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# Morphology And Phylogenetic Characterization Of Aspergillus Species Isolated From Rhizospheric Sediments Of Avicennia Marina Mangrove

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**Abstract**

Mangroves, a salt-tolerant coastal ecosystem, were known for their large deposits of organic matter that protected the niche of a large consortium of microorganisms. However, the role of mangrove and fungal dispersal needs to be studied for their interaction and importance in the estuary environment. Study of isolation and molecular characterization of fungi isolated from rhizospheric sediment of mangrove habitations of Ghogha coast. The fungal characterizations were based on PCR amplification and genomic sequencing of the internal transcribed spacer region (ITS), along with the morphological characterization. The sequences derived were subjected to BLAST analysis and were assigned species name after comparison with representative sequences and reported to be Aspergillus niger from mangroves sediments. This study highlights the important role of fungi in the mangrove ecosystem.

**Keywords:** Fungal diversity, mangrove, Aspergillus Niger, molecular characterization, internal transcribed spacer regions (ITS).

## 1. Introduction

In tropical and subtropical regions among which fungi are the key microbial community found in the sediments and the plant parts (Friggens et al., 2017; Rodriguez et al., 2013). Mangroves exhibit high microbial diversity and play important roles in bioregulation, promotion of plant growth, maintenance of nutrient availability, bioremediation activities (Thattoi et al., 2013), and in the carbon and nitrogen cycles. It is essential and is involved in the breakdown of lignocellulosic. The fundamental role of this ecosystem in productivity and biodiversity makes them an important transition zone between the terrestrial and marine environment (Sridhar et al., 2011). The fungi inhabit in mangrove forest as saprophyte, symbionts or parasites and important decomposers of organic matter. Diversity of fungi depends on their metabolism, changes in salinity; substrate availability. (Hrudayanath et al., 2013).

Mangrove distribution is strongly influenced by various abiotic factors such as temperature, humidity, and water availability. However, mangrove species are vulnerable to oil spills, deforestation, pollution, overexploitation of resources, heavy metal pollution by various chemicals, pollution of wastewater and other anthropogenic activities (Diaz, 2011). The sediments concentration increases as it accumulates on the surface. (Fernandez et al., 2014). Total 414 fungus belonging to 226 genera were recorded from the Indian mangrove habitates among which, Ascomycetes have

become the primary genus in a marine environment. Most of the genera of fungi isolated from Avicennia marina are Aspergillus sp., Biopolaris sp., Botrytis sp., Chaetomium sp., Cladosporium sp., Curvularia sp., Fusarium sp., Masoniella sp., Penicillium sp., Rhizopus sp., Helminthosporium sp., Gloeocercospora sp., Mortierella sp., Trichoderma sp., Torulla sp., Verticillium sp., Trichothecium sp. (Anjugam et al., 2019). Most of the identified fungi are endophytes and produce important bioactive compounds that are only important for host immunity and development (Sanchez et al., 2013). Aspergillus sp. Some fungi such as. And Penicillium sp. It is known to deposit heavy metals in intracellular compartments, suggesting alternatives to decontamination (Cardoso et al., 2010). Many fungi in the ascomycete family colonize mangrove trees (Pan et al., 2018), and the relationship between fungi and plants is unknown.

Aspergillus is widely distributed in nature and due to its small size and conidia having easy dispersal rate can make them more persistent in the environment; also known for its enzyme profiling and metabolites having many industrial application, model organisms and human pathogens (Kjaerbolling et al., 2018), which is also helpful in studying and improving the future omics (Shu- Lei et al., 2020) through which we can furthermore untapped bio resources for identification of novel products (Raghukumar et al., 2004; Tisthammer et al., 2016).

Advancements in molecular technique led to many new studies in microbial ecology, by using an important approach to explore microbial populations using Polymerase Chain reaction (PCR) amplification and sequencing of PCR gene products, which is used to molecularly characterization by regions internal transcribed spacer (ITS1- ITS4). In order to resolve the difficulties of fungal identification to species level, the internal transcribed spacer regions (ITS) are used as official universal DNA barcode for fungi (Yin et al., 2017; Rajeshkumar et al.,2019), for identification of yeasts and fungi.

