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Roksana Sultana
Department of Zoology
Jahangirnagar University
Savar, Dhaka, Bangladesh

Ismot Ara
Professor, Department of Zoology Jahangirnagar University Savar, Dhaka, Bangladesh

Ismath Jahan Chanda
Department of Zoology
Jahangirnagar University
Savar, Dhaka, Bangladesh

Nuhu Alam
Professor, Department of Botany
Jahangirnagar University
Savar, Dhaka, Bangladesh

Corresponding Author:
Ismath Jahan Chanda
Department of Zoology
Jahangirnagar University
Savar, Dhaka, Bangladesh

First report of *Lichtheimia hyalospora* from fresh water dried shrimp in Bangladesh

Roksana Sultana, Ismot Ara, Ismath Jahan Chanda and Nuhu Alam

Abstract
Dried shrimps are largely consumed as source of protein in Bangladesh. It has been established that dried shrimp can act as vehicle for transmission of some mycological pathogens. The present study investigated the fungal pathogen associated with dry shrimps. *Lichtheimia hyalospora*, was identified through morphological characterization based on mycelium, conidia, colony features as well as molecular characterization using internal transcribed spacer (ITS) region of fungal ribosomal DNA (rDNA). The genetic analysis of *Lichtheimia hyalospora*, the ITS region was amplified using ITS4 and ITS5 primers and sequenced. The length of polymorphism at the sequence level of *Lichtheimia hyalospora* was 721 bp. There is no published research article regarding the studies on the identification and molecular characterization of the associated fungi on shrimps in Bangladesh. To the best of our knowledge, the experimental result concluded that dry shrimps contaminated with saprophytic fungus, *Lichtheimia hyalospora* is the first report in Bangladesh.

Keywords: *Lichtheimia hyalospora*, dry shrimp fungus, fungus morphology, molecular identification

1. Introduction
The freshwater shrimps of the genus *Penaeus* are distributed throughout the tropical and subtropical regions of the world [1]. Shrimp is one of the most important sources of protein and it is popular for its unique test, flavor and texture [2]. Moreover, shrimp meat has micronutrients such as calcium and selenium. Lipids in prawns are largely made up of polyunsaturated fatty acids which are essential for human health [3].
Drying is one of the traditional methods in Bangladesh used as fish preservation. This dried fish is a major source of available animal protein with cheaper cost for the consumers. Consumption of dried fishes in Bangladesh is increasing due to its test and food values [4]. Drying methods dehydrate the fish tissue thereby inhibit microbial growth. The growths of fungus on dried shrimp products cause spoilage, discoloration, rotting, alter the flavour and textural quality of dry fish. So, fungus leads to loss not only the nutrient quality but also huge economic loss. Growth of fungus is mainly influenced by nutrients, temperature, moisture, relative humidity, pH, salt concentration and water activity during storage [5]. Chakrabarti and Varma [5] also reported that *Basipetospora halophila*, *Polypaecilum pisce*, *Eurotium amstelodami*, *E. repens* and *E. rubrum*, *Aspergillus flavus*, *A. niger*, *A. sydowii*, *A. wentii*, *A. penicilloides*, *Penicillium citrinum*, *P. thomii*. *Rhizopus* sp., *Mucor* sp., and *Curvularia* sp. were common fungi in fresh and marine water dried fish, prawn and shrimp.
Schipper [6] already recognized *Lichtheimia hyalospora* as distinct taxa on the basis of morphological features, referring to strains of *Lichtheimia sphaerocystis* as "*Absidia* aff. *blakesleeana*." The degradation of *Lichtheimia blakesleeana* to a synonym of *Lichtheimia hyalospora* is supported by the presence of zygospores. Isolates of *L. hyalospora* were isolated from fermented food (taosi or soybeans). Continued subcultivation of single strains for fermentation might have enhanced the occurrence of mutants whose phenotypes can be characterized by an increase in spore size [7].
Fungal morphology, such as spore-producing structures are very important to identification [8]. Fungal asexual structures, such as conidia/spore shape and size, can often show highly synthetic characters, making identification challenging. For sexual states (teleomorphs), ascomata are not often produced in culture, thus making it difficult to identify them based solely on morphology. As a consequence, polymerase chain reaction (PCR) and DNA sequence-based methods have emerged for identifying species within the mega diverse fungi.

