

Morphological and molecular characterization of *Aspergillus elegans* from small indigenous dry fish 'Jat Puti' *Puntius sophore* (Hamilton 1822) in Bangladesh

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Abstract

Small indigenous species (SIS) of fishes are available source of animal protein, vitamin and minerals, not commonly available in other foods in Bangladesh. These fishes are eaten both in fresh and dried condition. *Puntius sophore* was collected from different regions of Baikka Beel at Sreemangal Upazila, Moulvibazar district, Bangladesh to investigate mycoflora association with dried condition. Fungus was isolated from dried fish using tissue planting methods for classical and molecular characterization. *Aspergillus elegans* was identified through classical taxonomy and molecular approach based on ITS region of ribosomal DNA (rDNA) of fungi. For analyzing ITS4 and ITS5 were used. Mycelium of the identified fungus septated, hyaline, abundant and branched. Long, straight or flexous conidiophore were present. The molecular phylogeny in morphologically identified dry fish fungi of *P. sophore* such as *A. elegans* was studied based on their internal transcribed spacer (ITS). The sequences of ITS 600 bp region of *A. elegans* had 5.8S of rDNA sequences were identical. The highest mycelial growth was recorded in potato dextrose agar (PDA) medium for the growth and development of *A. elegans*. Temperature 30°C was optimum, this fungus grew well in pH 7. So, tested fungi grew well in neutral condition.

Key words: *Aspergillus elegans*, Dry fish fungus, ITS sequences, *Puntius sophore*.

INTRODUCTION

Fresh water SIS fish of Bangladesh is normally known to those, which grow to a length upto 25 cm in adult (Fahmida *et al.*, 2011). Bangladesh is considered one of the most suitable regions for SIS fish, with the largest aquatic biodiversity. SIS is the source of vitamin-A and vitamin-D that are important for building and strengthening our skeletons, eyes and skin (Ara & Nabi, 2013). Preservation of fish is necessary as spoilage occur very quickly. There are so many methods for fish preservation through out the world. Among them sun drying is the most favorite and less expensive methods in Bangladesh (Sultana *et al.*, 2020).

Shutki is the local term used for dried fish. Bangladeshi people are fond of shutki. The significant amounts of dried fishes are exported from Bangladesh and earn good amount of foreign currency (Ara *et al.*, 2020).

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Fungal growth on dried fish caused off flavors, soften the flesh and some can produce mycotoxins under certain circumstances (Pitt & Hocking, 1999). A number of fungi *viz.*, *Aspergillus* spp., *Penicillium* spp., *Fusarium* sp., *Alternaria* sp., *Rhizopus* sp., *Mucor* sp., *Acremonium* sp., *Walleria seba* and *Sporodendron epizoum* from India, Pakistan, Thailand, Malaysia and Hong Kong (Sivaraman *et al.*, 2015). Many fungal species especially *Aspergillus* sp. is known to produce various toxins such as aflatoxins, ochratoxins and sterigmatocystine, which has mutagenic effects in human health (Motalebi *et al.*, 2008). This causes considerable decrease in the consumption of dried fish. According to Sivaraman *et al.* (2015), halophilic moulds like *Basipetospora halophila* (Syn: *Oonspora halophila*), *Polypaecilum pisce*, *Eurotium amstelodami*, *E. repens* and *E. rubrum* were detected from salted shukti. *Aspergillus flavus*, *A. niger*, *A. sydowii*, *A. wentii*, and *A. penicilloides*, *Penicillium citrinum* *P. thomii*. *B. halophilica* were also common dried fish fungi. Most commonly encountered fungal genus in Veravel (Gujarat, India) is *Aspergillus*, *Rhizopus*, *Penecillium*, *Absidia* and *Mucor*. Presence of pathogenic fungi like *Aspergillus* sp, *Lichtheimia* spp, *Curvularia* spp may causes Aspergillosis, mucormycosis and phaeohyphomycosis to the handlers and consumers.

