Article

Molecular characterisation of oomycetes from fish farm located in Mymensingh sadar during summer

K. M. Mahfujur Rahman and Mohammad Nasif Sarowar*

Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

*Corresponding author: Dr. Mohammad Nasif Sarowar, Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. Phone: +88 01712 117963; E-mail: n.sarowar@bau.edu.bd

Abstract: Oomycetes, also known as water molds, can cause serious infection to plants and animals especially at lower temperature although they dwell in freshwater and moist ecosystem throughout the year. The aim of this research was to investigate the diversity of oomycetes inhabiting in small water bodies during summer. Three types of samples i.e. water, fish mucus and apparently infected muscle samples of fish were collected from a large fish farm consisting of over 100 medium to large ponds in Mymensingh during summer (March to June) in 2015. A total number of 385 samples (284 of water, 79 of mucus and 22 of apparently infected muscle samples) were collected in 15 ml sterile falcon tubes with baits in each. Eleven of the isolates were isolated in Potato Dextrose Agar (PDA) plates and were identified using molecular methods that included DNA extraction, PCR amplification and subsequent sequencing of the ITS region of the genomic DNA of the samples. BLAST analysis to GenBank revealed that two of the isolates were 99% similar to *Pythium* sp. (HQ643814), three of the isolates were 98-99% similar to *Pythium* sp. (KT247392), and each of the remaining four isolates was similar up to 99% to *Pythium* sp. (KF836354), 99% to *Pythium* sp. (EU544193), 99% to *Pythium rhizo-oryzae* (HQ643757) and 100% to *Pythium catenulatum* (KP862946). Two of the eleven isolates were not assessed due to sequencing error. Phylogenetic analysis revealed that six of the isolates are of clade B1 and three of the isolates are of clade B2 in the *Pythium* phylogeny. The results partially suggest that plant pathogenic oomycetes are more common in summer than animal or fish pathogenic isolates in the sampled farm however; intensive sampling with a broad range of freshwater ecosystems during summer can give a clearer view on oomycete diversity in Bangladesh.

1. Introduction

Mycotic diseases of fish are mainly caused by fungal-like organisms called oomycetes often referred to as water molds (van West, 2006). They are capable of infecting different hosts ranging from algae, protists, plants, fungi and arthropods to vertebrate animals (Phillips *et al*., 2008; Beakes *et al.*, 2012; Sarowar *et al.*, 2013). As a group oomycetes are well known for being pathogenic to plants causing serious economic losses to crops worldwide and also environmental damage in natural and captive ecosystem (van West, 2006). Among the plant pathogenic oomycetes, *Phytophthora* is the best studied genus (Phillips *et al.*, 2008); species in this genus cause devastating diseases on several crops, ornamental and wild plants (Kamoun, 2009). *Phytophthora infestans* causes late blight disease in potato and caused great Irish potato famine in mid-19th century (Fry *et al*., 2009). Soybean production is hampered greatly by *Phytophthora sojae* (Kaufmann and Gerdemann, 1958). *Plasmopara viticola* that infect wild grape plants was an important plant pathogen in Europe and North America in the late 19th century (Fry and Grünwald, 2010). *Phytophthora cinnamomi* is a threat to agricultural plants and around

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3000 plants can be affected by this pathogen (Hardham, 2005). *Phytophthora ramorum* is best known for causing sudden oak death on different oak species (Grünwald *et al*., 2008). *Sclerophthora rayssiae* var. *zeae* and *Peronosclerospora philippinensis,* the cause of downy mildew of maize in India and Philippines and agents of 20-90% crop loss annually (Fry and Grünwald 2010). *Pythium aphanidermatum* and *P. ultimum* are causal agents of seed rot, seedling damping-off, and root rotof several plants (Fry and Grünwald 2010). Moreover, *Pythium insidiosum* is the only member of oomycetes that infects mammals and birds (Dick 2001)**.** Animal pathogenic oomycetes are of recent concern in wild as well as in fish culture farms. The most devastating mycotic diseases in fish are saprolegniosis caused by *Saprolegnia* spp., and epizootic ulcerative syndrome (EUS) caused by *Aphanomyces invadans* which result severe mortalities in fish of both wild and captive environment (van West 2006).

In Bangladesh most study on mycotic diseases in aquaculture is based on morphological studies. However, most fungi and fungus-like organisms associated fish diseases are unidentifiable to species level using morphological methods, because they have very similar characteristics and often fail to produce sexual organs *in vitro* (Diéguez-Uribeondo *et al.*, 1996; 2007), or, if they do, it may happen only after a long development time (Xiao *et al*., 2009). Identification of asexual pathogenic isolates using classical taxonomic criteria is impractical and also difficult (Fernandez-Beneitez *et al.*, 2007; Diéguez-Uribeondo *et al*., 1996). Furthermore, species identification has been more challenging because it has required presence of the sexual structures (Petrisko *et al*., 2008). However, recent developments in molecular biology have made it possible to identify fish pathogenic oomycetes at species level which is more reliable, time saving and non-questionable (Robideau *et al*., 2011).