The polymerase chain reaction (PCR) is the most important and sensitive technique presently available for the detection of pathogenic fungi.

The internal transcribed spacer region of genomic DNA is very useful for assessing genetic relationships at lower taxonomic levels [9]. There is no record of fungal inoculums-*Lichtheimia hyalospora* associated with dry fish or shrimp from Bangladesh. Therefore, the present experiment was attempted to identify dry shrimp associated fungus through both morphological and molecular characterization.

2. Materials and Methods

2.1. Sample collection

Fresh water dry shrimps were collected from dry fish whole sale market at Savar, Dhaka with aseptic condition using sterile polyethylene bags (Fig. 1). After collection these samples were brought to the Limnology and Fishery Sciences Laboratory of the Department of Zoology, Jahangirnagar University (JU), Savar, Dhaka-1342. Fungi isolation, identification and pure culture experiments were conducted in the Laboratory of Mycology and Plant Pathology, Department of Botany, JU.

Fig 1: Fresh water dried shrimps.

2.2. Fungus isolation and morphological characteristics

Fungus was isolated through tissue planting method. Infected parts of dry shrimps were cut into small pieces about 0.5cm in length in such a manner so as to include both fungal infected and non-infected tissues in piece. Then sterilization was done using NaOCl (0.5%) solution for 5 minutes and rinsed with distilled water several times. Four pieces of samples were placed into potato dextrose agar (PDA) medium and were incubated under 12/12 hours dark and light condition at 25±2°C for 10 days. Mycelial growth of growing fungus colony was transferred to fresh PDA plates as well as PDA slants to obtain a pure culture. The pure culture of the isolated fungus was identified microscopically using standard methods [10].

2.3. Molecular characterization and amplification of the ITS region

For molecular characterization, fungus genomic DNA samples were extracted using DNA extraction Kit (Promega, USA). DNA concentrations were measured using Nano Drop Spectrophotometer (ND2000, Thermo Scientific, USA). The primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAA GG-3') were used for the PCR reaction [11]. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 25 µl reaction mixture by using a LA Taq (TAKARA BIO INC, Japan) as follows- activation of Taq polymerase at 94°C for 5 minute, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 5 minutes each were performed, finishing with a 10-minute step at 72 °C. The Maxwell® 16 DNA Purification Kits were used to purify the amplification products (Promega, USA). The purified PCR products of approximately 725bp sequenced by using two primers in *First BASE* Laboratories SdnBhd (Kuala Lampur, Malaysia). Amplified PCR products were electrophoresed on 1.5% agarose gel in 1 × TAE buffer for 1 hr at 100V with a 1kb DNA ladder as a size marker and then stained while agitated in an EtBr solution (0.5% µg/mL). The stained gels were visualized and photographed using a UV transilluminator (Kodak Image Station 4000R; Molecular imaging system, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608).

2.4. Analysis of sequences

DNA sequences were checked by BioEdit and MEGA6. Sequencing data were submitted to the NCBI, and received an accession number (MN886599). Multiple sequence alignments were done using MEGA6. Data was converted from fasta to MEGA format with Clustal W. The models of evolution were determined under the Akaike Information Criterion (AIC). The model selected was Tamura-3 parameter for analysis. Maximum likelihood (ML), Neighbor-joining (NJ), and Maximum parsimony (MP) analysis were done and robustness of the branches were determined with 1000 bootstrap replicates along with max-trees set at 1000. The number of replications was inferred using the stopping criterion. Bootstrap values greater than 60% were accepted [12].

