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Original Contribution

First Detection of *Batrachochytrium dendrobatidis* in Wild Frogs from Bangladesh

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Abstract: Global amphibian populations are facing a novel threat, chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), which is responsible for the severe decline of a number of species across several continents. Chytridiomycosis in Asia is a relatively recent discovery yet there have been no reports on *Bd*-presence in Bangladeshi amphibians. We conducted a preliminary study on 133 wild frogs from seven sites in Bangladesh between April and July 2018. Nested PCR analysis showed 20 samples (15.04%) and 50% of the tested taxa (9 species from 6 genera and 4 families) as *Bd*-positive. Eight of the nine species are discovered as newly infected hosts. Analysis of *Bd*-positive samples shows prevalence does not significantly vary among different land cover categories, although the occurrence is higher in forested areas. The prevalence rate is similar in high and low disturbed areas, but the range of occurrence is statistically higher in low disturbance areas. Maximum entropy distribution modeling indicates high probabilities of *Bd* occurrence in hilly and forested areas in southeast and central-north Bangladesh. The *Bd*-specific ITS1-5.8S-ITS2 ribosomal gene sequence from the *Bd*-positive samples tested is completely identical. A neighbor-joining phylogenetic tree reveals that the identified strain shares a common ancestry with strains previously discovered in different Asian regions. Our results provide the first evidence of *Bd*-presence in Bangladeshi amphibians, inferring that diversity is at risk. The effects of environmental and climatic factors along with quantitative PCR analysis are required to determine the infection intensity and susceptibility of amphibians in the country.

Keywords: Amphibian disease, *Batrachochytrium dendrobatidis*, *Bd* distribution, Chytrid fungus, Chytrid infection, Bangladesh

Introduction

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*), is considered one of the greatest threats to global amphibian diversity (Skerratt et al. 2007; Vredenburg et al. 2010; Bower et al. 2017). The first

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confirmed case of *Bd* outbreaks linked to frog population declines was reported in 1998 (Berger et al. 1998; Longcore et al. 1999), and to date, it is known to infect over 700 amphibian species globally (Lips 2016; O'Hanlon et al. 2018). Thus, the fungus has become a major conservation concern worldwide (Stuart et al. 2004). A significant loss of amphibian diversity has been discovered within restricted geographic regions, such as Australia (Berger et al. 1998), Central America (Muths et al. 2003; Lips et al. 2006), Europe (Garner et al. 2005), and western North America (Vredenburg et al. 2010). However, *Bd* in the continent of Asia is a post-2007 phenomenon as the first case was reported in December 2006 in Japan from captive amphibians (Une et al. 2008), and in 2007 from free-ranging amphibians in Java, Indonesia (Kusrini et al. 2008). The first report of chytridiomycosis in mainland Asia was done by Yang et al. (2009) in South Korea. Lips et al. (2008) showed that *Bd* can spread rapidly, moving across an entire continent in approximately 20 years. This rapid rate of dispersal and level of persistence does not guarantee the occurrence of *Bd* throughout the entire range because environmental variables also play a crucial role. In comparison with other continents, reports of chytridiomycosis in Asia are relatively recent (Kusrini et al. 2008; Une et al. 2008; Yang et al. 2009), but severe declines of the wild amphibian population have not been reported so far. This could be due to the lack of adequate and detailed research on chytrid fungus (Molur et al. 2015), and on amphibian demography in Asia (Lips 2016).