Oomycetes are phylogenetically very distant from the Kingdom Eumycota, the true Fungi, unlike their initial appearance would suggest. There are several developmental stages in the life cycle of oomycetes distinct from the true fungi (Beakes *et al*. 2012). Fishes are more susceptible to mycotic diseases such as saprolegniosis or EUS in winter with low and/or dropping water temperatures, related to impaired immune function at lower temperatures (Noga, 1993). For example, *A. invadans,* is very effective at 18-22 ℃ water temperature or less in infecting fish (Bondad-Reantaso *et al*., 1992). In low water temperatures oomycetes undergo asexual reproduction. During critical environmental conditions such as dryness or extreme temperature, the oomycetes undergo sexual reproduction for survival. It is one of the major evolutionary developments that define more advanced oomycetes (Beakes *et al*., 2012). The sexual phase is thought to enable survival until conditions become more favorable for germination (Sarowar *et al*. 2014). Strategies of survival employed by oomycetes in harsh conditions have been reported (Sarowar *et al*. 2014, Judelson 2009), however, there is no clear description about survival strategy of oomycetes in summer when infection in fish is less frequent.

Recent developments in molecular biology have made it possible to identify fish pathogenic oomycetes at species level which is more reliable, time saving and non-questionable (Robideau *et al*., 2011). The internal transcribed spacer (ITS) region of the rRNA gene of oomycetes is mostly used for DNA sequencing (Peay *et al*. 2008). By comparing ITS nucleotide sequences, it is possible to develop accurate phylogenetic relationships (Lee and Taylor 1992). Furthermore, as non-coding regions, ITS evolves more rapidly than coding regions such as large subunit rRNA (28S rRNA), small subunit rRNA (18S rRNA) and 5.8S rRNA, and can be useful for comparisons at the species or population level (Matsumoto *et al*., 1999). ITS region sequencing, therefore, is an excellent approach compared to morphological methods that can be used to identify and define oomycete species.

There are hardly any studies on the diversity of these pathogenic oomycetes during summer season which would reflect surviving strategies employed by the organism. The target of this current research is to identify diversity of oomycetes species in small water bodies during summer and subsequently construct a phylogenetic tree based on Internal Transcribed Spacer (ITS) region of rRNA gene for better understanding of the nature of the collected species compared to already reported species worldwide.

2. Materials and Methods
2.1. Sampling sites and sample collection
Samples were collected for 8 consecutive weeks from tilapia (*Oreochromis niloticus*), shing (*Heteropnesteus fossilis*) and koi (*Anabustes tudineus*) pond of a well-managed fish farm known as Reliance Aqua Farm of Mymensingh Sadar, Bangladesh in March to June 2015. Three types of samples were collected to investigate the diversity of oomycetes present in the selected ponds during the study period. Water samples from each sampling ponds, mucus samples of brood tilapia, and muscle samples from apparently infected koi and shing were collected in 15 ml sterile falcon tube separately. Water samples (10 ml) were collected from different ponds during each sampling in individual 15 ml falcon tubes containing an autoclaved rice grain that served as bait. Fish mucus samples were (2 ml) collected in the falcon tubes with autoclaved rice grain and was filled with

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autoclaved distilled water (8 ml). In case of apparently infected fish, a small cube of around 0.5 cm² from infected muscle were cut with a sterile scalpel, and washed with distilled water and soaked up in dry paper to reduce bacterial load before being stored in tube with 10 ml distilled water without placing the rice grain. During collection, all samples were stored in an ice box on the sampling site and immediately brought to laboratory and were kept in refrigerator at 5-6 °C for two days.

2.2. Laboratory culture

Inoculation of water samples and mucus samples were carried out by placing the bait into 9 cm diameter petri dish in 25 ml potato dextrose agar (PDA; 39 g in 1 liter dH_2O and autoclaved) medium supplemented with Vancomycin (200 mg/l), Ampicilin (500 mg/l) and Pimaricin (50 mg/l) while infected muscles (washed with dH_2O) placed directly in petri dish with PDA and antibiotics and were sealed with parafilm. Culture plates were maintained at room temperature and check daily for hyphal growth. Once the mycelium has grown into agar, a small agar plug was cut and re-inoculated to a new plate of PDA with antibiotics aseptically several times until no bacterial growth was observed in the oomycetes culture. Cultures were observed for contamination by placing an agar plug of mycelium in petri dish containing 20 ml pea broth (20 ml garden pea in 500 ml dH_2O and double autoclaved) without antibiotics. The plates were sealed with parafilm and incubated at 20°C for three days.