Several genetic markers were used for rapid identification and maintaining accuracy of fungi having conserved sequences using internal transcribed spacer regions (ITS) and other secondary markers such as Beta- tubulin genes (BenA), calmodulin (CaM) and RNA polymerase II gene (RPB2) (Raja et al., 2017; Das et al.,

2014; Rajeshkumar et al.,2019; Visagie et al., 2014). In the present study, identification of varied assembly of Aspergillus can be done by molecular identification, Phylogenetic analyses, and morphologically characteristics.

## 2. Materials and Methods

### Study area

The Ghogha coast was selected for this study. The coastline of Ghogha in Bhavnagar, Gujarat is about 4 km (21°40’32" to 21°41’18" N and 72°17’5" to 72°16’48" E). Ghogha has muddy habitats and a mangrove forests, mainly the Avicennia marina (Solanki et al. 2016) (Fig.1 & Fig.2).

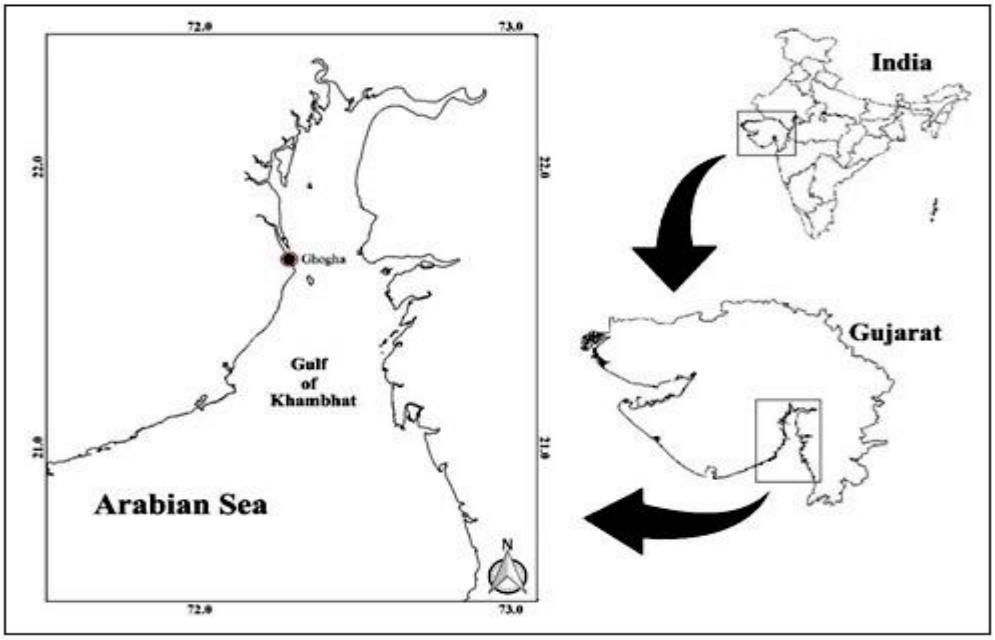


Figure 1: Map of study Site

**Sample collection**

Mangrove rhizospheric sediment sample was collected at 2-3m depth using a soil cover during low tides pre-cleaned glass bottles were used and stored at -20˚C before further analysis. Sediment samples were used to carry out a fungal analysis within 24 hours of collection. Samples were collected in 3 replicates (Petinataud et al., 2016).

Figure 2: Mangrove habitation of Ghogha

**Isolation of fungi from collected sediment sample**

Sediment samples were serially diluted upto $10^{-5}$ dilutions, by adding 1g in 10ml of 2% saline and suspended on the orbital shaker at 250rpm for 10min. 0.1ml of the residue of each sample was spread plated on sterile potato dextrose agar (PDA) plate and Malt extract Agar (MEA) plates in duplicate (containing 50µg/L of chloramphenicol), using sterile glass rod. All the plates were incubated at 30˚C for 7 days (Ezeonuegbu et al., 2014).

**Colony morphology and microscopic characterization of fungal isolates**

Each colony grown on the medium was characterized based on its appearances, color, zonation, and sporulation (Machido et al., 2014). The distinguishable colonies were sub-cultured on PDA slant at room temperature to obtain pure isolates. By the use of tape, the mycelium was separated and placed on a slide with a drop of lacto-phenol and sealed for microscopic characterisation examined under optical microscope using x40 objective lens. The isolates were characterized and identified using the mycobank (http://mycobank.org) and taxonomic keys (de Hoog et al., 2001) were used.