The distribution and prevalence of *Bd* fungus in South and South-East Asia will significantly contribute to the global distribution pattern of the fungus since these regions host more than 1,000 amphibian species including over 200 endemic species (Molur 2008) and more species presently being described (Howlader 2016; Saikia and Sinha 2019; Nguyen et al. 2020; Razi et al. 2020; Yao Li 2020). Swei et al. (2011) completed a comprehensive survey where the sampling sites spanned 15 South and South-East Asian countries. They found very low prevalence rates (2.35%) in over 3000 samples from 242 amphibian species, although their prediction model suggested that *Bd*-suitable areas exist throughout the entire range. More recently, Rahman et al. (2020) have revealed that *Bd*-suitable environmental conditions are widely distributed across Asia. As part of the Indo-Burma and the Himalayan biodiversity hotspots, Bangladesh hosts 50 amphibian species including new species discoveries on a regular basis (Howlader 2016; Razi et al. 2020). A comprehensive species assessment including cryptic species may contribute to increasing the total number of amphibian fauna in the country. Some *Bd* host species are common in the Western Ghats, India, and Bangladeshi amphibian fauna (Nair et al. 2011; Molur et al. 2015), and Bangladesh also shares some *Bd* host families and genera with South Asian and South-East Asian amphibians (Rowley et al. 2007, 2013; Mendoza et al. 2011; Swei et al. 2011; Bai et al. 2012; Gilbert et al. 2012). Despite the current diversity and environmental suitability, the potential threats from *Bd* or even its occurrence have never been studied. It is a key knowledge gap for Bangladesh and for *Bd* distribution (Rahman et al. 2020). This study attempts to address this through research into *Bd* infection in amphibian species from the country.

Bd infections on amphibian hosts are subject to environmental effects particularly land cover categories because both hosts and infective zoospores are free-living and the infections are naturally occurring (Van Rooij et al. 2015). There is evidence that areas with greater host species richness are more likely to be susceptible to *Bd* infection and transmission (Olson et al. 2013). Saenz et al. (2015) suggested that anthropogenic disturbed habitats may act as a refuge from diseases such as *Bd*, when the species are able to tolerate those environments. In contrast, there is also growing evidence suggesting that anthropogenically disturbed habitats and proximity to human habitation influence the amphibian distribution, susceptibility and exposure to diseases (Lips et al. 2008; Pauza et al. 2010; Thorpe et al. 2018b). A recent study has revealed that *Bd* occupancy may not depend on the presence of amphibian hosts in that locality (Chestnut et al. 2014), which intensifies the importance of using species distribution models (SDMs) to predict future *Bd* distribution without necessarily modeling their host distributions simultaneously. SDM is therefore considered to be a valuable tool for evaluating environmental suitability for *Bd*. The technique allows us to determine areas most suitable for *Bd* infection establishment, identify preventive measures and ensure resource prioritization (Murray et al. 2011). This study aims to collect baseline information about *Bd* prevalence, whether it varies according to different land cover categories, and to assess the environmental suitability for future *Bd* distribution throughout the country.

Methods

Study Sites and Sample Collection

We collected 133 swab samples of 18 frog species from seven sites in Bangladesh (Fig. 1) between April 2018 and July 2018. The sampling period covers the summer and the monsoon season of the country when frogs are naturally more active and do their breeding. Summer in Bangladesh (March to mid-June) is characterized by hot and humid followed by a cool, rainy monsoon season (mid-June to September). Climatic conditions are petty similar in throughout the country. April is the warmest (ranges from 32 to 38°C), and January is the coldest month (7.2 to