2.3. DNA extraction

Once a bacteria free culture was attained, genomic DNA from the mycelia was extracted using a Wizard® Genomic DNA Purification Kit following manufacturer's instructions with some minor modifications to the protocol. The mycelium from the pea broth was completely dried into filter paper (autoclaved). The dried mycelium was homogenized in 500 µl nuclei lysis solution supplied with the kit in a sterile tissue homogenizer and transferred into a 1.5 ml flip cap tube aseptically. Three (3) µl RNaseA solution supplied in the kit was added in the tube and inverted several times. The mixture was incubated at 65ºC for 45 minutes in hot water bath, with inversions every 15 minutes. After being cooled down to room temperature, 200 µl of mixture sample was transferred to a new 1.5 ml sterile eppendrof tube and 400 µl of protein precipitation solution supplied with the kit was added. The mixture was vortexed vigorously for 5 minutes to emulsify the 2 phases. The tube was centrifuged (Centrifuge 5415R) at $16,000 \times g$ for 5 minutes. The 500 µl supernatant containing the DNA was carefully pipetted to a new 1.5 ml sterile eppendrof tube that already contained 500 µl isopropanol. The tube was inverted until thread like strand of DNA appeared and incubated at 65°C for 5 minutes, before being centrifuged at 4°C, $16,000 \times g$ for 10 minutes. The supernatant was decanted carefully and the pellet was washed with 300 µl 70% ethanol several times. The pellet along with 70% ethanol was centrifuged at $13,000 \times g$ for 2 minutes. The ethanol was carefully air-dried and the pellet was re-suspended in 30 µl of DNA rehydration solution supplied in the kit. The tube was finally incubated at 65°C for 20 minutes, with gently tapping every 10 minutes. In order to determine the DNA concentration, samples were analyzed using an Eppendorf Bio Photometer plus. Each extracted sample was diluted as necessary to obtain a DNA concentration and checked further for quality by electrophoresis on 1% agarose gel.

2.4. PCR amplification and band visualization

PCR reaction was carried outin a thermal cycler (Eppendorf Mastercycler Gradient) to amplify the ITS region of the genomic DNA. To perform the PCR, 5 µl 2x GoTaq® G2 Colorless Master Mix (Promega®; contains DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffer) was added with 1 µl template DNA (~50 ng/µl), 0.3 µl (10 µM) forward primer (ITS 5 alt: 5' TGA AAA GTC GTA ACA AGG TT 3'), 0.3 µl (10 µM) reverse primer (ITS 4 alt: 5' TCC TCC GCT TAT TGA TAT G 3'), and 3.4 µl nuclease free water to make the final reaction volume of 10 µl. The 0.2 ml sterile PCR tubes were placed in a Mastercycler Gradient, and the PCR reaction was run under the following conditions: 1 cycle at 95°C for 5 minutes followed by 30 cycles each having 95°C for 30s, 57°C for 30s and 73°C for 1 minute finishing with a final elongation cycle at 73°C for 7 minutes. The reaction mixture was cooled down at 4°C for 5 minutes to end the reaction cycle.1% Agarose gel (0.2 g agarose/20 ml TAE buffer) containing 3 µl Ethidium Bromide (Promega®; conc. 100 µg/ml) was prepared to test the quality of the DNA. 6 µl of a mixture of PCR sample (5 µl PCR product and 1 µl 6× loading dye added together) was loaded into each well of the gel with a micropipette. A 1 kbp DNA marker (Promega, USA) was loaded into 1st well in the gel to study the band size. The agarose gel was flooded with 600 ml TAE buffer in a gel running chamber and was run for 70 minutes at 120 V before photographing under UV light (High Performance Ultraviolet Transilluminator) to visualize DNA bands.

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2.5. DNA sequencing and phylogenetic analysis
PCR amplicons were sent off for Sanger sequencing to 1st BASE, Malaysia. The PCR products were prepared according to the sequencing company's instruction following sample preparation guide. The generated sequences (fasta file) were aligned using Bio Edit software (Version 7.2.5) and consensual sequences introduced in Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find closest related species. The sequences obtained were compared with homologous sequences in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 6 (Tamura *et al.*, 2013). All sequences were aligned by ClustalW and a maximum likelihood phylogeny tree was constructed with 1000 bootstrap replications. *Phytophthora polymorphica* (GenBank accession no: DQ335636) was used as an out group.

3. Results
3.1. Isolation of oomycetes
A total of 11 samples showed oomycete like mycelial growth on PDA plates out of collected 385 samples (284 water samples, 79 mucus samples and 22 apparently infected muscles). Isolated 11 mycelia samples were fished out from 284 water samples. No growths of mycelium were observed in mucus and fish samples. Mycelia that were re-inoculated in a new plate showed growth within 12 hours in room temperature.

3.2. Molecular analysis
Extracted DNA samples of all isolates were run in a PCR reaction after quantification. The PCR productions were run in 1% agarose gel electrophoresis and approximately 850 bp band sizes were observed after UV visualization (Figure 1). The nucleotide sequence of ITS of 11 isolates indicated that nine (isolates no. 2, 3, 5, 6, 7, 8, 9, 10, and 11) of the isolates were *Pythium* (Table 1) while the chromatogram of 1 and 4 appeared very noisy with background peaks (Figure 2 A, B) which deterred identification of the isolates compared to the rest that had very little or no background peaks (Figure 2 C). For isolates 3 and 4, the bands appeared faint, a secondary PCR was run to get a desired concentration of target amplicon for sequencing. The sequences obtained were compared with homologous sequences in GenBank (table 1). An average of 860 bp of the rRNA ITS region of all the isolates, that is very similar number used in the phylogeny tree construction process, showed that nine (9) of the isolates were identified as the genus *Pythuim.*

ITS sequences of isolated *Pythium* species were compared with reference species adapted from Levesque and Cock (2004) and phylogenetic analysis (Figure 3) confirmed that the sequencing results of isolates 2, 3, 5, 8, 9 and 10 are closely related to *Pythium catenutalum*, *Pythium rhizo-oryzae*, *Pythium torulosum and Pythium folliculosum* and isolates 6, 7, and 11 could be *Pythium pachycaule* and/or *Pythium dissotocum* based on ITS sequences. ClastulW alignment of 2, 3, 5, 8, and 9 (Figure 4) in BioEdit software revealed that these five isolates are almost identical to each other based on ITS sequence except in four neucleotide pairs placed with some interval which also supports the phylogenetic tree.

