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Molecular identification of oomycete species affecting aquaculture in Bangladesh

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ABSTRACT

Fish mycotic disease outbreaks occur due to infections with oomycete pathogens such as *Saprolegnia* spp. and *Aphanomyces invadans,* and cause large-scale fish production losses. Despite its negative impact on aquaculture, little is known about the diversity of oomycete pathogens. The aim of this study was to identify the diversity of pathogenic oomycetes causing infections in major aquaculture zones of Mymensingh and Jessore regions in Bangladesh. A total of 449 water and infected fish samples were collected from 28 fish farms in both regions of which 29 samples were able to grow out with mycelia on the Potato Dextrose Agar (PDA)/Glucose Peptone Yeast Agar (GPYA) plates. Sequence database searches using the rRNA Internal Transcribed Spacer (ITS) region revealed that 15 belonged to *Pythium* spp., 12 were *Aphanomyces invadans* and two corresponded to *Saprolegnia parasitica*. Five isolates of *Pythium* spp. were identified to the species level: one was closely related to *Pythium catenulatum*, four to *Pythium rhizo-oryzae*, rest were identified up to genus. The *Pythium* spp. were only isolated from water samples whereas *A. invadans* and *S. parasitica* were found in fish lesions. Phylogenetic analysis revealed that a single *A. invadans* clone exists in the sampled area. The results obtained confirm the existence of pathogenic oomycetes in Bangladesh fish farms and this will pave future research on diversity, prevention and control measures.

1. Introduction

The oomycetes (phylum Heteronkontophyta) are among the most detrimental pathogens of freshwater farmed and wild fishes all over the world (van den Berg, McLaggan, Diéguez-Uribeondo, & van West, 2013). As a group oomycete are considered as decomposer of plants and animals debris in the aquatic ecosystem. However, some species such as *Saprolegnia parasitica* and *Aphanomyces invadans* are often responsible for serious disease outbreaks in fish farms and in the natural environment causing large economical losses by threatening industry production targets and long-term viability (Iberahim, Trusch, & van West, 2018; Sarowar, van den Berg, McLaggan, Young, & van West, 2013; van West & Beakes, 2014). Fish pathogenic oomycetes are mostly undervalued as secondary pathogens (van den Berg et al., 2013) in spite of highly specialized infection mechanisms similar to many primary pathogens (Tadiso et al., 2011; Young, Cooper, Nowak, Koop, & Morrison, 2008). The carbohydrates of the *S. parasitica* cell wall and prostaglandin E2 were shown to suppress fish immune response during the early stages of infection (Belmonte et al., 2014). Therefore, understanding pathogen diversity and host-pathogen interactions of oomycete infections in fish is necessary to combat the disease.

Oomycete infections are mainly spread through swimming zoospores (Beakes, Glockling, & Sekimoto, 2012; van West & Beakes, 2014). Because of their characteristic filamentous mycelial growth, oomycetes were previously grouped as Eumycota (the true fungi) but they are now classified in the Stramenopiles or Chromista group with the diatoms and brown algae due to the presence of biflagellate motile zoospores (Beakes et al., 2012). Infection outbreaks are common at the onset of winter season when the water temperature decreases and favors the pathogen to infect immune compromised hosts (Bly & Clem, 1992). Saprolegniosis on fish are typically manifested by visible white or grey patches of mycelia on the body and fins causing eventually hemodilution and death (Richards & Pickering, 1979). In case of epizootic ulcerative syndrome (EUS), the early infections show petechial hemorrhagic lesions on the skin developing into severe necrotic ulcers in the muscle tissues due to penetrating hyphae of *A. invadans* (Chinabut,

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1998; Noga, Levine, Dykstra, & Hawkins, 1988). The zoospore release is synchronized with environmental factors such as temperature and host-presence (Diéguez-Uribeondo, Cerenius, & Söderhäll, 1994).

Aphanomyces invadans is an important oomycete recognized as the primary pathogen responsible for EUS (Baldock et al., 2005); a detrimental fish disease that occurs in natural waters and commercial fish farms in Asia, Africa, parts of Australia and North America (Roberts, 2012; Sosa et al., 2007). The World Organization for Animal Health (OIE) listed that this disease has been responsible for large-scale mortalities of farmed and wild fish in more than 20 countries across four continents (Iberahim et al., 2018; Pradhan, Mohan, Shankar, & Kumar, 2008). The *A. invadans* was first reported in Ayu, *Plecoglossus altivelis* in Japan (Egusa & Masuda, 1971) but since then it has been reported from a number of fish species including carps, snakeheads, salmonids and estuarine species (Lilley et al., 2003; Oidtmann, 2012). Outbreaks of EUS in aquaculture occur regularly in Bangladesh although recent studies are rare (Khan & Lilley, 2001). In Bangladesh and India, outbreaks of EUS have been responsible for large scale fish mortalities (up to 20–50%) (Pradhan et al., 2014). However, this disease is not considered as seriously as other bacterial infections mainly due to lack of sufficient knowledge and available data. Currently, little is known about pathogen diversity and etiology of the disease that difficult the prevention and control measurements.