Research articles on the associated fungi in dried fish of Bangladesh is very limited. The present research will give comprehensive information on fungal presences in SIS dry fish caused by various fungi that affect the dry fish preservation and marketing. Thus, acquiring knowledge for the implementation of appropriate and effective control measures against the fungal diseases and the causal agent would play a vital role in quality dry fish production, good storage and transportation. Fungal growth on dried fish indicates the onset of spoilage and deterioration of the fish product. Again, fungi can produce dangerous mycotoxins under certain circumstances, which have mutagenic effects in human health. Therefore, the present experiment was to identify and investigate morphology, biology and cultural conditions from dry 'Jat Puti' i.e. *Puntius sophore* using classical fungal taxonomy and ITS sequence analysis.

MATERIALS AND METHODS

Fresh and dried 'Jat Puti' *Puntius sophore* (Fig. 1.); a kind of small indigenous species (SIS) of fish were collected from different regions of Baikka Beel at Sreemangal Upazila, Moulvibazar district, Bangladesh for the isolation and identification of selected fungi through classical taxonomy and molecular techniques. Fungal morphology, biology and physico chemical condition was established of the isolated pathogens.

Collection and sterilization of infected fish samples: Sterilized polyethylene packed were used to avoid secondary infection in collected fishes and kept them to the Laboratory of Mycology, Jahangirnagar University, Savar, Dhaka for further investigations.

Dried fishes were washed with running tap water and soaked in 5% NaOCl (sodium hypochloride) for 2-3 minutes for surface disinfection. After that samples were washed three to four times with sterilized distilled water and air dried into the laminar airflow cabinet. Tissue planting method was applied to isolate fungal pathogens; subculture was

cultivated on PDA medium; the pure culture was preserved in refrigerator at -4°C . Standard manuals were used to identify the isolated fungi on the basis of colony morphology, morphological characteristic of conidia and conidial measurements.

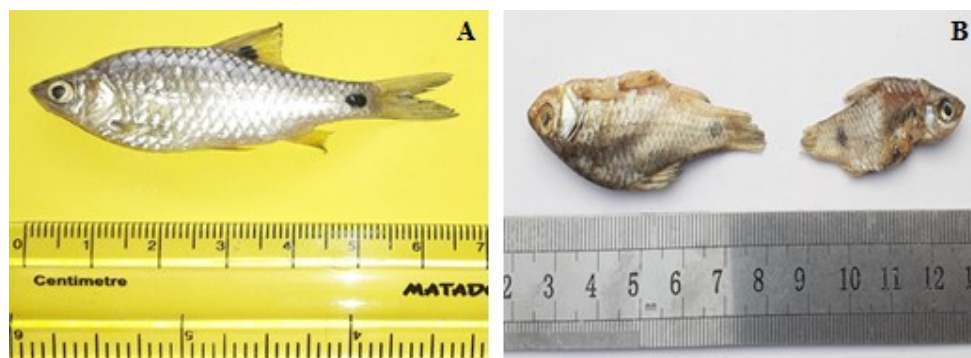


Fig. 1. Fresh (A) and dried (B) condition fish of *Puntius sophore* was collected from different regions of Baikka Beel at Sreemangal Upazila in Moulvibazar District, Bangladesh for present study

Molecular Characterization: Molecular identification was carried out by using the ITS sequence of the dry fish fungal genome (Alam *et al.*, 2009). The fungal genomic DNA was extracted using the Maxwell Cell Kit (AS1030, Promega, USA). The primer forward ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and reverse ITS5 (5'-GGAAGTAAAAGTCG TAACAAGG-3') were used for the PCR amplification. The PCR was performed in a total volume of 25 μl reaction mixture by using 5 μl DNA template (20ng/ μl) and GoTaq® G2 Hot Start Green Master Mix (Promega, USA) as pre-heat at 94 $^{\circ}\text{C}$ for 5 min, 35X (94 $^{\circ}\text{C}$ for 30 s, 57 $^{\circ}\text{C}$ for 30 s, 72 $^{\circ}\text{C}$ for 5 min), and 72 $^{\circ}\text{C}$ for 10 mins (Sultana *et al.*, 2020). The purified PCR products were sequenced bi-directionally in First BASE Laboratories SdnBhd (Malaysia). DNA sequences were analyzed by BioEdit and MEGA6 software. Sequencing data were submitted to the NCBI, under accession number JUF0054. A BLAST search with the ITS sequences were used to reveal the closest matching taxa. Data was converted from fasta to MEGA format with Clustal W. Maximum likelihood, Neighbor-joining, and Maximum parsimony trees were generated (Tamura *et al.*, 2013).