Table 1. Isolated species that are closely related to the species enlisted in GenBank and their source of origin.

Isolates No	Closely related species (From GenBank)	Source of species in the study (fish pond)	GenBank accession No. of the closely related species	Isolation sources (Country of origin)	Similarity to GenBank accession No.
1, 4	Sequencing error	*H. fossilis*	-	-	-
2	*Pythium catenulatum*	*O. niloticus*	KP862946	Certified organic soil (Colombia basin, WA, USA)	100%
3, 5	*Pythium* sp.	*A. testudineus, H. fossilis*	HQ643814	Unknown (Tamberma Land Togo)	99%
6, 7, 11	*Pythium* sp.	*H. fossilis*	KT247392	Greenhouse recycled water irrigation tank (Pennsylvania, USA)	98-99%
8	*Pythium rhizo-oryzae*	*H. fossilis*	HQ643757	Soil of paddy field (Gorakhpur, India)	99%
9	*Pythium* sp.	*A. testudineus*	KF836354	Unknown (France)	99%
10	*Pythium* sp.	*A. testudineus*	EU544193	Sediment (USA)	99%

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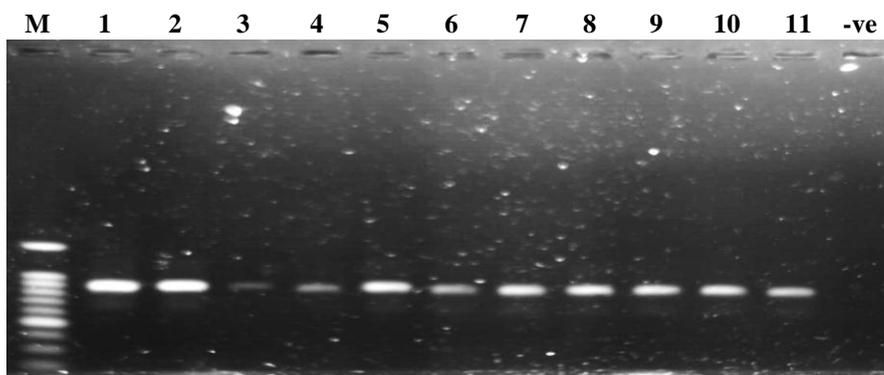
Figure 1.Agarose gel (1%) run of the PCR products of isolates (1 to 11) produce an approximate band size of 800 - 900 bp that is visualize under UV; M = 1 kbp molecular marker, -ve = negative control (DNAse/RNAse free water).
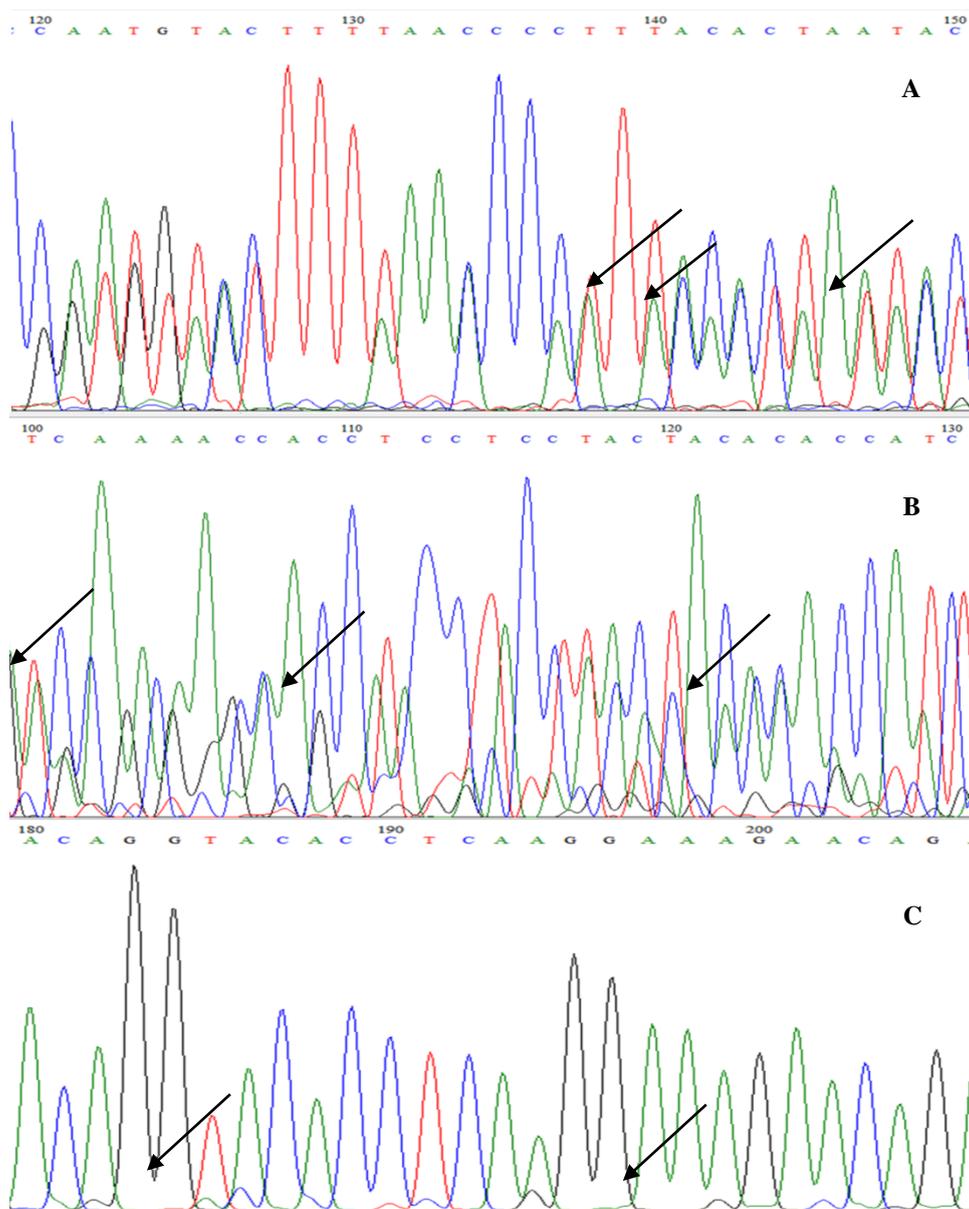
Figure 2. Partial chromatogram of ITS sequencing as seen in the software. Isolate 1 (A) and isolate 4 (B) with noisy backgrounds and isolate 10 (C) with better sequencing data. Arrows indicate some of the background noises.

