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Fungal phylogenetic diversity in estuarine sediments of Gautami Godavari River, Andhra Pradesh, India

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Abstract

Mangroves are a unique ecosystem that experiences fluctuations in salinity which is influenced by terrestrial and marine environment, tidal waves and wind velocity. The aforesaid factors provide a suitable environment for phylogenetically and physiologically diverse microorganisms to grow in a mangrove ecosystem. The present study was initiated to characterize the phylogenetic diversity of fungi in the sediments collected from Bhairavapalem estuary situated on the mouth of Godavari River in Andhra Pradesh, India. Fungi were isolated using serial-dilution method on potato dextrose agar medium amended with chloramphenicol. The isolated fungi were identified to belong in *Aspergillus allahabadi*, *A. sydowii*, *A. terreus* and *Penicillium shearii* clades in an ITS-based phylogenetic tree. Further studies employing a polyphasic approach are required to improve our understanding of genetic diversity, including cryptic species, of fungi those survive and flourish in dynamic marine niches such as estuaries in India.

Key words – DNA barcoding – Estuary – Identification – Mangrove – Taxonomy

Introduction

Mangrove forests are an ideal habitat for many detritus-dependent fungi and bacteria, as sufficient amount of detritus materials like leaf litter, wood debris and inflorescence are generated there (Wafar et al. 1997). The stability of mangrove environment is critical for the survival of several species of flora and fauna, which are native to this environment (Krishnamurthy et al. 1987). Mangroves provide a supportive environment for several practices such as fish farming etc. Fungal biodiversity, especially in dynamic systems such as mangroves and estuaries, can be viewed from a variety of perspectives, including physiological, intraspecific, genetic and phylogenetic diversities of species and higher taxa (Delong et al. 1997). It is a large untapped biodiversity reservoir which has a huge potential for biotechnological products such as new medicines, enzymes, novel pathways in the organisms (Jensen & Fenical 1994).

Our literature review (detailed in the discussion section) suggested that there have been few studies on fungal diversity in sediments of Gautami distributary of Godavari River, which passes through Bhairavapalem in Andhra Pradesh state of India. Bhairavapalem is a village closer to Gautami Godavari River, which is a distributary of the river Godavari (Sarma et al. 2011). It is situated at

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16°44'35"N and 82°18'59"E location (Fig. 1). The present study was initiated with the following objectives: 1) to collect sediment samples from Bhairavapalem estuary (BE) and isolate fungi from them using serial-dilution method, and 2) to characterize the phylogenetic diversity of the isolated fungi based on ITS sequence analysis.

Materials & Methods

Sample collection

Sediment samples were collected at 3.711 m depth, 27 °C temperature and 27 ppt salinity (Personal communication, Mr. Sampath Kumar, CSIR-NIO Regional Centre, Visakhapatnam) of Bhairavapalem estuary (Fig. 1). Sampling was done by Mr. Sampath Kumar and his team on 8th January, 2016 and sediment samples were generously provided for this study. The sediment samples were collected with the help of a sediment grab and transferred into a clean Zip-lock bag and labelled. The samples were further processed in the laboratory at CSIR-NIO Regional Centre, Visakhapatnam.

Fig. 1 – Bhairavapalem estuary aerial view (Image source: Google map)

Isolation of fungi

Sample processing was done aseptically in a laminar airflow. Approximately, 1 g of sediment sample was introduced into a test tube containing 100 ml sterile sea water. 1 ml of this mixture was transferred to another test tube for serial dilution. 1 ml of the selected dilution was taken and plated onto the potato dextrose agar (PDA) medium amended with chloramphenicol (100 mg/l). A sterile L-shaped rod was taken and the sample was spread evenly on the medium in petri-dish. These plates were maintained at 28 ± 2 °C in an incubator for growth. Within 48–72 hours of incubation, the inoculated plates began to exhibit fungal growth. The plates exhibited mixed fungal growth. So, the fungal colonies were sub-cultured consecutively a number of times to obtain pure cultures. The time range for the complete growth of all the fungal cultures was 3–14 days. The cultures were maintained at 28 ± 2°C in cooling incubator and sub-cultured at regular intervals. The colour, thickness and the appearance of the fungal colony were recorded to aid in fungal identification.