Effect of culture media, temperature and pH : Potato dextrose agar (PDA), carrot agar (CA), potato sucrose agar (PSA), Richard agar (RA), Honey peptone agar (HPA), Honey agar (HA) culture media were used to investigate the mycelial growth characteristics of the dry fish fungus (Sultana *et al.*, 2020). Before autoclave the media will be adjusted to pH 6.5. To find out the effect of temperature on mycelial growth of dry fish fungus was inoculated on PDA medium and incubated at 15, 20, 25, 30 and 35 $^{\circ}\text{C}$ for seven days (Alam *et al.*, 2010). The effect of pH on the growth of the pathogen was assayed on PDA medium. Five different pH levels *viz.*, 5.0, 6.0, 7.0, 8.0 and 9.0 were selected to evaluate the mycelial growth and development of the tested dry fish fungus. Before autoclave the PDA medium was adjusted to pH 5, 6, 7, 8 and 9 with the addition of 1 N NaOH or HCl

and it will be incubated at 30°C for 7 days. Radial growth of mycelia on each Petri dish would be measured at 3 directions (Alam & Rahman, 2020).

RESULTS AND DISCUSSION

Morphological Characteristics of *Aspergillus elegans*: Colonies of *A. elegans* were yellowish, effuse. Mycelium well developed, septate, profusely branched and hyaline. Cells are multinucleate. Conidiophores were very long, often with a foot cell, straight or flexuous, swollen at the apex into a spherical vesicle. Surface of vesicle covered by closely packed more or less clavate branches. A closer look will revealed that the conidial heads of the organism to be globose, catenulate, dry, echinulate or biseriolate conidial heads and yellow to ochre conidia and sclerotia were dark brown in color (Fig. 2).

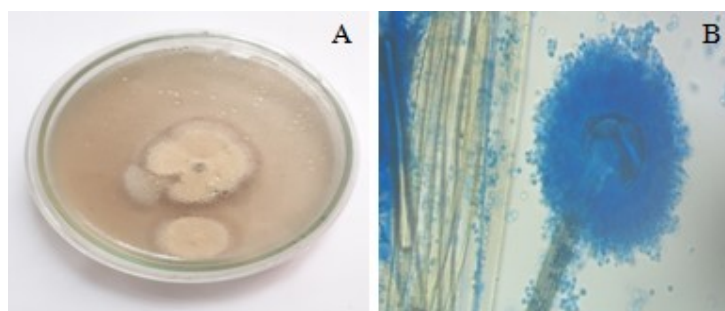


Fig. 2. Morphological view of *Aspergillus elegans*. A, Vegetative growth of *A. elegans* on PDA medium B, Microscopic view of spore of *A. elegans* (40 x 10x)

Molecular Characterization of *Aspergillus elegans*: The ITS region of 700bp was amplified using ITS4 and ITS5 primers and sequenced (Fig. 3). Recent molecular phylogenetic studies have demonstrated that the internal transcribed spacer (ITS) region of genomic DNA is very useful for identification of fungi at lower taxonomic levels. The internal transcribed spacer of rDNA is considered as a variable region among the species and even among the strains (Alam *et al.* 2010).