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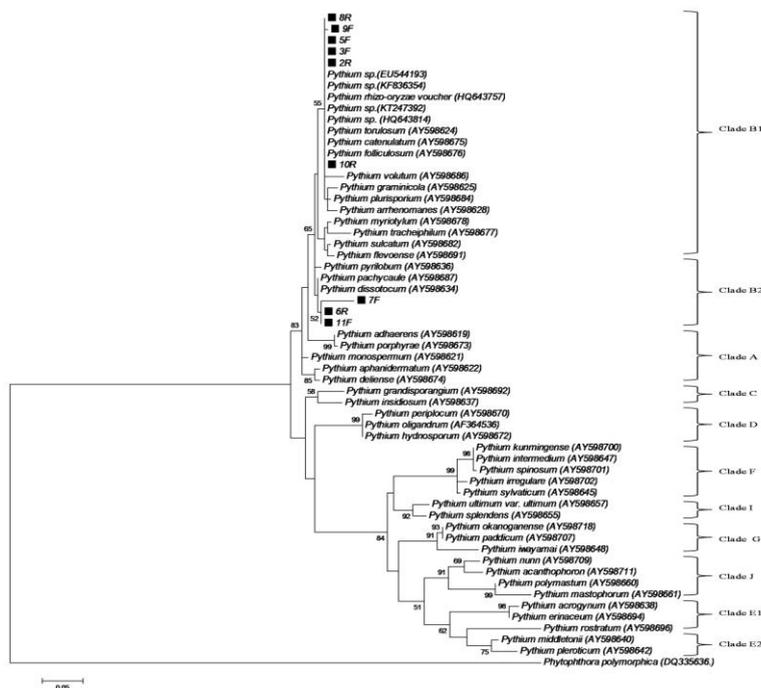
Figure 3. Maximum likelihood phylogram based on ITS showing phylogenetic relationships of the oomycetes isolated (2R, 3F, 5F, 6R, 7F, 8R, 9F, 10R, and 11F) from the fish ponds that are compared with the homologous sequences adapted from Levesque and Cook (2004) sequence of are retrieved from GenBank. Accession numbers of the sequences are given in bracket. Numbers within the tree represent bootstrap value (1000 replications); values less than 50% were not noted. Clades in the phylogram represent the species of same groups that was obtained by alignment of the ITS sequences from Genbank and from the current study. *Phytophthora polymorphica* was used as out-group.
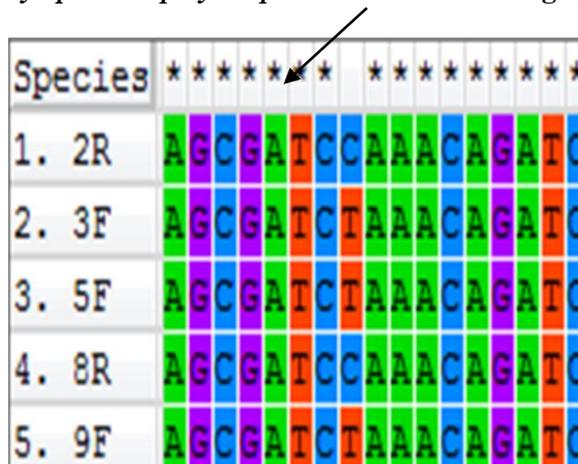
Figure 4. Alignment of 5 isolates (2, 3, 5, 8, and 9) by Clastul W showed that isolates were almost identical except differences in four nucleotide base of which one is showed with an arrow. The identical nucleotides are marked by stars at top of the figure. 2R, 3F, 5F, 8R, and 9F are the alias no of the isolates 2, 3, 5, 8, and 9, respectively.