DNA extraction and PCR amplification of the ITS region

Selected pure cultures were further processed to extract DNA using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Catalogue number D6005) according to manufacturer's protocol. DNA samples were subjected to PCR amplification of the ITS region in a Mastercycler. The reactions were carried out in 50 µl of volume, containing 5 µl of (10X) PCR buffer (containing 15 mM $MgCl_2$; Genei), 40 µl of nuclease-free water, 0.5 µl of 10 mM dNTPs, 1 µl of (20 picomole) forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG) (White et al. 1990), 1 µl of (20 picomole) reverse primer ITS4 (5′-

TCCTCCGCTTATTGATATGC) (White et al. 1990), 1 µl of (1 unit/µl) Taq polymerase (Chromous) and 1.5 µl of DNA template. The following PCR cycling conditions were used: initial denaturation at 95 ℃ for 2 minutes, denaturation at 95 ℃ for 1 minute, annealing at 52 ℃ for 30 seconds, extension at 72 ℃ for 1 minute and final extension at 72 ℃ for 10 minutes. The denaturation, annealing and extensions steps were repeated 35 times (White et al. 1990). The PCR products were purified using QIAquick PCR purification kit (QIAGEN, Catalogue number 28106) following manufacturer's protocol. The sequencing of the ITS region was performed using Genetic Analyzer 3130xl (ABI) based on the Big Dye terminator v 3.1 (Chain terminator) chemistry at Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa, India.

Sequence alignment and phylogenetic tree construction

The forward and reverse sequences obtained from each primer were aligned in MEGA version 7 (Kumar et al. 2015) to generate a consensus sequence. A multiple sequence alignment was prepared in MEGA using newly-generated ITS sequences and the reference sequences retrieved from NCBI-GenBank (Fig. 2). The evolutionary tree was inferred in MEGA using Neighbour-Joining method.

Results

Isolation of fungi from sediment samples

The culture morphology of 15 fungi isolated from the estuarine sediment samples is presented in Table 1.

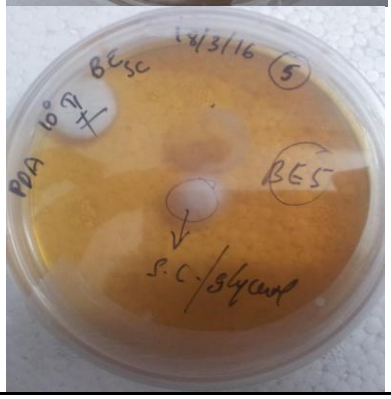
ITS-based phylogenetic tree

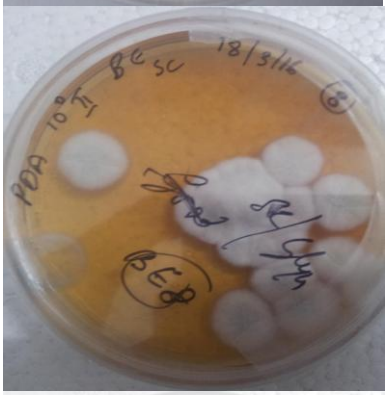
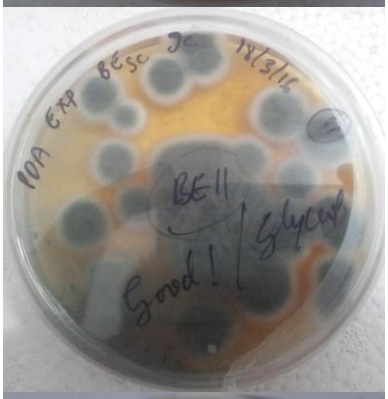
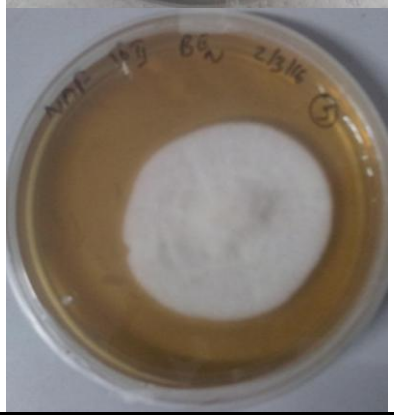
The evolutionary relationships of selected 7 fungi are presented in Fig. 2. The tree details retrieved from MEGA software are reproduced here, with minor modifications: "The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.63763121 is shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 17 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 637 positions in the final dataset. Evolutionary analyses were conducted in MEGA7". In the phylogenetic tree (Fig. 2), isolates #BEF12, #BENF13, #BENF22 and #BEF3 clustered within *Aspergillus terreus* clade. Isolates #BEF4 and #BEF18 clustered within the *A. allahabadi* clade and *A. sydowii* clade, respectively. One isolate, #BENF21 clustered within the *Penicillium shearii* clade. *Caliciopsis indica* NR119752 is the designated outgroup (Fig. 2).