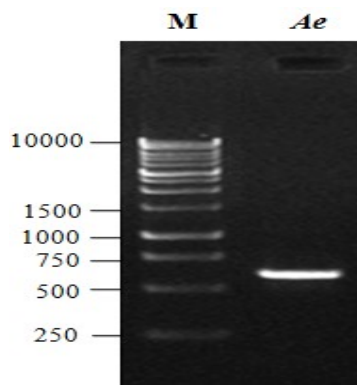


Fig. 3. ITS region of *Aspergillus elegans* (*Ae*). M, molecular size marker (1 kb DNA ladder)

Phylogenetic tree was constructed based on the nucleotide sequences of the ITS regions in thirty seven fungal taxa were downloaded from the NCBI database for phylogenetic analysis. Percent homology of rDNA sequence of ITS region (JUF0054) was compared with formerly identified fungi *A. elegans* under accession number EF661413.1. In maximum parsimony tree, there are seven different clades were found in the phylogenetic tree (Fig. 4). Reciprocal homologies of the ITS region sequences ranged from 98 to 100%. The sequencing data of the selected NCBI GenBank strain (AM981238.1 *Penicillium chrysogenum*) was used as out group for the comparative studies on phylogenetic relationship with the selected strain of *A. elegans* (JUF0054). The results indicated that all the individual species of *A. elegans* belong to single cluster. Alam *et al.* (2010) reported that ITS sequences are genetically constant or show little variation within the species, but vary between species in a genus. Based on molecular evidence, it is clearly indicated that our studied fungus is *Aspergillus elegans*.

