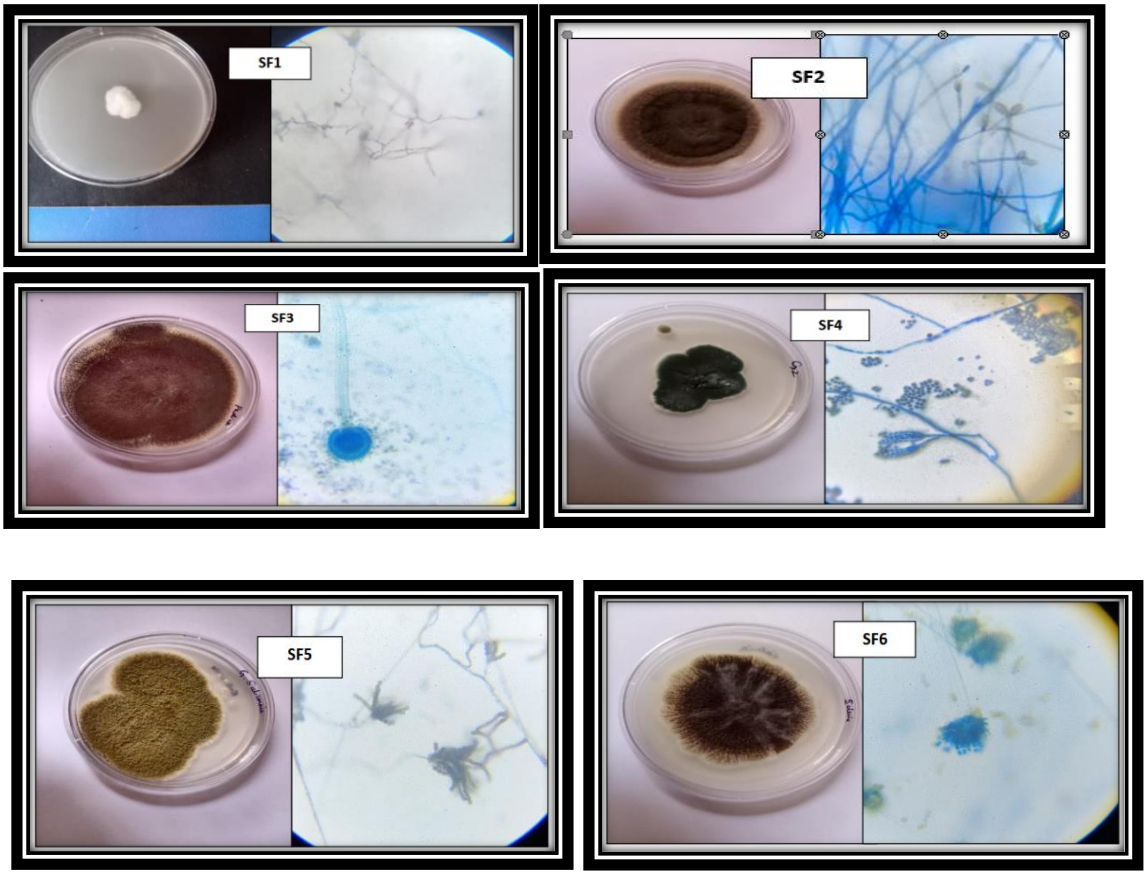
Fig. 3 Isolated strains of entophytic fungi