3. Results and Discussions

The genus *Lichtheimia* belongs to the family Lichtheimiaceae of the zygomycetous fungus is isolated from dried shrimp food. The genus *Lichtheimia* consists of saprotrophic fungi inhabiting soil or dead organic material, may cause fulminate infections in patients with impaired immunity. *Lichtheimia hyalospora* produces small, dark spores inside pear-shaped (pyriform) sporangia. The species is characterized by a conically shaped columella and a short, pronounced projection, a funnel-shaped apophysis, on the top. The sporangia-bearing stalks are hyaline to slightly pigmented, sometimes branched, and arising from stolons in groups of three to seven. The zygospores are naked with equatorial rings, have opposed suspensors, lack appendages and limited production of rhizoids (Fig. 2). Its thermotolerance enables the fungus to survive the digestive tract of mammals and it is often found in animal intestinal contents and feces. It can cause 'self-heating' of animal feed stored under moist conditions.

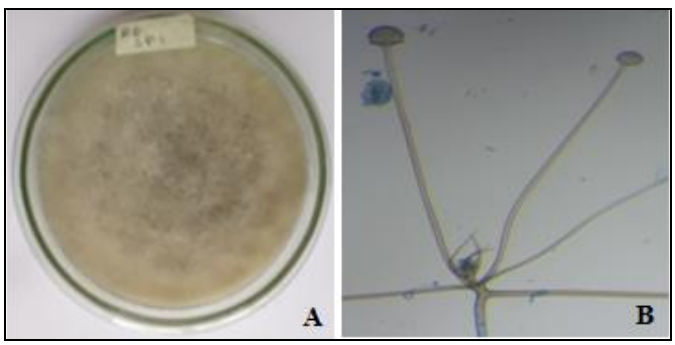

Fig 2: Morphological characteristics of *Lichtheimia hyalospora*. A: Vegetative growth of *Lichtheimia hyalospora* on PDA medium, B: Microscopic view of conidia and conidiophores and mycelium of *Lichtheimia hyalospora* (40 X 10x).

The internal transcribed spacer regions of fungal rDNA are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. The complete set of primers was useful for studies of fungi. The size of the internal transcribed spacer region as measured by gel electrophoresis of PCR products, amplified by primers ITS4 and ITS5, was 721 bp for *Lichtheimia hyalospora*, but other species of this genus had a shorter ITS region, making this characteristic potentially useful in the identification of *Lichtheimia hyalospora* (Fig. 3). Recent molecular phylogenetic studies have demonstrated that the internal transcribed spacer region of genomic DNA is very useful for identification of fungi at lower taxonomic levels [13].

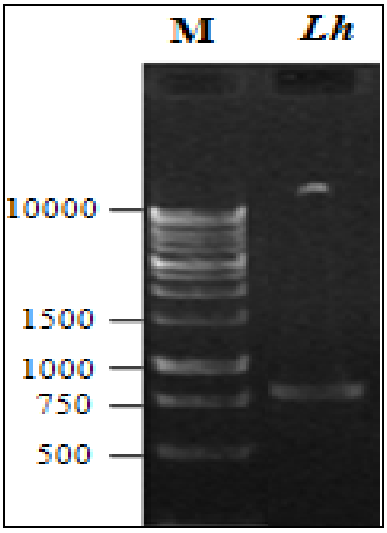

Fig 3: PCR products of the ITS region of *Lichtheimia hyalospora*. M, molecular size marker (1 kb DNA ladder); lane *Lh*, *Lichtheimia hyalospora*.