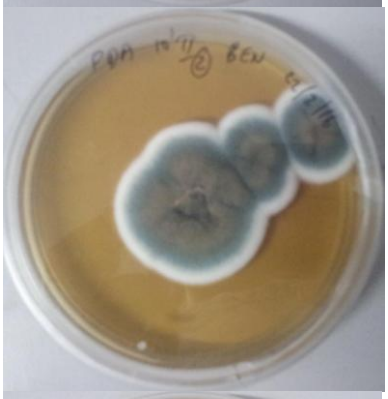
Discussion

There have been a few studies on fungal diversity in mangrove soil collected from India. Pawar & Thirumalachar (1966) compared the growth of pure fungal cultures of marine and terrestrial isolates belonging to the same species of soil fungi and concluded that most marine isolates grew better on sea water agar than on a distilled water medium, while the terrestrial isolates showed opposite reaction. Chinnaraj (1993) reported 63 species of higher marine fungi from the Andaman and Nicobar Islands. Ravikumar & Vittal (1996) reported 48 fungal species belonging to 37 genera from mangroves at Pichavaram coast, Tamil Nadu. Sarma & Vittal (2000) carried out the research on fungal diversity of proproots, seedlings and wood of *Rhizophora apiculata* and wood, roots and pneumatophores of *Avicennia* spp. in mangroves of Godavari and Krishna Rivers, on the east coast of India. Great numbers of fungi were reported on proproots (61), while fungal occurrence on wood (24) and seedling (21) was comparatively low according to their study.

Table 1 Colony morphology of 15 fungal cultures associated with estuarine sediment samples from this study

Strain no.	Taxon name (based on ITS analysis)	Colony morphology on PDA medium (from top)	Colony characteristics
BEF1	Unknown (not sequenced)		White colony with wave-like appearance
BEF3	*Aspergillus terreus*		Light brown, with white margins
BEF4	*Aspergillus allahabadi* (indicated by a red arrow)		White, fluffy with thick mycelia
BEF5	Unknown (not sequenced)		White, Long filaments, very smooth

Strain no.	Taxon name (based on ITS analysis)	Colony morphology on PDA medium (from top)	Colony characteristics
BEF7	Unknown (not sequenced)		Yellow with white margins
BEF8	Unknown (not sequenced)		Pale ash coloured colonies
BEF11	Unknown (not sequenced)		Dark green with white margins
BENF12	*Aspergillus terreus*		White colony with thick growth

Strain no.	Taxon name (based on ITS analysis)	Colony morphology on PDA medium (from top)	Colony characteristics
BENF13	*Aspergillus terreus*		Light brown with white margin, sporulating
BENF14	Unknown (not sequenced)		Slightly ash coloured, white filamentous colony
BENF16	Unknown (not sequenced)		Olive green center with white margins, sporulating
BENF17	Unknown (not sequenced)		Velvety center with pale-white margin

Strain no.	Taxon name (based on ITS analysis)	Colony morphology on PDA medium (from top)	Colony characteristics
BENF18	*Aspergillus sydowii*		Dark green with white margin, sporulating
BENF21	*Penicillium shearii*		Green coloured with white margin, sporulating
BENF22	*Aspergillus terreus*		White, filamentous with yellowish tinge, sporulating

Thamizhmani & Senthilkumaran (2012) carried out fungal diversity studies in four stations, *i.e.*, Ariyankuppam, Thenkaithettu, T.R. Pattanam and Muthukuda located along the East Coast of India. During their study, 80 fungal species were obtained from 4 sampling stations with the help of plating and baiting techniques. Forty-three species of fungi were isolated from sediment samples, 40 species from water samples and natural substrates yielded 37 species. Recently, Saravanan & Sivakumar (2013) carried out a study in five sampling stations in Mammallapuram, Tamil Nadu. Water, sediment and natural substrates of plants were collected. The study was carried out for four months and a total of 41 fungal species were enumerated by plating and baiting techniques including 24 species from sediment samples, 30 species from water samples and 24 species from natural substrates.