Comparisons of the genomes of different fish pathogenic oomycetes such as *S. parasitica, S. diclina, A. invadans* revealed potential differences in the molecular and biochemical mechanisms of infection. For example, *S. parasitica* contains a large repertoire of proteases, kinases and disintegrins that are expressed at different stages of the infection (Jiang et al., 2013; Makkonen et al., 2016) and different *Saprolegnia* species use distinct infection strategies (Songe et al., 2016). In addition, several *S. parasitica* strains are linked to pathogenicity on specific life stages such as egg, alevin, juvenile and parr of Atlantic salmon (Sandoval-Sierra, Latif-Eugenin, Martín, Zaror, & Diéguez-Uribeondo, 2014). Therefore, accurate identification of the pathogen for saprolegniosis is an essential prerequisite for disease management. Knowledge and understanding of pathogens diversity is essential to formulate sustainable prevention and control of mycotic diseases. The objective of this study was to understand the diversity of oomycetes species causing infections in the aquaculture farms of Mymensingh and Jessore region of Bangladesh.

2. Materials and methods

2.1. Sample collection

Water samples and tissues from apparently infected fish (Fig. 1) were collected from different aquaculture farms located in Jessore and Mymensingh districts during the winter season from 15th of November 2015 to 09th of February 2016. Water temperature and pH of sampling sites were measured. All samples were collected from 28 commercial fish farms of which 16 were from Jessore and the remaining from Mymensingh region (Table 1). The exact location of the farms are not disclosed due to the confidentiality agreement. A total of 449 samples were collected of which 248 samples (55.23%) were from Mymensingh and 201 (44.77%) from Jessore. There were 399 (88.86% out of 449 total samples) water samples (217 from Mymensingh and 182 from Jessore) collected from the farms and 50 (11.14%) samples (31 from Mymensingh and 19 from Jessore) were tissue taken from the apparently infected fish. Water samples (10 ml) were collected in 15 ml sterile falcon tubes. Each tube contained an autoclaved adult mosquito (*Anopheles* sp.) as bait. All water samples were kept at 18 °C temperature for seven days to enable the colonization of the baits. For tissue samples, the infected fish were euthanized using 1 g/L Tricaine Methanesulfonate (MS222; Sigma-Aldrich) bath until the opercula and bodily movements were ceased completely in order to comply with institutional experimental animal ethics. A small sample (around

Fig. 1. Photographs showing EUS infected fish. A) and B) Rohu (*Labeo rohita*) and C) Tilapia (*Oreochromis niloticus*). White arrows indicate the eroded muscles due to *Aphanomyces invadans* infections.

0.5 cm^3) of infected tissues or small portion of fin (0.5 cm) was cut with a sterile scalpel and scissor, and washed with autoclaved distilled water, to reduce the unwanted microorganisms load adhered to the surface. The collected samples were placed aseptically on Potato Dextrose Agar (39 g/L PDA in dH_2O and autoclaved) and GPYA plates (glucose 3 g/L, peptone 1 g/L, yeast 0.5 g/L, agar 0.5 g/L, Nacl 0.5 g/L) plates. All the collected tissue samples were incubated for a week at 18 °C. The samples that showed positive growth were used for further investigation.

2.2. Laboratory culture of collected samples

The bait (mosquito) within the collected water samples were inoculated on Petri dishes (90 mm diameter) filled with 25 mL growth medium supplemented with antibiotics (100 mg/L Vancomycin, 500 mg/L Ampicillin and 20 mg/L Natamycin) to reduce bacterial growth. Two different growth media were used; Potato Dextrose Agar and GPYA. For inoculated fish tissues on plates, a small portion of mycelia growing out from the inoculum was aseptically inoculated on new PDA and GPYA plates supplemented with antibiotics. All the plates were sealed with parafilm and incubated at 18 °C. The isolates were re-inoculated several times to eliminate bacterial contamination.

2.3. DNA extraction

A small agar plug with mycelia was aseptically placed in 20 mL Pea broth (124 g/L green pea autoclaved in dH_2O). The liquid media containing inoculum were incubated at 18 °C for 3 days to allow mycelia to grow. DNA was extracted from the grown mycelia using the Phenol: Chloroform: Isoamyl alcohol (with the ratio of 25:24:1) method according to Zelaya-Molina, Ortega, & Dorrance, 2011, quantified (Nanodrop, Thermo Scientific, USA) and stored at −20 °C for downstream analysis.

Table 1
Average temperature and pH in different sampling sites at the time of sample collection and species of the fish sampled in the farms.