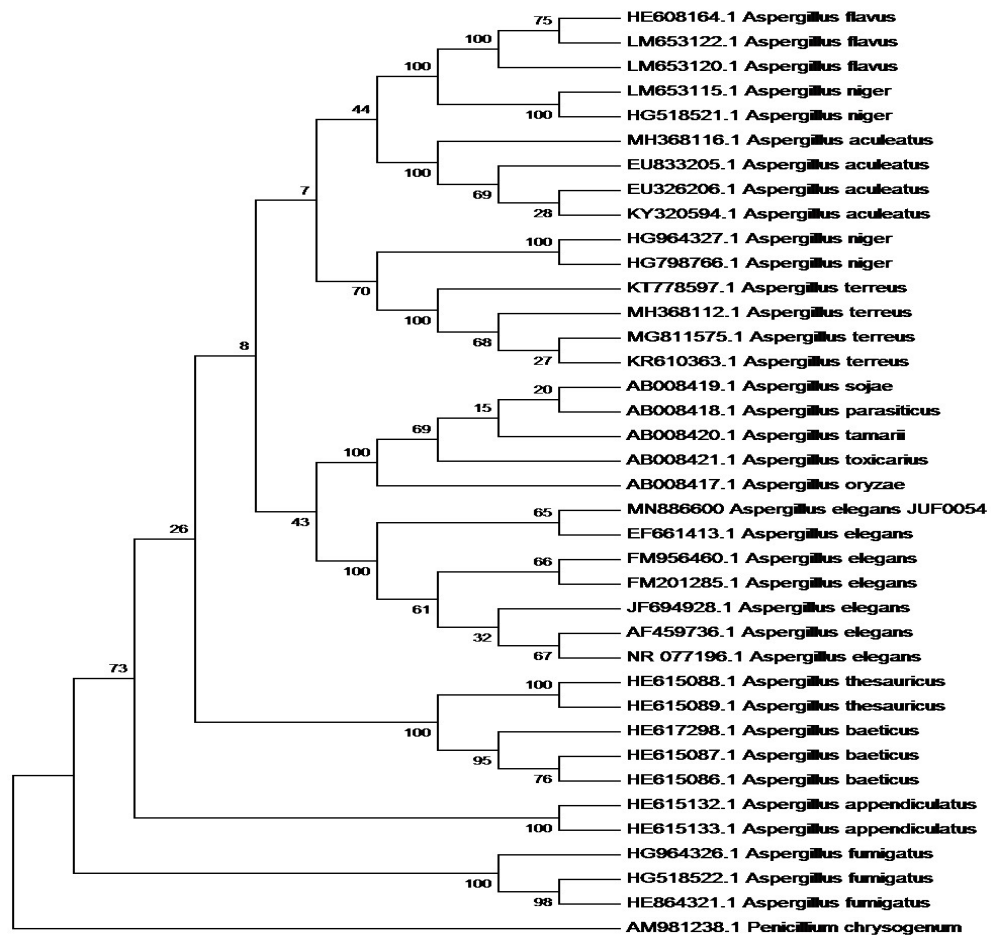


Fig. 4. Maximum likelihood tree of *Aspergillus elegans* from analysis of ITS sequence with bootstrap value. Our organism marked as MN88660 JUF0054

Effect of culture media, temperature and pH of *Aspergillus elegans*: The results of five different culture media such as PDA, CA, RA, PSA, HPA and HA for the mycelial growth of *Aspergillus elegans* have been presented in Fig. 5. The highest mycelial growth (95.33mm) of *A. elegans* was recorded on PDA medium which was followed by PSA medium and the lowest growth (65.50 mm) was measured in HPA medium. The current results of *A. elegans* is supported by Koley & Mahapatra, (2015) who reported mycelial growth and development of *Alternaria solani* very well in potato dextrose agar culture medium among the tested solid culture media. Result suggested that mycelial growth pattern was the best on PDA medium under *in vitro* condition. Similar results also reported by Kumar *et al.* (2008) who working with *Alternaria solani*.

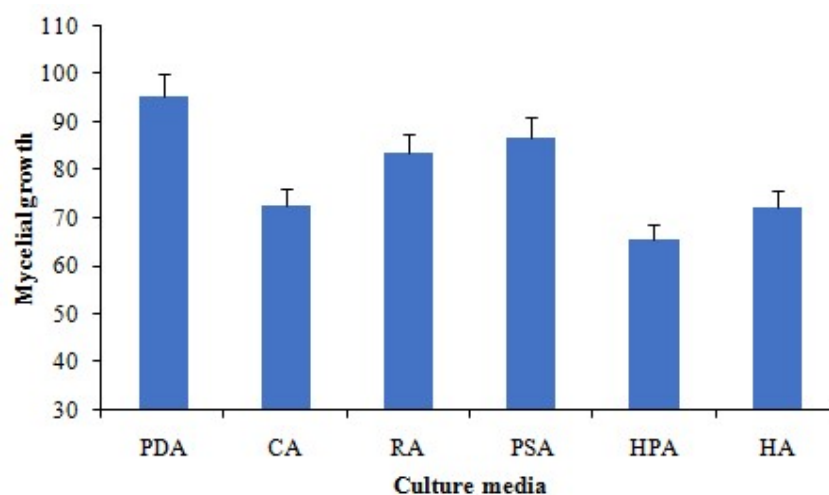


Fig. 5. Effect of culture media of *Aspergillus elegans* at 7 dpi. PDA, potato dextrose agar; CA, carrot agar; PSA, potato sucrose agar; RA, Richard agar; HPA, honey peptone agar; HA, honey agar

Present study probed the effect of temperature on mycelial growth of *Aspergillus elegans* on PDA media were incubated at five different temperature such as 15°C, 20°C, 25°C, 30°C and 35°C and the results have been presented in Fig. 6. The data informed that the highest growth of *A. elegans* was recorded at 30°C, followed by 25°C. In our experiment, *A. elegans* grew maximum at 30°C which is consistent with the previous findings of Iwen *et al.*, (2007) who cited that the highest mycelia growth and sporulation of *A. elegans* registered at 30°C. The result also showed that temperature has no effect on the mycelial growth of *A. elegans* and that their appropriate temperature can be used to inhibit the growth of the studied fungus to maintain the quality.

