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Research Article
Molecular Characterization Reveals the Presence of Plant Pathogenic *Pythium* spp. Around Bangladesh Agricultural University Campus, Mymensingh, Bangladesh

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Abstract

Background and Objective: Oomycetes (water molds) are ubiquitous organism thriving in moist or aquatic environment. They can cause devastating infections to animals and plants. Farmers, both crop and fish, count huge losses every year due to outbreak of oomycete infections. However, the lack of study in Bangladesh makes it harder to address the problem in the country. This study was conducted to investigate the diversity of oomycetes in the surrounding crop fields and water bodies of Bangladesh Agricultural University campus, Mymensingh, Bangladesh. **Materials and Methods:** The sampling took place during the winter season. A total of 356 water samples were collected out of which only seven came positive with oomycete growth. The seven isolates were grown in potato dextrose agar (PDA) plates. The isolates were identified using molecular methods that included DNA extraction, PCR amplification and subsequent sequencing of the internal transcribed spacer (ITS) region of the genomic DNA of the samples. **Results:** The BLAST analysis of the retrieved sequences to GenBank revealed that four of the isolates were *Pythium catenulatum*, two were *Pythium rhizo-oryzae* and the remaining isolate was *Pythium torulosum* strain. *Pythium* spp. are known to be plant pathogens of both crops and vegetables. **Conclusion:** The results suggest the evidence of plant pathogenic oomycetes around the study area that were causing damage to the crops and extend understanding of the diversity of the genus *Pythium*.

Key words: *Pythium*, internal transcribed spacer, plant pathogenic, oomycete, polymerase chain reaction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycotic diseases of plants and animals are caused by a group of organism called oomycetes. Oomycetes are available in assorted environments as they have evolved the mechanism to infect a wide range of host from plants to animals, fungi and other eukaryotic microbes[1]. Among the plant pathogenic oomycetes, *Phytophthora* is the best studied genus due to its economic importance[2-4]. Another widely distributed plant pathogenic genus is *Pythium*. For example, *Pythium aphanidermatum* and *Pythium ultimum* are agents of seed rot, seedling damping-off and root rot of many plants[5]. On the other hand, *Saprolegnia* spp. and *Aphanomyces* spp. are known as animal pathogens infecting range of fishes and amphibian[1]. In Bangladesh, there have been frequent outbreaks of mycotic disease in crops and fishes. However, the lack of comprehensive study to identify the causative agent of these infections deterred its prevention and control.

Traditionally oomycetes were taxonomically identified through morphological characteristics which were based on sexually or asexually produced spores, the mode of sporulation etc. Recent developments in molecular biology have made it possible to identify oomycetes at species level in more dependable and time saving manner[6]. The internal transcribed spacer (ITS) region of the rRNA gene of oomycetes is mostly used for DNA sequencing[7]. By comparing ITS nucleotide sequences, it is possible to develop accurate phylogenetic relationships[8]. 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[9]. The ITS region sequencing, therefore, is an excellent approach compared to morphological methods that can be used to identify and define oomycete species.

Therefore, the aim of this research was to study the diversity of oomycetes around Bangladesh Agricultural University (BAU) campus, through molecular characterization of ITS region for better understanding of the species/strains present compared to already reported species worldwide.

MATERIALS AND METHODS

Sample collection: A total of 356 water samples were collected from 20 water bodies such as small ponds, ditches around the crop fields of BAU campus, Mymensingh, Bangladesh. To meet the research objective of the study, 9 samplings were carried out within the span of 79 days between 27th November, 2015 and 15th February, 2016. Water samples (10 mL) were collected during each sampling in individual 15 mL falcon tubes containing a sterile (autoclaved) mosquito as bait. Water samples with the pond water in falcon tube were kept in room temperature for 2 days.

Mycelia culture in laboratory: All of the samples were cultured in a vertical laminar air flow cabinet (cleaned with 70% ethanol and treated in UV for 15 min before each use). Inoculation of colonized baits from water samples were carried out by placing it on 9 cm diameter petri dish of 25 mL potato dextrose agar (PDA, 39 g in 1 L dH_2O and autoclaved) medium supplemented with Vancomycin (200 mg L^{-1}), Ampicillin (500 mg L^{-1}) and Pimaricin (50 mg L^{-1}) and sealed with parafilm before being incubated at 18°C. Agar plug with mycelial growth were re-inoculated repeatedly on new plates still they were 100% bacteria free.