Region	Sampling No.	Sampling Area	Name of the cultured fish species	Average Temperature of water (°C)	Average pH of water	Sampling Dates
Mymensingh	1	Muktagacha	*Mystus cavasius*	22.4	8.5	15-Nov-15
	2	Dapunia*	*Anabas testudineus*	23.0	7.8	22-Nov-15
	3	Muktagacha	*Mystus cavasius*	23.8	7.6	28-Nov-15
	4	Netrokona	*Oreochromis niloticus*	22.1	7.7	05-Dec-15
	5	Dapunia*	*Anabas testudineus*	20.2	7.9	17-Dec-15
	6	Dapunia	*Anabas testudineus*	20.8	7.5	23-Dec-15
	7	Muktagacha	*Anabas testudineus*	19.3	9.3	24-Dec-15
	8	Natrokona	*Mystus cavasius*	14.6	8.4	24-Dec-15
	9	Dapunia*	*Anabas testudineus*	18.7	7.5	31-Dec-15
	10	Muktagacha*	*Labeo rohita*	16.5	9.5	31-Dec-15
	11	Dapunia*	*Labeo rohita*	21.4	7.0	06-Jan-16
	12	Muktagacha*	*Mystus cavasius*	23.5	8.4	16-Jan-16
	13	Bhaluka*	*Channa striata*	22.5	8.4	22-Jan-16
	14	Netrokona	*Anabas testudineus*	19.4	7.8	05-Feb-16
	15	Dapunia	*Anabas testudineus*	22.9	7.4	06-Feb-16
	16	Dapunia	*Anabas testudineus*	21.3	8.1	06-Feb-16
	17	Muktagacha	*Anabas testudineus*	22.0	7.8	08-Feb-16
	18	Nalitabari	*Channa striata*	20.4	7.5	09-Feb-16
Jessore	1	Jamtola, Kazipur	*Anabas testudineus*	22.1	8.3	28-Nov-15
	2	Jamtola, Kazipur	*Anabas testudineus*	23.9	7.9	28-Nov-15
	3	Kazipur	*Heteropnesteus fossilis*	24.1	9.5	29-Nov-15
	4	Turminal More, Kazipur*	*Oreochromis niloticus*	21.5	8.8	22-Dec-15
	5	Malancha, Jessore Sadar*	*Anabas testudineus*	22.4	8.5	22-Dec-15
	6	Jessore Sadar*	*Oreochromis niloticus*	22.5	8.4	23-Dec-15
	7	Jessore Sadar*	*Channa striata*	22.2	8.6	23-Dec-15
	8	Jessore Sadar	*Cyprinus carpio*	22.0	8.8	06-Jan-16
	9	Dupdia, Jessore*	*Labeo rohita*	20.7	7.9	06-Jan-16
	10	Malancho, Jessore Sadar	*Anabas testudineus*	23.1	8.0	18-Jan-16
	11	Malancho, Jessore Sadar	*Anabas testudineus*	22.2	9.0	18-Jan-16
	12	Noapara, Jessore	*Anabas testudineus*	22.9	8.0	18-Jan-16
	13	Noapara, Jessore*	*Anabas testudineus*	22.1	8.4	18-Jan-16
	14	Malancho, Jessore Sadar	*Pangasius hypophthalmus*	23.3	8.7	18-Jan-16
	15	Malancho, Jessore Sadar	*Oreochromis niloticus*	20.3	8.2	19-Jan-16
	16	Noapara, Jessore*	*Anabas testudineus*	21.1	8.8	02-Feb-16

The (*) mark indicates the positive samples that yielded fish pathogenic oomycetes.

2.4. PCR amplification

PCR amplification of the internal transcribed spacer (ITS) region between 18S and 28S ribosomal subunits of the rDNA was performed using the primers ITS 5 alt (5′ TGA AAA GTC GTA ACA AGG TT 3′) and ITS 4 alt (5′ TCC TCC GCT TAT TGA TAT G 3′) modified from (White, Bruns, Lee, & Taylor, 1990). The PCR was performed for a total final volume of 10 µL containing 5 µL 2x GoTaq® G2 Colorless Master Mix (Promega®; contains DNA Ploymerase, dNTPs, $MgCL_2$ and reaction buffer), 1 µL of template DNA (~50 ng/µL), 0.3 µL of each primers (10 µM) and 3.4 µl of nuclease free water. The reaction was performed using a Mastercycler Gradient (Eppendorf, Germany), as follows: 95 °C for 5 min followed by 30 cycles (95 °C for 30 s, 57 °C for 30 s, 73 °C for 1 min), final extension at 73 °C for 7 min. PCR products were visualized in a 1% agarose gel electrophoresis stained with 1 µL Ethidium Bromide (Promega®; conc. 10 mg/mL).

2.5. DNA sequencing and analysis

Sanger sequencing of the amplified PCR products (~750 bp - ~800 bp) was done at the BIONEER, South Korea using the PCR amplification primers. For each amplicon, two reads (forward and reverse) were performed. The consensus sequence was searched at GenBank repository using Basic Local Alignment Search Tool (nucleotide BLAST) to confirm.

2.6. Phylogenetic analysis

Phylogenetic analysis was carried out using the Maximum Likelihood (ML) and Bayesian inference (BI). The MEGA v7 program (Kumar, Stecher, & Tamura, 2016) was used to align and construct the ML tree (Tamura & Nei, 1993). The *Aphanomyces* and *Saprolegnia* analysis involved 25 and 31 nucleotide sequences (retrieved sequences available in Appendix A). The sequences were aligned with ClustalW (Thompson, Higgins, & Gibson, 1994) in MEGA v7 and were manually adjusted and, positions containing gaps and missing data were eliminated. For the ML analysis the general time reversal (GTR) + Gamma (G) model was considered the best substitution model based on the lowest Bayesian information criterion (BIC) and Akaike information criterion value (AIC), and tree was built using 1000 bootstrap replications. For BI, the analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012) using the same model as the ML tree. The BI analysis included four parallel running of one and half million generations carried out separately, for each run eight Markov chain Monte Carlo (MCMC) were done having one cold and seven hot chains (temperature 0.10) for both *Aphanomyces* and *Saprolegnia* sequences. The datasets were samples every 1000th generation. The burn-in threshold was analyzed in the Tracer v1.5.0 (http:/tree.bio.ed.ac.uk/software/tracer/). The first 25% trees were discarded for each run, the remaining trees were used for estimating posterior probabilities (pp).