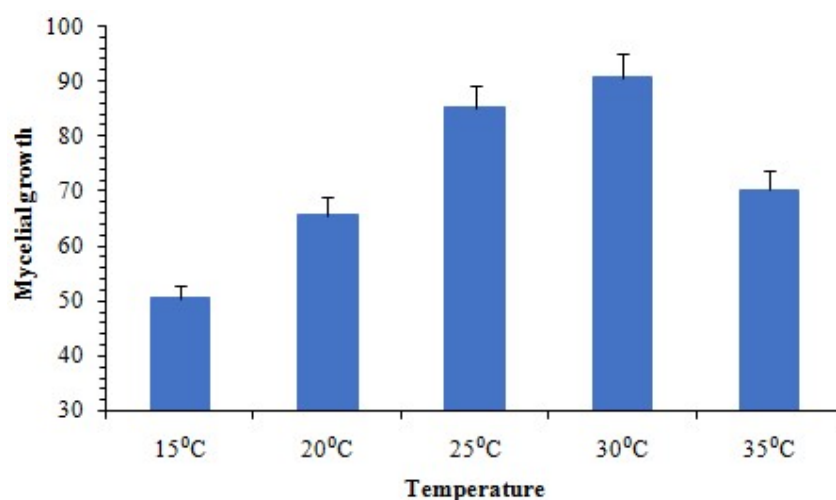


Fig. 6. Effect of different temperature on mycelial growth of *Aspergillus elegans*

pH is an important parameter to understand the fungal ecology. However, five different pH level viz., 5, 6, 7, 8 and 9 were used for the incubation of the experimental plates. Maximum mycelial growth (88.25mm) of *A. elegans* was recorded at pH 7 and followed by pH 8 and pH 6, while minimum mycelial growth was found at pH 9 (Fig. 7). Sonyal *et al.* (2015) reported that the maximum growth of *Ceratocystis fimbriata* at pH 7.5, followed by pH 7.0 and pH 8.0, which showed different from our experimental findings. Yadahalli *et al.* (2007) noticed the maximum mycelial growth of *Ceratocystis paradoxa* when the pH of the media was between 6.0 and 7.5. So, the result suggested that neutral pH condition is favorable for *A. elegans*.

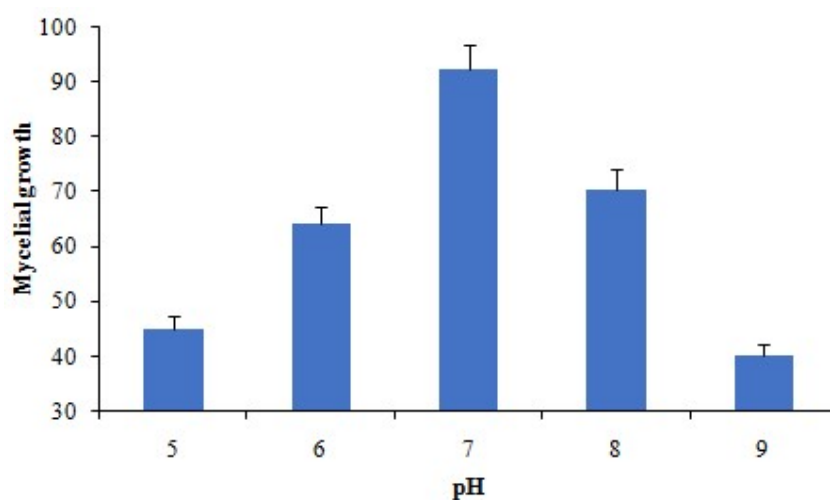


Fig. 7. Effect of different pH on the mycelial growth of *Aspergillus elegans*

This research showed that most of the dried fish samples from the selected areas were contaminated with fungi. It is necessary to conduct pathogenicity test of the above fungi as the dried small indigenous fish species considered an important position as a popular food item for the people of Bangladesh. The present study serve as a pioneer research materials in the investigation of associated fungi of dried small indigenous fish species in Bangladesh and helpful for further investigation.

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