4. Discussion

This is the first effort on the molecular identification of oomycetes occurring in small water bodies in Bangladesh. The identified nine oomycetes isolates of *Pythium* genera (7 isolates of *Pythium* sp., 1 isolate of *Pythium catenulatum*, 1 isolate of *Pythium rhizo-oryzae*) were isolated from the collected water samples of the fish farm. In general, *Pythium* spp. are known as either plant pathogens or saprotrophs (Alcala, 2013; Robideau *et al.*, 2011). Typical fish pathogenic oomycete such as *Saprolegnia* spp. were not found in the current study conducted. *Pythium* spp. have been reported from bony fishes; however, it is yet to be established whether they are primary pathogens of fish (Czeczuga *et al.*, 2002a). In 2000, Czeczuga *et al.* (2002a) isolated eleven species of *Pythium* from the muscles of monkey goby (*Neogobius fluviatilis*), Chinese sleeper (*Perccottus glenii*), and

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stone morocco (*Pseudorasbora parva*) inhabiting in the ponds, rivers and spring waters in Poland e.g. *Pythium afertile, P. aquatile, P. arrhenomanes, P. butleri, P. dissotocum, P. hemmianum, P. intermedium, P. myriotylum, P. ostracodes, P. periplocum,* and *P. tenue*. These *Pythium* species have been reported from both dead and live fishes. Sati and Khulbe (1983) reported growth of *Pythium diclinum*as a parasite on the gills of a few fish species in India; however, *P. diclinum* failed to infect fish in experimental condition. Moreover, Czeczuga (1996) isolated a number of *Pythium* spp. that grew on the eggs of freshwater fishes sporadically. He assumed that species of *Pythium*, apart from *Saprolegnia* and *Achlya*, contributes significantly to mycosis which may lead to the mass development of pathogenic oomycetes and cause damage to a large number of incubated eggs. Moreover, other than fish, *Pythium* species were also reported from a number of crustaceans (Czeczuga *et al.*, 2002b). Czeczuga *et al.* (2002b) reported forty-three species of *Pythium* of which most common were *Pythium acanthicum*, *Pythium butleri* and *Pythium myriotylum* in appearance and abundance. Majority of the works that reported *Pythium* spp. from fish (Czeczuga, 1996; Czeczuga *et al.*, 2002a; 2002b; 2004; Czeczuga and Muszyfska, 1996) were conducted in the water bodies surrounded by forests. It could be that the reported *Pythium* spp. took the water route to spread throughout the forests and were found on fish. Similar scenario was evident in our sampling site where it was surrounded by paddy and vegetable fields. It might be that the resulted *Pythium*spp. in our samplings have originated from the surrounding crop fields and were flushed out to the culture ponds with rains.

The isolated species that gave 100% similarity with GenBank accession no. KP862946 was *P. catenulatum* that was isolated from the water samples of the tilapia brood pond. *P. catenulatum* was first cited by Matthews (1931) from turf grasses; however, Bala*et al.* (2006) stated that the identified species of Matthews were *P. rizho-oryzae* based on ITS sequencing of their collected isolates. He concluded that the presence of numerous catenulate hyphal bodies led the misconception to develop. In 1976, Plaats-Niterink (1981) isolated *P. catenulatum* from plant debris in water, and also from soil and turf grasses in the USA. The first record of *P. catenulatum* in Morocco was cited by Androusse *et al.* (2007) from the water samples that were collected from different places of dam reservoir. Roudsary *et al.* (2010) isolated *P. catenulatum* from diseased grass and soil samples from green spaces and sport fields in between 2005-2006 and published as a scientific short note that was the first record of the identified species in Iran. The second species of our study, *P. rhizo-oryzae*, was collected from the brood shing fish pond of the fish farm. *P. rhizo-oryzae* was identified as novel species in 2001 from the northeastern India (Bala *et al.*, 2006). They collected some soil samples from paddy fields and studied on the morphological and molecular identification of the isolates. They finally mentioned that *P. rhizo-oryzae* is closely related to the members producing a filamentous inflated type of sporangia like *P. catenulatum*, *P. torulosum*, and *P. inflatum* based on ITS sequence.