3. Results

3.1. Isolation of oomycetes

A total of 29 samples (around 6.5%) out of the 449 samples that were collected during the sampling period grew positive with mycelial outgrowth. Of the 29 samples, 15 were water samples and 14 were from tissue samples. The highest temperature (23.9 °C) in sampling ponds was recorded from Jamtola, Jessore on 28th November 2015 and the lowest temperature (14.6 °C) was recorded from Natrokona, Mymensingh on 24th December 2015 (Table 1). The mean water

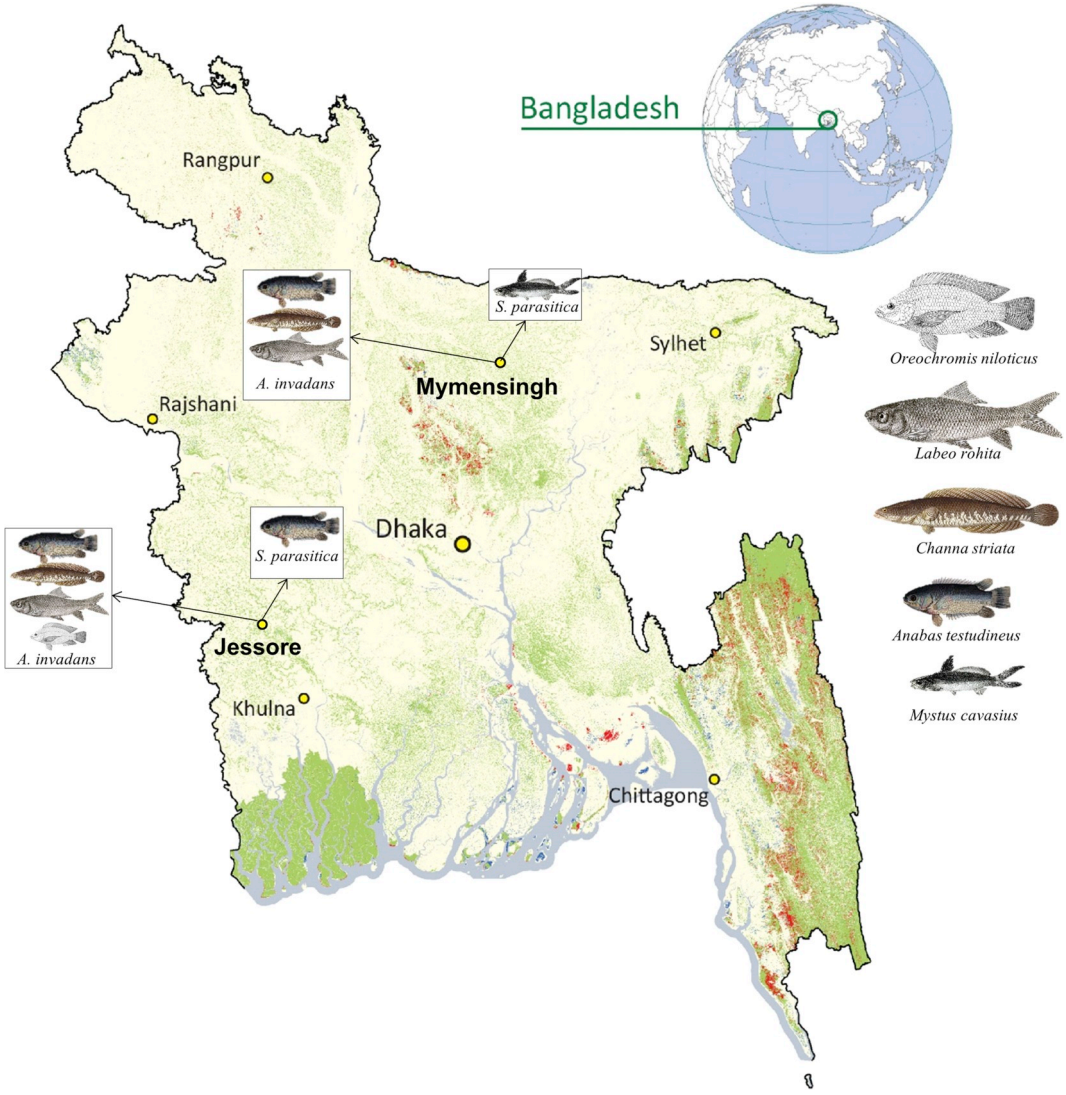

Fig. 2. Figure showing different fish species found to be infected by *Aphanomyces invadans* and *Saprolegnia parasitica* in the study area of Mymensingh and Jessore regions.

temperatures of the ponds from which samples were collected in Mymensingh and Jessore were 20.8 ± 2.36 °C and 22.3 ± 1.02 °C, respectively. The temperature between the two regions did not vary significantly during the study period. Similarly, the average water pH in Mymensingh (8.0 ± 0.63) and Jessore (8.5 ± 0.43) were not significantly different between the two regions. There was also no significant correlations between the positive growth from collected samples (water and apparently infected tissues) and different water temperatures or pH during the sampling period.