DNA extraction: Once a bacteria free culture was attained, genomic DNA from the mycelium was extracted for further identification. Genomic DNA from the mycelia samples was extracted using a Wizard® Genomic DNA purification kit following manufacturer's instructions with some minor modification. The mycelium were grown in from GPY (GPY, glucose 3 g L^{-1}, yeast 0.5 mg L^{-1}, peptone 1 g L^{-1}, agar 0.5 mg L^{-1}, NaCl 0.5 mg L^{-1}) broth for 1 day at 18°C in incubator. The mycelia were completely dried on sterile filter paper inside laminar air flow cabinet. 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. The RNase A solution supplied in the kit was added in the tube and inverted several times. The mixture was incubated at 65°C for 45 min in heat block, with inversions every 15 min. After being cooled down to room temperature, 200 µL of mixture sample was transferred to a new 1.5 mL sterile microfuge tube and 400 µL of protein precipitation solution supplied with the kit was added. The mixture was vortexed vigorously for 5 min to emulsify the 2 phases. The tube was centrifuged (Centrifuge 5415R, eppendorf) at 16,000 rpm for 5 min. Five hundred microliters supernatant containing the DNA was carefully pipetted into a new 1.5 mL sterile microfuge 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 min, before being centrifuged at 4°C at 16,000 rpm for 10 min. 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 rpm for

2 min. 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 min, with gently tapping every 10 min. Each extracted sample was diluted as necessary to obtain 50 ng μL^{-1} DNA concentration and checked further for quality by electrophoresis on 1% agarose gel.

PCR amplification and band visualization: The PCR reaction was carried out in 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^{-1}), 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 min followed by 30 cycles each having 95°C for 30 sec, 57°C for 30 sec and 73°C for 1 min finishing with a final elongation cycle at 73°C for 7 min. The reaction mixture was cooled down at 4°C for 5 min to end the reaction cycle. About 50 mL of 1% agarose gel containing 3 µL ethidium bromide (Promega®, conc. 100 µg mL^{-1}) was prepared to test the quality of the DNA. Six microliters 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 min at 120 V before photographing under UV light (High Performance Ultraviolet Transilluminator) to visualize DNA bands.

DNA sequencing: The PCR amplicons were prepared according to the instruction of the sequencing company and sent off for sequencing to Bioneer, Korea. The PCR samples were cleaned up and diluted down to 40 ng μL^{-1} that was a requirement outlined by the sequencing company. The generated sequences (fasta file) were aligned with the Sequence Alignment Editor Software (BioEdit) and consensual sequences introduced in Basic Local Alignment Search Tool in order to find matching species. The sequences obtained were compared with homologous sequences in the GenBank.

RESULTS

Isolation of oomycetes: A total of 7 samples out of collected 356 water samples showed oomycete like mycelial growth out of collected 356 water samples. Water samples were collected within 7-11 days interval throughout the study period. Temperature range was between 19.27±0.52°C to 25.77±0.50°C and pH was between 8.14±0.44 to 8.48±0.32.

Sequence analysis: Extracted DNA samples were run in 1% agarose gel electrophoresis to observe the quality of DNA. Approximately 800 bp band sizes of the isolates were observed comparing to the DNA marker after UV visualization (Fig. 1). Basic Local Alignment Sequence Tool (BLAST) analysis of the nucleotide sequence of isolates (B-5, B-19, B-20, B-21, B-25, B-44, B-45) revealed that the isolates were of *Pythium* genus (Table 1). The ITS sequence results of the isolate B-19,

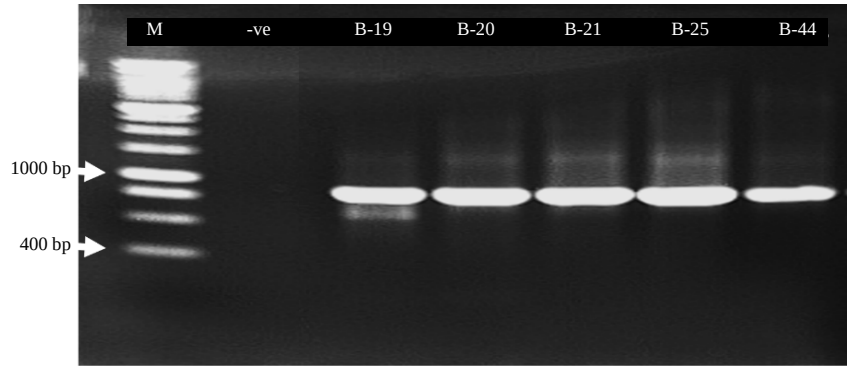

Fig. 1: Agarose gel (1%) run of the PCR products of isolates produce an approximate band size of 800 bp that was visualized under UV. M: 1 kbp molecular marker, -v: Negative control (DNAse/RNAse free water), lane B-19 to B44 represents the positive amplicons of the isolates in PCR reaction