Phylogeny tree analysis revealed that six of the isolates (2, 3, 5, 8, 9 and 10) were species of clade B1 (Levesque and Cock, 2004) based on ITS sequence. They stated that species of the clade B1 consist of filamentous-inflated sporangia. Their distribution is worldwide and has mostly been isolated from monocotyledons, bryophytes, green algae and soil, occasionally from dicotyledons and conifers (Levesque and Cock, 2004). According to the databases of GenBank, reported closest match of the isolate 2 was found from certified organic sediment as well as isolate 10 was from sediment and both isolates was collected form USA. Isolate 8 found in our study was reported from soil of paddy field in India (Bala *et al.*, 2006). However, isolate 3 and 5, in the current research, both were *Pythium* sp. that were sequestered from unknown origin in Togo and isolate 9 were also found from unknown source in France. The tree suggests that all the isolates of clade B1 are *Pythium rhizo-oryzae,* except isolate 10 in this work which could be *Pythium folliculosum* or *Pythium catenulatum* based on ITS sequence. It could also be *Pythium rhizo-oryzae* since these three species are closely related. Full length ITS as well as CoXII sequences might give a clearer resolution up to species level in this sub-clade of *Pythium* phylogeny. The search sequence lengths e.g. between 675-810bp added with quality of the sequences can make the difference in the position of a taxa in the phylogenic tree. The results based on phylogenic tree and existing data indicate that the identified isolates of clade B1 collected in this study might had transferred to the fish pond water from the soil of paddy field around the farm.

The other three of the isolates (6, 7 and 11) belonged to the species of clade B2 of *Pythium* genus based on ITS phylogeny (Levesque and Cock, 2004). This group consists of species with filamentous non-inflated to slightly inflated sporangia, smooth oogonia, and a moderate growth rate. Except for *P. dissotocum*, which is commonly found in several greenhouse crops such as lettuce and tomato, most species seem to be uncommon (Levesque and Cock, 2004). Information on GenBank database suggests that the closest match of the isolates 6, 7 and 11 were separated from greenhouse recycled water irrigation tank in USA. From the tree, the results indicate that the isolates could be *Pythium dissoticum*. The vegetable plants in the farm are the possible source of *P.*

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dissoticum in the water samples collected from fish ponds of the farm. The result might be also hampered as the search sequence length of the isolates 6, 7, and 11 were only 705 bp, 660 bp and 710 bp respectively.

A total of 79 mucus samples of fish were collected to observe the diversity of oomycetes occurring in fish farm in summer. Mucus samples of brood tilapia were collected during the study period while mucus of koi and shing were not collected because handling could infect the fishes and could result in economic losses of the fish farm. There were no oomycetes found in the fish mucus samples collected from the brood tilapia. Lilley *et al*. (1998) stated that tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*) and milk fish (*Chanos chanos*) is somehow naturally resistant to *A. invadans*. Afzali*et al.* (2015) also suggested that tilapia is EUS resistant. They found no mortality in tilapia against EUS. In their work, live zoospores of EUS were injected to show the histopathological responses of tilapia, however, no visible inflammatory response was found. Afzali *et al*. (2015) stated that in Nile tilapia, fully mature granulomata of varying sizes fused with multinucleated giant cells and formed large granuloma, surrounded with dense fibrous layers and melanin pigments that filled the whole affected area in very early stages of infection. Thompson *et al*. (1999) suggested that giant cell formation did not seem to be an indicator of resistance to EUS while the actual impact of this type of response in respect to fungal infection is still unclear. According to Afzali *et al*. (2015), mycotic granulomatous reaction could prevent and halt *A. invadans* growth only in resistant fish such as Nile tilapia, however in susceptible fish, for example snakehead, gouramies, koi carp, catfish, goldfish, and climbing perch, the rate of hyphal growth was more rapid than granulomatous reaction. Granulomatous reaction could not be an indicator of susceptibility or resistance to the EUS as it varied to the cellular responses of different fish species. Another research of Khan *et al*. (1998) stated that out of 16, only 2 tilapias died at 20 and 21 days post inoculation of *A. invadans*. The histology of the dead tilapia revealed hyphae inside the granulomas and were surrounded by epithelioid cells. Dramatic granulomatic response with fibrous tissue layer formation and a few lymphocyte aggregations along with foreign body type giant cells were also apparent. They concluded as the injection of spores inside the muscles of tilapia circumvented the means of protection. Thus, it is difficult to trap oomycetes in the mucus samples of EUS resistance Nile tilapia.

Apparently infected muscle samples of koi (10 samples) and shing (12 samples) were collected during the research. The infected koi and shing samples were collected in 24th April and 12th May respectively in 2015 that represent summer season in Bangladesh. However, fish or plant pathogenic oomycetes were not found in apparently infected muscle samples collected from fish farms in the summer season indicated that the infection in koi and shing may be of bacterial origin.

All of the 11 identified *Pythium* species were found in water samples during 3rd sampling (11th April, 2015) and 8th sampling (8th June, 2015). There was heavy rainfall before the 3rd and 8th sampling days resulting in lower water temperature (15 °C) which could induce the formation of *Pythium* zoospores and might be baited with the water samples. *Pythium* species might also be washed out into the fish pond from the plant root and were collected during sampling.