3.2. Molecular analysis

Of the 29 samples (9 from Mymensingh and 20 from Jessore) analyzed fifteen were identified as *Pythium* spp., twelve as *A. invadans* and two were *S. paraisitca*. The *A. invadans* and *S. paraisitca* were recovered from infected/moribund fish species collected from both regions (Fig. 2) while the *Pythium* isolates were recovered from water samples. On the basis of ITS sequences of the samples from Mymensingh region, six isolates e.g. M-FR-1, M-FR-2, M-87, M-223, M-106 and M-shol were identical to *A. invadans* (KC137251) (Fig. 3; Table 2), and isolate M-38 was identical as *S. parasitica* (JN400038). Isolate M-24 and M-19 were similar (99% similarity to GenBank sequence) to *Pythium catenulatum* (FJ415899) and *Pythium rhizo-oryzae* (HQ643757).

On the other hand, ten isolates from Jessore were identified up to species level. Isolate J-37, J-168 and J-181 were similar (99% similarity to GenBank sequence) to *Pythium rhizo-oryzae* (LC150560); J-78, J-116, J-133, J-Koi, J-Rui and J-Shol isolate were identical to *A. invadans* (KC137251), and J-120 was identified as *S. parasitica* (JN400038). The *S. parasitica* isolates found in both regions shared identical sequences. In addition, ten isolates from Jessore were only identified up to genus level *i.e. Pythium* sp. (J-18, J-25, J-29, J-129, J-132, J-138, J-140, J-141, J-173 and J-175). Majority *Pythium* species found during the study were isolated from the Jessore region (Table 2).

3.3. Phylogenetic analysis

The phylogenetic analysis of the ITS rDNA sequence collected from the Jessore and Mymensingh region with retrieved homologous sequences confirmed that the collected isolates of M-FR-1,M-FR-2, M-87, M-223, M-106, M-shol, J-133, J-78, J-116, J-Rui, J-Koi and J-Shol are identical to the reported *A. invadans* (Fig. 3) with high bootstrap values and posterior probabilities (pp = 1, bs = 100). No difference was observed between the isolates from both Mymensingh and Jessore regions. Moreover, it appears that the animal pathogenic isolates of different *Aphanomyces* species/strains clustered apart from the plant pathogenic/saprotroph supported by bootstrap values and posterior probabilities

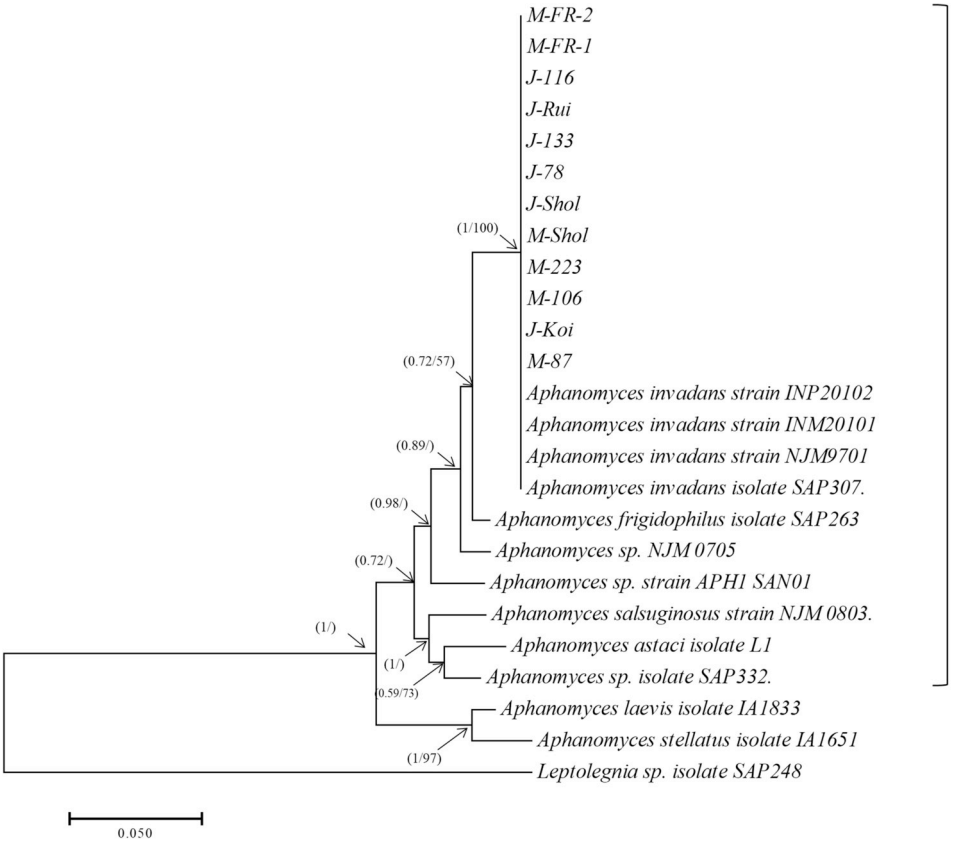

Fig. 3. The figure is a summary of the phylogenetic tree inferred by Bayesian inference and Maximum Likelihood analyses based on the internal transcribed spacer (ITS) regions of nrDNA. The analyses comprise all the *Aphanomyces invadans* isolates obtained in the study and closely related GenBank sequences. Isolates having J or M in the beginning of their name indicate the region where they were recovered from; M-Mymensingh, J-Jessore. Numbers in the branches represent the posterior probability values and bootstrap support (values smaller than 50% are not shown).

(pp = 1, bs = 97; Fig. 3). In *Saprolegnia* tree, the M-38 and J-120 isolates are identical to the reported *S. parasitica* (Fig. 4). No difference were observed between the Mymensingh and Jessore isolates.