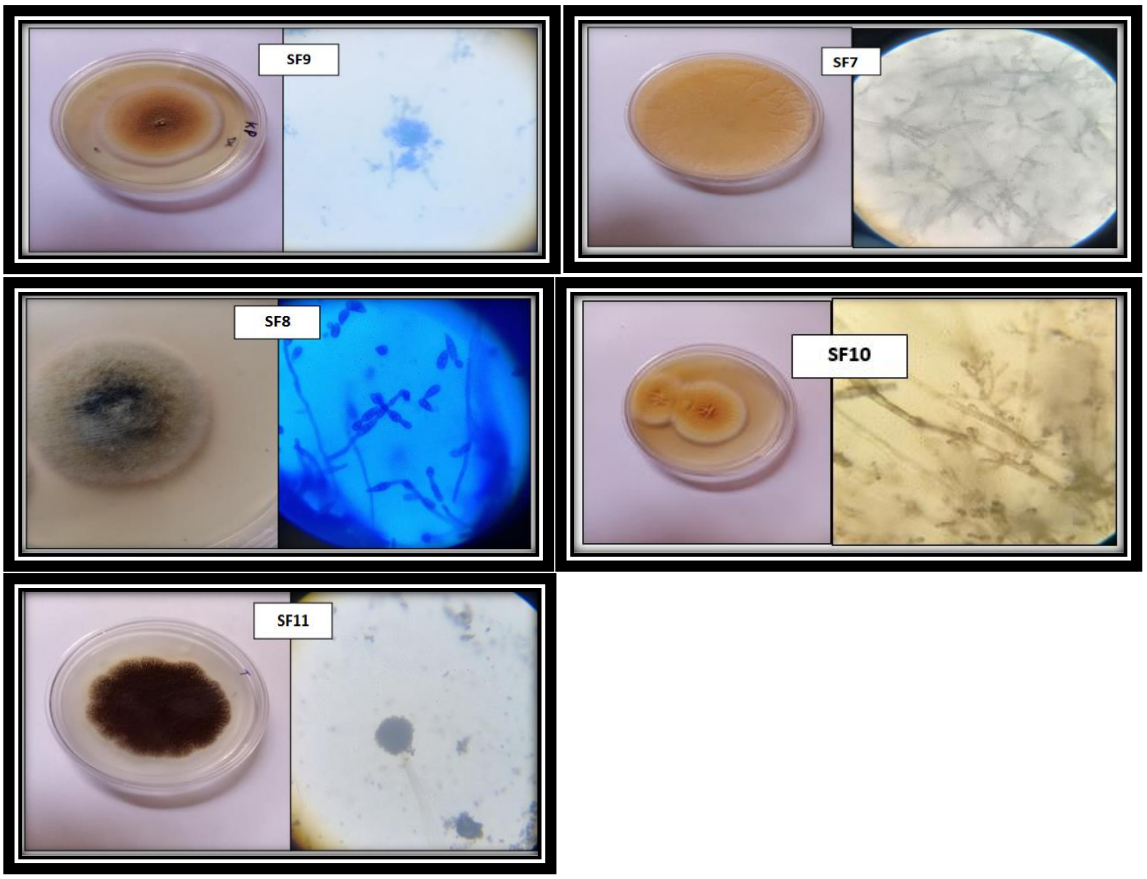


Table 3. ISOLATED STRAINS OF ENDOPHYTIC FUNGI			
Sample No.	**Fungal Strains**	**Seaweeds**	**Fungal Isolated**
1	**SF 1**	***Laurancia dendroidea***	**Sterile *Mycelia***
2	**SF 2**	***Sargassum polycystum***	***Curvularia species***
3	**SF 3**	***Padina Pavonica***	***Aspergillus niger***
4	**SF 4**	***Sargassum wightii***	***Penicillium species***
5	**SF 5**	***Gracilaria Corticata***	***Aspergillus species***
6	**SF 6**	***Gracilaria crassa***	***Aspergillus species***
7	**SF 7**	***Padina Pavonica***	***Aspergillus species***
8	**SF 8**	***Gracilaria edulis***	***Alternaria alternata***
9	**SF 9**	***Kappaphycus alvarezii***	***Aspergillus species***
10	**SF 10**	***Ulva fasciata***	***Aspergillus species***
11	**SF 11**	***Turbinaria conoides***	***Aspergillus species***

Molecular characterization (Flewelling- et al., 2013a,b,c)

The extraction of DNA

- Isolated fungi were cultured upon the Potato dextrose agar (PDA) till 5 days before being transferred with a sterile toothpick into sterile 100L of distilled water in 1.5mL microcentrifuge tubes.
- After vigorously vortexing the liquid for a full minute, we centrifuged it with 10,000 rpm for 3 min.
- After removing these supernatants, 100L of lysis solution (TE buffer, 10mM Tris-HCL, 1mM EDTA, and pH8) is poured into a microcentrifuge tube.
- Primers were used, and the 5'-3' direction sequence is indicated by the primer name. - TCCTCCGCTTATTGATATGC in the forwards' strand and TCCGTAGGTGAACCTGCGG in the reverse strand of ITS1.
- Applied Biosystems' Gene Amp PCR System 9700 was employed as PCR thermal cycler for the amplification.
- PCR amplification profile of the ITS region: Approximately 35 cycles 94°C for 5.00 min, 94°C till 50 sec, 54°C till 50 seconds, 72°C till 50 sec, 72°C till 10 min, 4°C till 5.00 min. Electrophoresis of PCR products in an Agarose gel.
- 1.0% Agarose gels having 0.5X TBE solutions comprising 0.5 g/mL ethidium bromide were used to analyze the PCR products.
- The resultant gels has been electrophoresed at 75V for about 1-2 hours, when the intermediate bromophenol blue front was moving nearly to the bottom within the gel, using 0.5X TBE to function as the electrophoresis buffer.
- To provide a molecular benchmark, we employed a 2-log DNA ladder (NEB).
- A UV trans-illuminator (Gene-i) was used to evaluate the gels, and a photograph was taken of them with the help of Gel documentation system (Bio-Rad) and UV light.