The sampling for tracing oomycetes was carried out in summer when water temperature was high (25-30 °C) which enable fish to fight infection much better (Bly *et al.*, 1994). Fish are not known to be infected during high temperature as the oomycetes produce oospores (Beakes and Bartinicki-Garcia, 1989) for survival during the harsh environment. Oospores are highly resistant to heat, drying, and disinfectants (Klinger and Francis- Floyd, 1996). Favourable conditions like low water temperature (van West, 2006), low circulated water, low dissolved oxygen, high ammonia content (Klinger and Francis-Floyd, 1996) are responsible for germination of the spore and produce zoospore which is mainly responsible for infection in fish. Moreover, oomycetes infection can take place in fish when the physiological condition becomes minimal and rush handling (Bruno and Wood, 1999) of fish occur. However, high water temperature increases the physiological activity of fish.

Apart from environmental conditions, the baiting method used in this particular research was also very important. The bait, rice grain, used in this research is a plant origin that could have favoured plant pathogenic oomycetes. However, due to natural rarity or a failure to provide suitable conditions for oomycetes capture, certain species of oomycetes may not found frequently using baiting (Johnson *et al.*, 2002).

The fish farm, used to study the diversity of oomycetes, is a very well managed aquaculture farm. They monitor the cultured and brood fish regularly for growth and to observe if there is any infection. Water supply process of the farm is also properly operated. They completely dry out their pond and clean the bottom mud after every culture interval and if any infection ensued. Moreover, they use antibiotics with the supplemented feed to prevent different bacterial diseases. Thus it is possible that fish pathogenic oomycetes may not be found from such a properly handled fish farm.

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During the sampling period, all of the collected water, fish mucus and apparently infected fish muscle samples were preserved at 4ºC to favour the growth of oomycetes than bacteria (Lechmere, 1910). Fish pathogenic oomycetes can grow better at 18-22ºC and can even grow at 4ºC, however, growth of bacteria become slower at such lower temperature. All of the samples contained a huge amount of bacteria which could result in loss of oomycetes. Thus to initiate the mycelium growth faster than bacteria it was necessary to provide such an environment.

Isolate 1 and 4 could not be identified as the sequence data had some background peaks in the chromatogram that GenBank could not trace the closest homology. These background noises are either the result of contamination during the study period or problem of the sequencing company as a result of low template concentration. ITS sequences of some *Pythium* species had not enough nucleotides to compare the species ideally with GenBank databases, for example, isolate 7 (identified as *Pythium* sp.) and 9 (*Pythium* sp.) had only 680 bp and 660 bp search sequence length. The constructed phylogeny tree also show that the alias of isolate 7 and 9, respectively 7F and 9F, produce some differences. Moreover, these differences can also arise due to sequencing error or overlapping of nucleotides. After investigating the phylogeny tree an alignment of isolate 2 (identified as *Pythium catenulatum*), 3 (*Pythium* sp.), 5 (*Pythium* sp.), 8 (*Pythium rhizo-oryzae*) and 9 (*Pythium* sp.) were generated to check whether these species are identical or not. The alignment data completely agreed with the hypothesis; however, four nucleotides of those species were not identical which could be the result of short search sequence length of isolate 9. The above hypothesis indicates that these isolates are different strains of a particular *Pythium* species based on ITS sequence.

All of the 11 isolates of *Pythium* spp. were compared with the recorded species based on ITS sequencing of GenBank databases. One of the major limitations of the databases is that there is a lack of biodiversity studies on the oomycetes. To confound this there are a number of incorrectly identified DNA sequences in the available public databases. Inevitably, this situation will result in incorrect identification of species (Bridge *et al*., 2003) and makes it difficult to understand the true extent of all possible oomycetes species that are pathogenic to fish (Hulvey *et al*., 2007). The result suggests that fish pathogenic oomycetes such as *Saprolegnia*, *Aphanomyces* may be absent in well managed fish farms in summer, however, this concept is still unclear. Larger research and intense sampling could provide a holistic picture of the whereabouts of the fish pathogenic oomycetes in summer.

5. Conclusions

The research work was partially successful in determining the diversity of oomycete species present in the fish ponds of Reliance Aqua Farm located in Mymensingh in summer. Eleven isolates were separated from the water samples and were sent off for sequencing to 1st Base in Malaysia. No oomycete species was present in the mucus samples of tilapia and apparently infected muscles of koi and shing. Analogy of the separated isolates with the existing data in GenBank based on ITS sequences revealed that nine of the isolates were *Pythium* spp.; Phylogeny tree analyses reveal that six of the isolates are placed within clade B1 and other three of the isolates are of clade B2. The results of the present study partially suggest that plant pathogenic oomycetes of *Pythium* genera were found in the fish farm during the summer in Mymensingh, Bangladesh. This identification process is recommended to be continued for the same farm as worldwide the prevalence of fish pathogenic oomycetes increases in winter. Moreover, a clear concept on fish pathogenic and also non-pathogenic oomycetes can be acquired through intensive sampling for oomycetes in a broad circumstance in future. None the less, this piece of research work lays a platform for future research in the field of oomycete studies in Bangladesh.

Conflict of interest
None to declare.

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