The ITS region was amplified using ITS4 and ITS5 primers and sequenced. Phylogenetic tree based on the nucleotide sequences of the ITS regions in thirty two fungal taxa were selected from the NCBI database for phylogenetic analysis. In maximum parsimony tree there are eight different clusters were found in the phylogenetic tree. Per cent homology of rDNA sequence of ITS region (MN886595) was compared with formerly identified fungi MH855719.1 *Lichtheimia hyalospora*, GQ342895.1 *Lichtheimia hyalospora*, MN423319.1 *Lichtheimia hyalospora*, GQ342894.1 *Lichtheimia hyalospora*, GQ342894.1 *Lichtheimia hyalospora* and JQ912656.1 *Lichtheimia hyalospora* (Fig. 4). Reciprocal homologies of the ITS region sequences ranged from 99 to 100%. The sequencing data of the selected NCBI GenBank strain JN205840.1 *Dichotomocladium elegans* was used as control for the comparative studies on phylogenetic relationships with the identified fungus of *Lichtheimia hyalospora* (MN886595, JUF0050). The results indicated that all the individual species of *Lichtheimia hyalospora* belong to major cluster. The ITS region is relatively short and can be easily amplified by PCR using universal single primer pairs. Genetic distance exhibited high level of similarity with identical ITS sequences. The size variation was caused by differences in the number of nucleotides, revealing that these strains are clearly distinguishable from each other based on the ecological distribution, substitution, and insertion or deletion polymorphisms of the base position [13]. Alam *et al.* [14] reported that ITS sequences are genetically constant or show little variation within the species, but vary between species in a genus. The genetic diversity detected within groups is probably due to an efficient gene flow and to a high genetic compatibility within the strains tested [15, 16]. Based on the molecular evidence, it is clearly indicated that our studied fungus is *Lichtheimia hyalospora* under the family- Lichtheimiaceae.

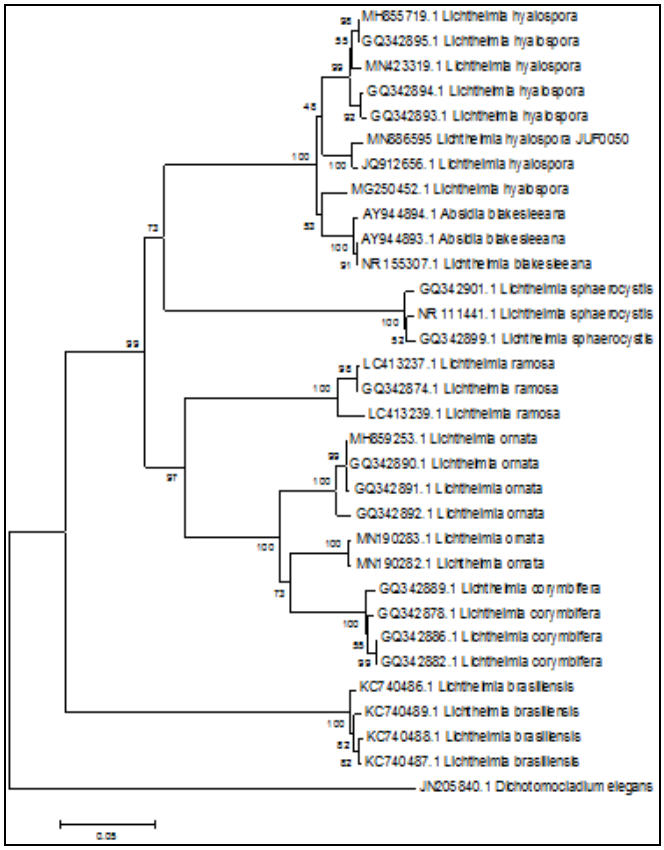

Fig 4: Maximum likelihood tree derived from analysis of ITS sequence dataset of the studied organism with bootstrap value (Bootstrap replication=1000). Our organism (MN886595) marked with 'JUF0050'.

4. Conclusions

The experimental findings of the study concluded that dried shrimps are contaminated with *Lichtheimia hyalospora* is first report in Bangladesh. Phenotypic traits and molecular characterization assured the pathogen identity. Phylogenetic tree was generated with ITS sequence data analysis by maximum likelihood, neibor-joining and maximum parsimony and demonstrates the position of *Lichtheimia hyalospora* under the family- Lichtheimiaceae.

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