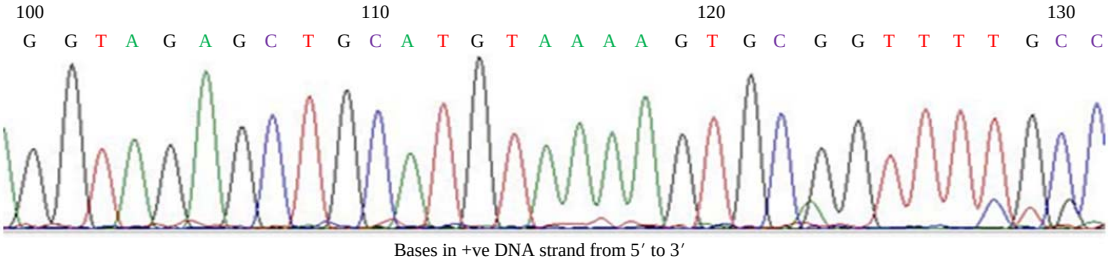

Fig. 2: Sequence data (chromatogram) of isolate B-21 showing clean sequencing result. The alphabet A, T, G and C represents the bases of nucleic acid. The peaks represents specific base at its position

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

Sample No.	Closely related species (From GenBank)	GenBank accession No. of the closely related species	Country of origin	Isolation sources	Similarity to GenBank accession No.
B-19, B-44, B-45	*Pythium catenulatum*	KJ865234.1	Columbia basin, WA, USA	Certified organic soil	99-100%
B-25	*Pythium catenulatum* isolate	KU210465.1	AR, USA	Soybean seedling	100%
B-5, B-20	*Pythium rhizo-oryzae* strain	KU751877.1	Gorakhpur, India	Paddy soil of field	99%
B-21	*Pythium torulosum* strain	KT716814.1	Algeria, Africa	Grasses, waters	99%

B-44 and B-45 showed 99-100% similarity to the *Pythium catenulatum* (GenBank accession No. KJ865234). On the other hand, the ITS sequence of isolate B-25 exhibited 100% similarity to another *Pythium catenulatum* isolate (GenBank accession No. KU210465). Sequences of B-5 and B-20 were almost identical (99%) to that of the *Pythium rhizo-oryzae* strain (GenBank accession No. KU751877). Finally, B-21 isolate's sequence shared 99% resemblance *Pythium torulosum* strain having GenBank accession No. of KT716814. The chromatogram with clean peaks helped to determine sample sequencing (Fig. 2).

DISCUSSION

Mycotic diseases of plants and animals have received little attention in Bangladesh despite its devastating impact on crops and Aquaculture. Mycotic diseases are caused by a group of organism called oomycetes, more commonly known as water molds. Plant pathogenic oomycetes of *Pythium* genera were identified from the water bodies being sampled surrounded by crop fields. *Pythium* spp. are reported to be either plant pathogens or saprotrophs[6,10,11]. Rahman and Sarowar[11] conducted similar studies in a large fish farm surrounded by crop fields reported 11 isolates of *Pythium*. Similar to their study, none of the typical fish pathogenic oomycetes such as *Saprolegnia* spp., *Aphanomyces invadans* were found in this study. Nonetheless, there have been reports of fish infections linked to *Pythium*[12]. Czeczuga *et al.*[12] reported as many as eleven *Pythium* spp. being isolated from a number of fish species, such as monkey goby (*Neogobius fluviatilis*), Chinese sleeper (*Perccottus glenii*) and stone morocco (*Pseudorasbora parva*). The listed *Pythium* spp. by Czeczuga *et al.*[12] found from both dead and live fishes. There are reports of *Pythium diclinumas* infection on the gills of a few fish species in India[13]. Moreover, a number of *Pythium* spp. reported to grow on the eggs of freshwater fishes sporadically[14]. It was concluded that *Pythium* spp. contributes significantly to mycosis of fish apart from *Saprolegnia* and *Achlya*. In addition, there were reports pointing out *Pythium* infections on a number of crustaceans[15]. However, it is yet to be established whether *Pythium* spp. are primary pathogens of fish and other aquatic animals[12]. Majority of the studies that found *Pythium* infections on fish[12,14,15] were conducted in the water bodies surrounded by forests. It could be that the reported *Pythium* spp. were using the water route to spread throughout the forests and were found on fish purely by chance. Similar scenario was evident in sampling sites where it was surrounded by paddy and vegetable fields. It might be that the resulted *Pythium* spp. in these samplings have originated from the surrounding crop fields and were flushed out to the water bodies with rains.