**DNA extraction, PCR amplification, sequencing and Phylogenetic analysis**

For extraction of genomic DNA, fresh fungal mycelium was collected in a PDA plate using Cetyltrimethyl ammonium (CTAB) method (Heinig et al., 2013). The internal transcribed spacer (ITS3-ITS4) regions of the fungi were amplified with the universal ITS primers, ITS3F (5' GCATCGATGAAGAACGCAGC 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Qadri et al., 2014), using the polymerase chain reaction (PCR). The amplified products were submitted in order to obtain a consensus sequence for sequencing and alignment by Basic Local Alignment Search Tool (BLAST) programs to find out homology for closed relatedness of organisms (≥ 98% similar to the query sequence). Fungal ITS- rDNA sequences of isolates were detected, Phylogenetic analyses of the isolates were carried out using MEGA X software (Felsenstein, 1985). The Neighbor- Joining (NJ) method was used to infer the evolutionary history of the fungal isolates (Saitou and Nei, 1987), and the bootstrapping was carried out, the evolutionary distances were computed using the Kimura 2 parameter method (Kimura, 1980).

**3. Results & Discussion**

Growing in solid culture medium, white mycelia was formed which later turned black (Fig. 3). When observing microscopically, theses isolates have smooth hyphae, the conidial heads are radiated with conidiogenous cells biseriate, by classical identification method fungi belongs to Aspergillus genus using taxonomic keys (de Hoog et al., 2001) and Mycobank database (http://mycobank.org). By ITS analysis, sample labelled as NF in red was related to Aspergillus niger.

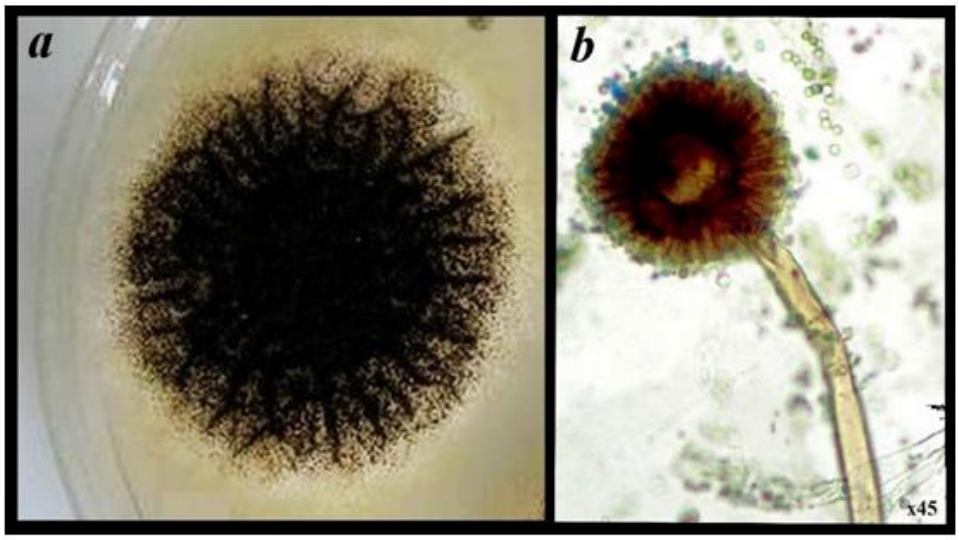


Figure 3: a. colony and b. microscopic observation of Aspergillus niger

**Determination of nucleotide sequence homology of the 18S rDNA amplicons of Aspergillus niger**

After sequencing, the nucleotide sequences were compared with ITS sequence data strains available on the NCBI GenBank database by using the BLASTN sequence match routines. 320bp of ITS region consensus sequence was generated using aligner software.

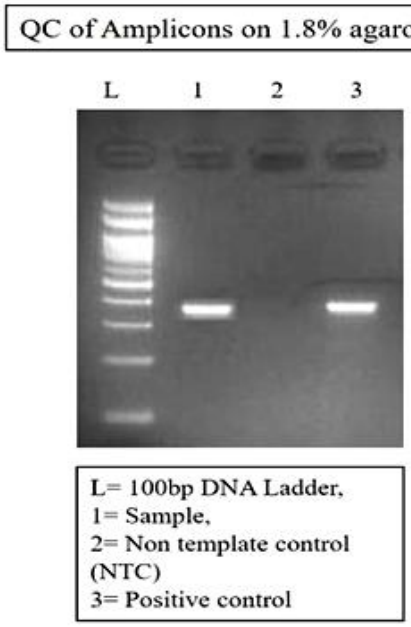


Figure 4: Quality Check on 1.8% Agarose gel (PCR product) (1.8% Agarose gel showing single 350 bp of ITS amplicon).