4. Discussion

Mycotic diseases of fish, cause by oomycete pathogens, are a real threat to the sustainability of a developing aquaculture industry in Bangladesh. Molecular identification of oomycetes is rare in the aquaculture farms of Bangladesh and detail information and knowledge about the mycotic diseases is absent. During the span of the research period, large number of water samples were collected to unveil the diversity of the oomycetes in the study area. However, only few samples showed mycelial growth *in vitro* which reflects the limitation of the traditional baiting technique to study fish pathogenic oomycetes (Arcate, Karp, & Nelson, 2006). The water sampling with bait only includes species that produced zoospores under the given conditions during sampling, leaving non-zoosporic species or species not developmentally in a state to release zoospores, to go undetected. For example, water samples collected with bait during the study did not show any *A. invadans* growth in laboratory conditions. Similar scenario were reported by Willoughby et al. (Willoughby, Roberts, & Chinabut, 1995) and Lilley et al. (Lilley, Beakes, & Hetherington, 2001) where water sampling for *A. invadans* zoospores were unsuccessful. In addition, the bacterial load in the collected water might have actively deterred zoospore settlement and germination on the provided bait. Using methods established to detect very low concentration of environmental DNA could helpful to overcome this problem (Thomsen & Willerslev, 2015). However, the methods are expensive and require expertise compared to the traditional water sampling with baits. Moreover, the later can result in an actual live isolate that can be used for further downstream analysis. Therefore, a combination of both baiting and environmental DNA techniques is expected to result in better resolutions to study oomycete pathogen diversity.

Advances in molecular biology have helped fungal taxonomy and diagnostics of mycotic diseases in fish. The rRNA gene internal transcribed spacer (ITS) region is a source of sequence variation among closely related species when compared to rRNA-coding genes (Belbahri et al., 2008; Windsor, Macfarlane, & Clark, 2006). The ITS region is comprised by three sub-regions, ITS1 and ITS2 and the 5.8S gene in between, of which two (ITS1 and ITS2) show a higher rate of evolution and are typically species-specific (Bruns & Shefferson, 2004; Kõljalg et al., 2005). Developments in molecular phylogeny studies for species delimitation based on DNA analysis allowed a greater resolution of taxonomically difficult oomycete species (Göker, García-Blázquez, Voglmayr, Tellería, & Martín, 2009) and can uncover new species (Sandoval-Sierra, Martín, & Diéguez-Uribeondo, 2014). In this study, isolation of ITS region provided resolution down to species level which helped to depict the diversity of oomycetes in the sampled area.

The free-swimming zoospores of *A. invadans* initiates the infection and causes spread of the pathogen from host to host. The zoospores are released from the sporangium that is formed at the hyphal tip. The swimming zoospores attach on the host, encysts and germinate to develop filamentous aseptate hyphae invading and ramifying through cutaneous tissues to form ulcerative granuloma (Kiryu, Shields, Vogelbein, Kator, & Blazer, 2003). However, it is difficult to identify *A. invadans* from water samples (Lilley et al., 2003; Willoughby et al., 1995) as it could be that the zoospores only germinate in presence of specific live hosts. In addition, *Aphanomyces invadans* are difficult to isolate from infected fish mainly because of the fact that it is slow-growing on agar plates compared to other saprophytic fungal species (not pathogenic) collected from infected fish (Willoughby et al., 1995). Therefore, samples from EUS infected fish can be easily overgrown with contaminating bacteria or other fungal species present in the water or on fish during sample collection. Employing molecular assays to detect *A. invadans* directly from infected fish (Vandersea et al., 2006) coupled to plating infected tissues on agar plates would be a more suitable approach to confirm EUS infections on fish.

Table 2
List (species, source, accession number and origin) of the isolate homologues available from GenBank.