One of the isolates gave 100% similarity whereas, two others gave 99% with GenBank accession No. KJ865234 and that was *Pythium catenulatum*. The GenBank isolate of *P. catenulatum* (KJ865234) was originally collected from the pond water surrounded by crop fields[16]. Another isolate was 100% similar to the GenBank accession No. KU210465 and that was *Pythium catenulatum* isolate which was originally collected and reported from wet soybean seedling bed[17,18]. This species was first cited by Matthews[19] from turf grasses, however, Bala *et al.*[20] reported that the identified species of Matthews was *Pythium rizho-oryzae* based on ITS sequencing

of their collected isolates. It was concluded that the presence of numerous catenulate hyphal bodies led the misconception to develop. *Pythium catenulatum* was isolated in the USA from plant debris in water and later also from soil and turf grasses[21]. El Androusse *et al.*[22] first recorded *P. catenulatum* in Morocco from the water samples that were collected from different places of dam reservoir. Roudsary *et al.*[23] isolated *P. catenulatum* form 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. Rudsari *et al.*[24] identified *Pythium* spp. and tested their pathogenicity on cool season turf grass in Tehran province.

Pythium rhizo-oryzae is another species identified in this study and was collected from a pond of the study area. *Pythium rhizo-oryzae* was identified as novel species in 2001 from the Northeastern India[20]. 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. The third species of the present study is *P. torulosum*. During the 1994 and 1995 seasons *P. torulosum* was isolated from soil and diseased soybean seedlings in Iowa state, USA[25]. El Androusse *et al.*[22] reported morphological features of *Pythium torulosum*. Zoospores were formed plentifully in water cultures at room temperature (18-24°C). Zoospores of *P. torulosum* might find the water of the present study area suitable to survive. *Pythium catenulatum*, *P. rhizo-oryzae* and *P. torulosum* originally belong to the clade B1a of the *Pythium* genera phylogeny[26]. Other species included in this clade are *P. folliculosum, P. aphanidermatum* etc. Species included in the clade B1a have mostly been isolated from crop fields, seed beds, soil and water having worldwide distribution[26]. Therefore, it is evident that the crop fields in the study area harbour several species of *Pythium*. However, the selective pressure of these pathogens on specific crop is still not clear in the study area.

The seven isolates were identified in species level by amplifying the internal transcribe spacer (ITS) region of the genomic DNA with the forward primer (ITS 5 alt: 5'-TGAAAAGTCGTAACAAGGTT-3') and reverse primer (ITS 4 alt: 5'-TCCTCCGCTTATTGATATG-3'). Van Buyten *et al.*[27] identified the *Pythium* spp. associated with growth reduction in Philippine aerobic ricefields using DNA sequencing of the (ITS) region and *b-tubulin* gene. Amplification of the ITS region was performed with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [28]. The new primers differe only few bases however, resulted in better PCR products in this study.

Eberle *et al.*[29] tested the pathogenicity of *P. arrhenomanes* and *P. catenulatum* in Arkansas ricefields, in USA. The number of surviving plants was greater in the warm environment compared to the cool environment, indicating that disease caused by these *Pythium* species is favoured by a cooler environment. Pivonia *et al.*[30] artificially inoculated *Pythium* in pepper plants and maintained at temperatures of 20, 14, 10.5 and 8.6°C in the Arava region of southern Israel. Highest wilting was at 8.6 and 10.5°C, respectively. As the present study was conducted during winter season and the lowest temperature was 19.27±0.52°C, the availability of *Pythium* spp. in water could be from plants of rice and grass fields that were attacked by the plant pathogens due to cold weather.

One of the major limitations 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[31] and makes it difficult to understand the true extent of all possible oomycete species. Only a very limited area was selected for study during the short period of work. However, the study provided credible insight of the presence of *Pythium* spp. in the study area.

CONCLUSION

Pythium spp. are devastating pathogens of plant roots and vegetables. Despite its huge economic importance, these oomycete pathogens are widely neglected. The lack of studies into the diversity of the pathogen have been one of the major issues contributing to the present sceranrio. Therefore, findings of study confirms the presence of *Pythium* spp. in the study area. Hence, it will contribute to future research on finding specific prevention and control measure of *Pythium* spp. in the crop field.

SIGNIFICANCE STATEMENTS

The study identifies the presence of the oomycete pathogen of the genus *Pythium* in the study area. In this study, for the first time confirms the presence of the pathogen using robust molecular tools like PCR and DNA sequencing. It also provides strong evidence of the pathogen in the study area and outlines the importance of specific prevention and control of the disease.

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