ExoSAP-IT Treatments

ExoSAP-IT (USB) serves as a buffer designed to remove undesirable dNTPs as well as primers having the PCR mixture product without affecting subsequent applications. It contains two Exonuclease I, hydrolytic enzymes, as well as Shrimp Alkaline Phosphatase (SAP).

After incubating the mixture at 37 degrees Celsius for 15 minutes, researchers inactivated the enzyme by heating it to 80 degrees Celsius for the same amount of time.

Used Big Dyes Terminator V3.1 to sequence. Big Dye Terminator V3.1. A cycle sequencing kit (Applied BioSystems, USA) was employed in a PCR thermal cycler (Applied BioSystems, Gene Amp PCR system 9700) to carry out the sequencing reaction as per the protocol.

The PCR solution contains the further Parts as discussed below;

- ExoSAP treated PCR product: 10-20ng primer: 3.2 pM (reverse/forward).
- 1.86 ul of reaction buffer; 0.28 ul of sequencing mix are included. We may sterilize up to 10 liters of water by distilling it.
- Starting with 2 minutes at 96 degrees Celsius, the PCR temperature profile included There should be 30 cycles for each primer, each lasting 30 sec at 96 ^{o}C, 40 sec at 500 ^{o}C, and 4 min at 60 ^{o}C.
- PCR cleanup following sequence analysis.
- Two master mixes were prepared: one containing 10 ul milli of milliQ and 2 ul 125 mM EDTA per reaction (master mix I) and the other containing 2 ul 3M sodium acetate pH 4.6 and 50 ul ethanol (master mix II).
    - 12 l among master mix I is mixed into every reaction, diluting the reaction mixture by 10%.
    - 52 l among master mix II is included with every answer.
    - .
- After 30 minutes of inversion and room temperature incubation, the contents were analyzed.
- This resultant mixture was spun at 14,000 rpm (30 min).
- After decanting, we washed it once again in 70% ethanol and added 100 l of the solvent.
- The pellet was air-dried after the supernatant was drained.
- To sequence the product which is prepared and air-dried, an ABI 3730 DNA Analyser (Applied Biosystems) was used.
- Sequencing of the purified PCR product. The submission of a DNA sequence is mandatory.
- A phylogenetic tree was constructed from the obtained sequences using genetic distance and bootstrap values calculated in MEGA6 (Tamura et al., 2013).
- The search result revealed that the sequence was identical 100% to that of *Curvularia geniculate*

Fig. 4- BLASTn

BLAST® » blastn suite » results for RID-1W6ZF57301R

Job Title	0122_188_031_PCR_EF2_IR_B01.ab1 ...
RID	1W6ZF57301R Search expires on 03-02 20:13 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_261415
Description	0122_188_031_PCR_EF2_IR_B01.ab1 ...
Molecule type	dna
Query Length	511

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Curvularia geniculata isolate PAK11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Curvularia geniculata	944	944	100%	0.0	100.00%	572	KR269820.1
Cochliobolus geniculatus isolate A155-D25 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	Curvularia geniculata	944	944	100%	0.0	100.00%	572	KJ767887.1

Curvularia geniculata strain NLEF2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank: OP321580.1
FASTA Graphics

Go to:

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LOCUS       OP321580                511 bp    DNA     linear   PLN 03-SEP-2022
DEFINITION  Curvularia geniculata strain NLEF2 small subunit ribosomal RNA
            gene, partial sequence; internal transcribed spacer 1 and 5.8S
            ribosomal RNA gene, complete sequence; and internal transcribed
            spacer 2, partial sequence.
ACCESSION   OP321580
VERSION     OP321580.1
KEYWORDS    .
SOURCE      Curvularia geniculata (Cochliobolus geniculatus)
  ORGANISM  Curvularia geniculata
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Pleosporomycetidae; Pleosporales; Pleosporineae;
            Pleosporaceae; Curvularia.
REFERENCE   1  (bases 1 to 511)
  AUTHORS   Nasreen Banu,J. and Kathireshan,A.K.
  TITLE     Endophytic fungi from Seaweed
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 511)
  AUTHORS   Nasreen Banu,J. and Kathireshan,A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-AUG-2022) Department of Microbiology, Vels
            University, PV Vaithiyalingam Rd, Velan Nagar, Krishnapuram,
            Pallavaram, Chennai, Tamil Nadu 600117, India
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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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ORIGIN
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       61 gctggagtat tttattaccc ttgtcttttg cgcacttgtt gtttcctggg cgggttcgct
      121 cgccaccagg accaccaaat aaacctttt tatgcagttg caatcagcgt cagtacaaac
      181 aatgtaaatc atttacaact ttcaacaacg gatctcttgg ttctggcatc gatgaagaac
      241 gcagcgaaat gcgatacgta gtgtgaattg cagaattcag tgaatcatcg aatctttgaa
      301 cgcacattgc gcccttggt attccaaagg gcatgcctgt tcgagcgtca tttgtaccct
      361 caagctttgc ttggtgttgg gcgtttttgt ctttggtcgc ccaaagactc gccttaaagt
      421 gattggcagc cggcctttct ggtttcgcag cgcagcacat ttttgcgctt gccatcagca
      481 aaacggcaat ccatcaagcc tccttctcac g
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Discussions

The current work intends to isolate and identify the endophytes generated by marine algae to discover secondary metabolites produced by the endophytes with antibacterial and anticancer activities. Brown, red and green algal samples were taken from the rocky beach of Mandapam in Tamil Nadu's Rameswaram district.

The following are the algal samples that were collected: *Laurancia dendroidea, Sargassum polycystum, Padina pavonica, Gracilaria crassa, Gracilaria corticata, Ulva lactuca, Gracilaria edulis, Kappaphycus alvarezii,* as well as *Turbinaria conoides (Table 1.1- Table 1.3).* The materials were collected and delivered to the lab using the appropriate procedures for further analysis. Dr.B.Babu, Assistant Professor, Department of Botany, Madras Christian College, validated all of the algal samples (Fig. 1).

The algal portions were carefully cleaned with flowing tap water. The algal segments were cut to around 6-9mm in length and surface sterilized with 70% ethanol for 5 seconds before being submerged in the item for thirty seconds using 4% sodium hypochlorite before washing it in sterilized in double-distilled water. After that, the algal segments were inoculated on potato dextrose agar. The inoculated plates were cultured in a light room for three weeks to allow endophytic fungi to develop. The shape and structure of conidial connections as well as the anatomy of the fruit allowed for the identification of endophytic fungus. The evolutionary screening was carried out in MEGA11 (Fig. 2).

Purity was achieved by subculturing isolated endophytic fungal strains using a PDA medium along with flooding pure mother cultures using 20% glycerol while keeping them at -20 ℃. A loop of spore suspension of endophytic fungal isolates was distributed at the intersection of the edges of the casein starch agar medium on the glass slide and sealed with the coverslip. The media were maintained at 28˚C for 4-8 days. These coverslips are detached every 2-4 days and examined under a light microscope. Aerial mycelium morphology, sporogenous hyphae organization, and spore structures were studied.

The DNA of fungal species was extracted and amplified using a thermal cycler employing a polymerase chain reaction (Fig.3). Electrophoresis on an Agarose gel was used to identify the byproducts of PCR. A UV transilluminator has been employed for illuminating the gel substances, as well as a Gel Documentation System (Bio-Rad) adopted for taking photos with UV light.

A Blast search of the EF2 strain's sequence against the sequences revealed a maximum similarity of 100% with *Curvularia geniculate* issued to the Gene Bank under the accession code OP321580 (Fig.4).

Conclusion

The identification of endophytic fungi was conducted through the examination of conidial attachments and the structure of the fruiting body. The evolutionary analysis was performed using the MEGA11 software. The fungal DNA was extracted via polymerase chain reaction utilizing a thermal cycler.

Upon conducting a Blast search of the sequence, it was observed that the maximum similarity of 100% was found with Curvularia geniculate, which has been previously submitted in the gene bank and can be identified through the accession code OP321580.

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