Isolates found in this study	Closely related species from GenBank	GenBank Accession number	Source of the isolates (Fish species/water)	Accession no. of the closely related species from GenBank	% Similarity	Country of Origin of the GenBank isolate	Source of the GenBank isolate
M-FR-1	*Aphanomyces invadans*	MK072625	*Anabas testudineus*	KC137251	100	India	*Puntius sophore*
M-FR-2	*Aphanomyces invadans*	MK072626	*Anabas testudineus*	KC137251	100	India	*Puntius sophore*
M-87	*Aphanomyces invadans*	MK072627	*Labeo rohita*	KC137251	100	India	*Puntius sophore*
M-106	*Aphanomyces invadans*	MK072628	*Anabas testudineus*	KC137251	100	India	*Puntius sophore*
M-223	*Aphanomyces invadans*	MK072629	*Labeo rohita*	KC137251	100	India	*Puntius sophore*
M-Shol	*Aphanomyces invadans*	MK072630	*Channa striata*	KC137251	100	India	*Puntius sophore*
J-78	*Aphanomyces invadans*	MK072631	*Oreochromis niloticus*	KC137251	100	India	*Puntius sophore*
J-116	*Aphanomyces invadans*	MK072632	*Anabas testudineus*	KC137251	100	India	*Puntius sophore*
J-133	*Aphanomyces invadans*	MK072633	*Oreochromis niloticus*	KC137251	100	India	*Puntius sophore*
J-Shol	*Aphanomyces invadans*	MK072634	*Channa striata*	KC137251	100	India	*Puntius sophore*
J-Rui	*Aphanomyces invadans*	MK072635	*Labeo rohita*	KC137251	100	India	*Puntius sophore*
J-Koi	*Aphanomyces invadans*	MK072636	*Anabas testudineus*	KC137251	100	India	*Puntius sophore*
M-38	*Saprolegnia parasitca*	MK072637	*Mystus cavasius*	JN400038	100	China	*Pelteobagrus fulvidraco*
J-120	*Saprolegnia parasitca*	MK072638	*Anabas testudineus*	JN400038	100	China	*Pelteobagrus fulvidraco*
M-24	*Pythium catenuatum*	MK072639	Pond waters	FJ415899	99	South Africa	-
J-37	*Pythium rhizo-oryzae*	MK072640	Pond waters	HQ643757	99	India: Gorakhpur	Rice seedlings from paddy fields
J-168	*Pythium rhizo-oryzae*	MK072641	Pond waters	HQ643757	99	India: Gorakhpur	Rice seedlings from paddy fields
J-181	*Pythium rhizo-oryzae*	MK072642	Pond waters	HQ643757	99	India: Gorakhpur	Rice seedlings from paddy fields
M-19	*Pythium rhizo-oryzae*	MK072643	Pond waters	HQ643757	99	India: Gorakhpur	Rice seedlings from paddy fields
J-18	*Pythium* sp	MK072644	Pond waters	KF836354	99	France	-
J-25	*Pythium* sp.	MK072645	Pond waters	KF836354	99	France	-
J-29	*Pythium* sp.	MK072646	Pond waters	KF836354	99	France	-
J-129	*Pythium* sp.	MK072647	Pond waters	KF836354	99	France	-
J-38	*Pythium* sp.	MK072648	Pond waters	KT247392	99	USA: Pennsylvania	Green house recycled water irrigation tank
J-132	*Pythium* sp.	MK072649	Pond waters	KT247392	99	USA: Pennsylvania	Green house recycled water irrigation tank
J-141	*Pythium* sp.	MK072650	Pond waters	KT247392	99	USA: Pennsylvania	Green house recycled water irrigation tank
J-173	*Pythium* sp.	MK072651	Pond waters	HQ643814	99	Togo: Tamberma Land	-
J-175	*Pythium* sp.	MK072652	Pond waters	HQ643814	99	Togo: Tamberma Land	
J-140	*Pythium* sp.	MK072653	Pond waters	EU544193	99	USA: Indiana	Sediment

The *A. invandans* isolates collected from the two geographical locations and from several different fish species during the study appeared to be identical and are also identical to those present in the databases. Phylogenetic analysis reinforce that there is only a single clone of *A. invadans* broadly distributed worldwide (Lilley & Roberts, 1997). Animal pathogenic *Aphanomyces* isolates spend energy to asexual reproduction (Diéguez-Uribeondo et al., 1994, 2009) to disseminate quickly across the water column and this may explain the clonality observed in *A. invadans* across the different continents. Nonetheless, *A. invadans* appears to be the dominating oomycete fish pathogen in the samples collected.

The phylogenetic relationship between the three lineages of the genus *Aphanomyces* appears to be very distinct based on ITS region. However, Diéguez-Uribeondo et al., 2009 placed few saprotroph species such as *A. stellatus* in the animal pathogenic clade and this could be an ongoing evolutionary trend which is not reflected in the ITS region. Nonetheless, the Bayesian inference and maximum likelihood analysis based on ITS sequences showed that *A. stellatus* branches with *A. laevis* with high confidence (Fig. 3) grouping the animal pathogenic species in a separate branch.

Saprolegnia parasitica, a fish pathogenic oomycete, was also recovered from fish samples. The *S. parasitica* is one of the most devastating fish pathogens in fish farms (van West & Beakes, 2014); and in Europe is second in economic importance to sea lice in salmonids (van den Berg et al., 2013). The genus *Saprolegnia* contains a number of pathogenic species to different freshwater fish and eggs worldwide and it is responsible for at least 10% mortalities in salmon hatcheries and farms (Phillips, Anderson, Robertson, Secombes, & van West, 2008). It was a long-held belief that *Saprolegnia* was a minor secondary pathogen (Diéguez-Uribeondo et al., 2009), however in the recent years this view has been categorically rejected as it actively suppress host immunity during the infection process (Belmonte et al., 2014; de Bruijn et al., 2012; Minor et al., 2014; Wawra et al., 2012). In addition to *S. parasitica*, other species such as *S. diclina*, *S. australis*, *S. delica*, *S. ferax* have been responsible for saprolegniosis in fish and eggs, especially in salmonid aquaculture (Sandoval-Sierra et al., 2014). *Saprolegnia* spp. have multiple hosts and are capable of infecting different aquatic organisms such as crustaceans and amphibians (Sarowar et al., 2013). In this study only two isolates were successfully obtained from infected fish tissues. Unlike *A. invadans*, *S. parasitica* is relatively easy to grow from pathological tissues. Therefore, the lack of number of isolates suggests that *S. parasitica* is not the most prevalent isolate in the study area. Several reports describe that different strains of *S. parasitica* vary significantly in pathogenicity (Thoen, Evensen, & Skaar, 2011) and it could also be that the isolate strain was non-pathogenic hence resulted in very low infections. Nonetheless, our study provides a baseline for the diversity of animal pathogenic oomycetes in the study area.