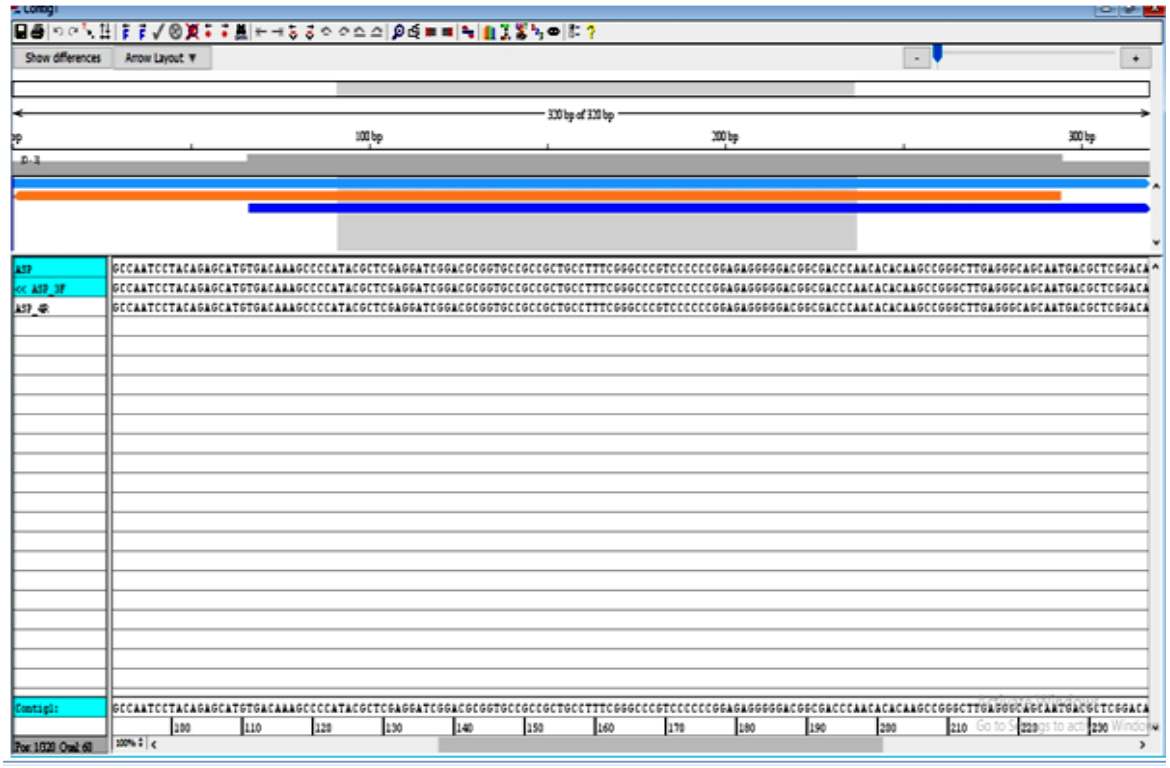

Figure 5: Snap-Shot view of Consensus Sequence

This analysis involved 11 nucleotide sequences by removing all ambiguous positions by pairwise deletion option and there were a total of 344 positions in the final dataset. The analysis is supported by Bootstrap 500 iterations and by the neighbour joining algorithm and dendrogram showed the detection of Aspergillus niger (Fig.4, 5 & 6; Table 1 & 2).