In the present study, 15 fungi were isolated from the estuarine sediment samples of Bhairavapalem. Among these, 7 isolates were selected and characterized using ITS-based phylogenetic tree. The phylogenetic analysis suggested that, these 7 isolates belong to 4 species and 2 genera. There are few reports on DNA-based phylogenetic analysis of fungal diversity from sediment samples of Gautami Godavari River. To the best of our knowledge, this is the first report of ITS-based phylogeny of fungi from estuarine sediment samples from Gautami Godavari River at Bhairavapalem estuary. It is highlighted here that the fungal identification is tentative, as further studies are to be carried out using a polyphasic approach, including mycotoxin analysis, to understand the genetic diversity and cryptic species present in the estuarine samples.

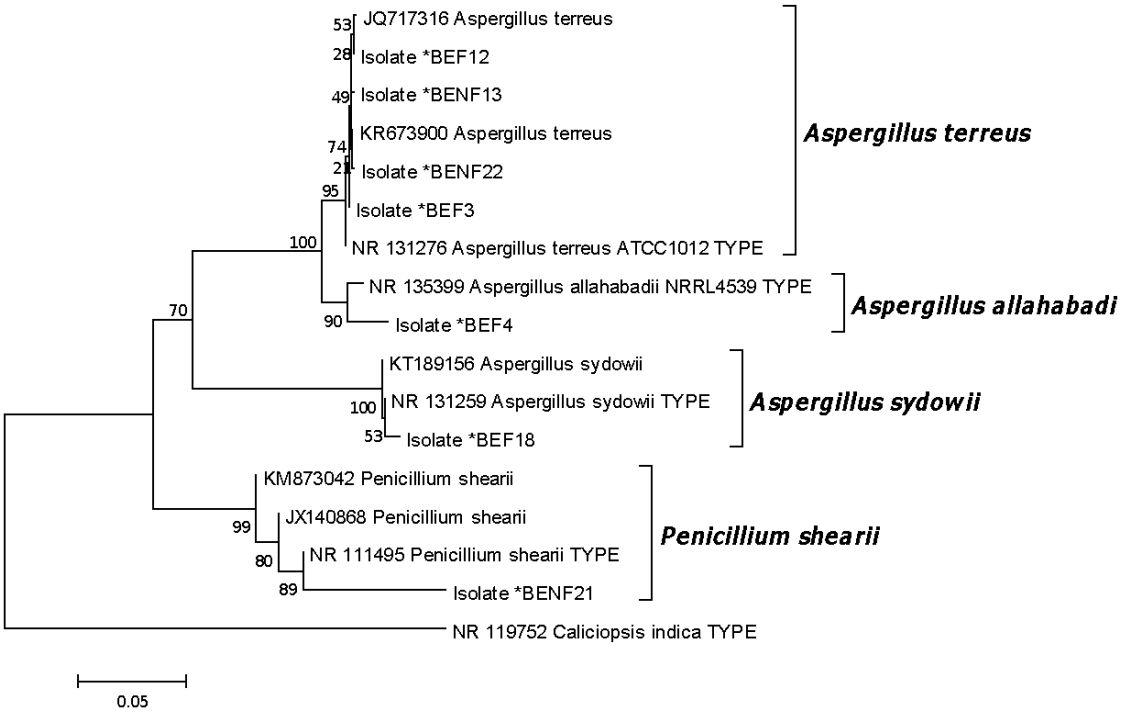


Fig. 2 – Evolutionary relationships of select 7 fungi (marked with *BEF3, *BEF4, *BEF12, *BENF13, *BEF18, *BENF21, *BENF22) from Bhairavapalem estuarine sediment samples as inferred from ITS sequence analysis using NJ method in MEGA

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