This study has also found a number of *Pythium* isolates from different fish farms that were solely recovered from water samples. The *Pythium* spp. are ubiquitous and present in almost every agricultural soil and they are known to be either plant pathogen or saprotrophs and to infect the root systems of various hosts reducing crop yield and quality in infected plants (Schroeder et al., 2013). The isolates found in our study are similar to *P. catenulatum*, *P. rhizo-oryzae* and a number of other *Pythium* sp. These were previously reported from farm soil, paddy field and greenhouse irrigation tanks from different parts of the world

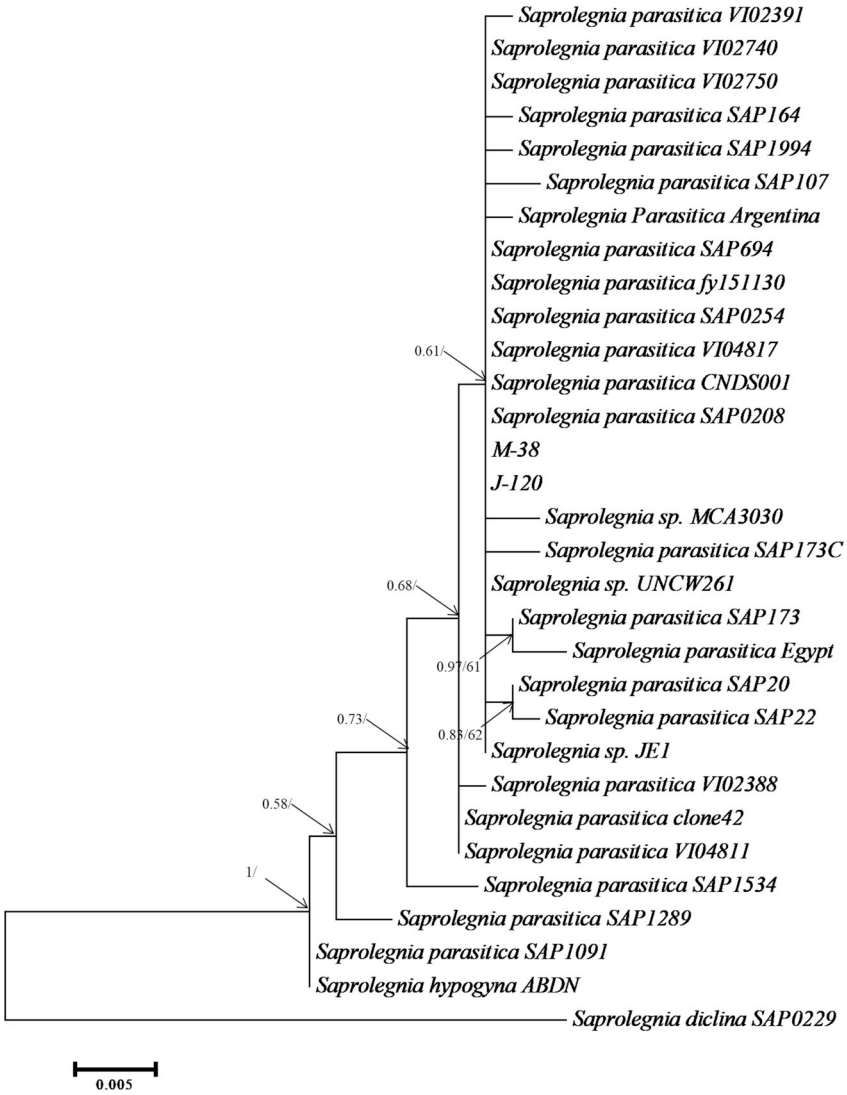

Fig. 4. The figure is a summary of the phylogenetic tree of a Bayesian inference and Maximum Likelihood analyses based on the internal transcribed spacer (ITS) regions of nrDNA. The analyses comprise the *Saprolegnia parasitica* isolates obtained in the study and closely related GenBank sequences. Isolates having J or M in the beginning of the name indicate the region where they were recovered; M-Mymensingh, J-Jessore. Numbers in the branches represent the posterior probability values and bootstrap support (values smaller than 50% not shown).

(Alcala et al., 2016). Most fish farms and ponds studied were surrounded by crop fields and *Pythium* spp. usually uses water routes to spread throughout the forests and crop fields (Czeczuga, Mazalska, Godlewska, & Muszyńska, 2005; Naznin, Hossain, Nasrin, Hossain, & Sarowar, 2017). In fact, *Pythium* spp. has been isolated from moribund and dead fish and crustaceans (Czeczuga, Kiziewicz, & Danilkiewicz, 2002; Czeczuga, Kozłowska, & Godlewska, 2002; Rahman & Sarowar, 2016) but it has not been yet established if *Pythium* spp. are primary pathogens of fish or of other aquatic organisms (Czeczuga et al., 2002). It might be that the traditional baiting technique resulted in the identification of *Pythium* spp. Since *A. invadans* zoospores are difficult to isolate from water samples and they grow slow on agar plates, *Pythium* spp. might have easily grown on the baits as it is prevalent in the study ponds.

5. Summary and conclusion

The results suggests that fish pathogenic oomycetes *A. invadans* and *S. parasitica* prevailed in fish farms during the winter and this indicates that fish mortality occurs due to EUS and saprolegniosis. Intense sampling could provide a clear idea on oomycetes diversity involved with fish infections throughout the year and would be helpful to identify pathogenic isolates from fishes that are commercially important.

Declarations of interest

None.

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