**Table 1: Blast Results**

| Accession ID | Description | Max Score | Total Score | Query cover (%) | E Value Ident. | Per. (%) |
|---|---|---|---|---|---|---|
| MH511140.1 | Aspergillus niger strain S.F-6 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT497445.1 | Aspergillus sp. isolate SS_16 small subunit ribosomal RNA gene | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT447497.1 | Aspergillus niger strain GFRS29 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT446087.1 | Aspergillus niger strain ZMQR6 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MK646029.1 | Aspergillus sp. strain F2826 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |

| | | | | | | |
|---|---|---|---|---|---|---|
| MK640609.1 | Aspergillus niger voucher HQU AR4 small subunit ribosomal RNA gene, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT106901.1 | Aspergillus niger isolate DR12 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT102656.1 | Aspergillus sp. isolate 20 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT102655.1 | Aspergillus sp. isolate 19 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |
| MT065679.1 | Aspergillus niger strain OOSF9 internal transcribed spacer 1, partial sequence | 549 | 549 | 100% | 5e-152 | 97.26 |

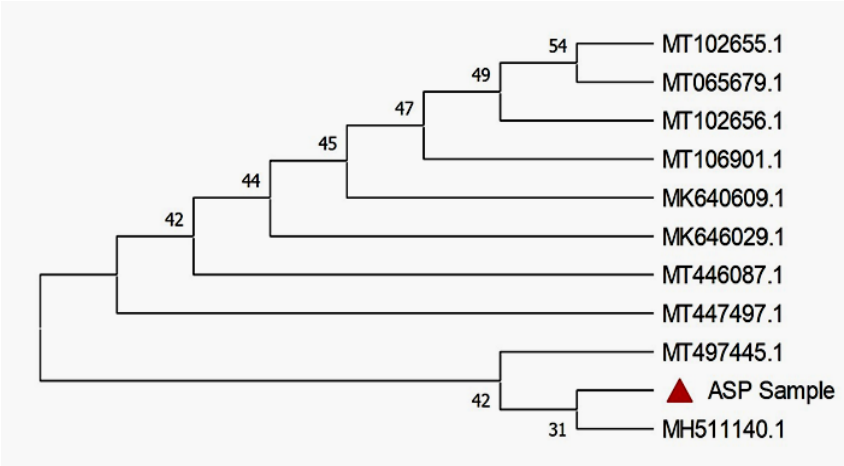


Figure 6: Phylogenetic Tree

**Table 2: Distance matrix**

| | ASP Sample | MH511140.1 | MT497445.1 | MT447497.1 | MT446087.1 | MK646029.1 | MK640609.1 | MT106901.1 | MT102656.1 | MT102655.1 | MT065679.1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ASP Sample | | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 | 0.128 |
| MH511140.1 | 1.032 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| MT497445.1 | 1.032 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MT447497.1 | 1.032 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| MT446087.1 | 1.032 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| MK646029.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| MK640609.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 | 0.000 |
| MT106901.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 | 0.000 |
| MT102656.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 | 0.000 |
| MT102655.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.000 |
| MT065679.1 | 1.032 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | |

**Discussion**

The relevance of this study is related as a baseline of a mycobiome approach in mangrove fungi identification. Aspergillus niger was previously recorded in other mangrove forest in China, Indonesia etc. (Sathiya et al., 2009; Deng et al., 2009; Lumbreras – Martinez at al., 2018). Traditionally, identification and classification of Aspergillus niger was based on the morphological features (Patki et al., 2015). Nevertheless, molecular characterization has proved to be an indispensable tool, since it allows distinguishing the Phylogenetic features (Samson et al., 2014). Our result are consistent with many previous research, amplification of ITS regions is widely accepted as a DNA barcode for fungi (Conrad et al., 2012). The Phylogenetic trees revealed the clear and well resolved classification and evolutionary history of the isolates related species are clustered together (Asan et al., 2019; Ozdil et al., 2017; Samson et al., 2014).

The importance of this study also relies in biotechnological capacity of the mangrove ecosystem and fungi producing the specific protein and metabolites in a high salinity environment (Nicoletti et al., 2018). Bioremediation activity in areas with heavy metal contamination, degrading the cellulose and hemicelluloses activity is reported by Aspergillus sp. (Araugo et al., 2016; Hauchi et al., 2014; Marraro et al., 2012).

Briefly this study was focused on the fungal isolates, as a potential candidate for the future applications and for more investigations and their rehabilitation and restoration purpose to preserve and maintain the ecology of mangrove forest. Therefore, diversity of fungi and other endophytes from mangrove forest ecosystem are necessary to be investigated and explore.

## 4. Conclusion

This study report the molecular and morphological identification of fungi isolated from the rhizospheric sediments of mangrove belonging to Clade Nigri, Aspergillus niger. This works is to understand the distribution of species and to emphasise its importance in the mangrove ecosystem.

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