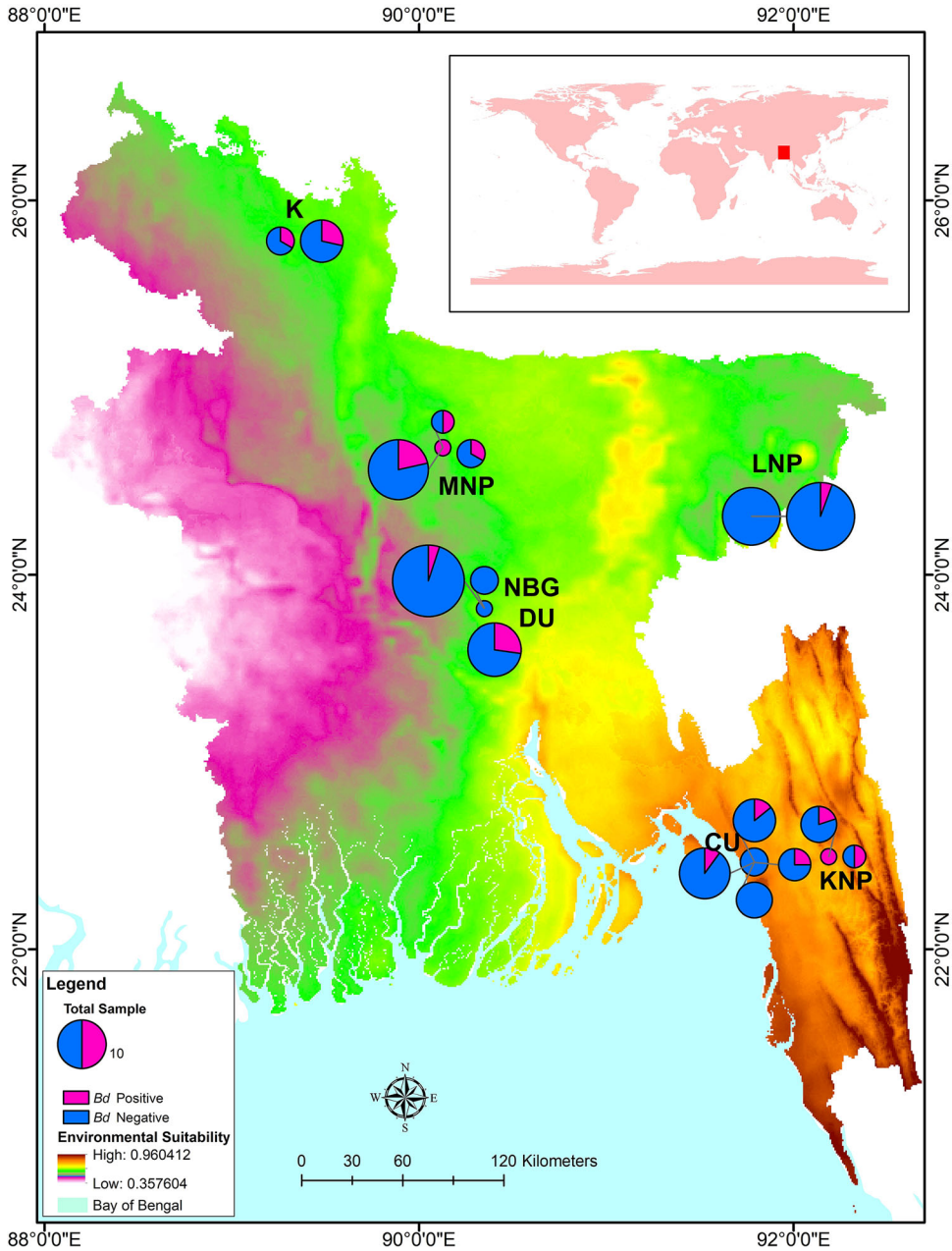

Figure 1. Prevalence of *Batrachochytrium dendrobatidis* and Maxent predicted distribution of the fungus from low to high environmental suitability. Colors indicate different levels of environmental suitability, while the size of the circles represents the sample size. Division of the circle indicates the percentage of positive and negative samples. MNP = Madhupur National Park, CU = Chittagong University campus, K = Khatkhatia, DU = Dhaka University campus, KNP = Kaptai National Park, NBG = National Botanical Garden, LNP = Lawachara National Park).

12.8°C). Most part of the country receives more than 2000 mm annual rainfall except the northern region. There is a little variation of temperature and rainfall among the sampling sites. Our sampling sites include protected and non-protected areas; Madhupur National Park (MNP), Lawachara National Park (LNP), Kaptai National Park (KNP), and National Botanical Garden (NBG) are protected areas, whereas Chittagong University campus (CU) is semi-urban, Dhaka University campus (DU) is urban, and Khatkhatia (K) is a rural village under Rangpur district (Fig. 1). Our sampling locations fell into four different land cover categories: forests, croplands, (natural) vegetation and waterbodies. We divided our sampling sites into high and low disturbance areas by following disturbance factors (modified to fit our context) described by Thorpe et al. (2018a). Disturbance factors recorded were: surfaced road, unsurfaced road, surfaced road within 200 m, domesticated animal grazing, tourism, adjacent built structures and agricultural activities. Sites with 0–3 factors were considered to have low levels of disturbance, and sites with more than 3 factors considered as high disturbance areas. According to these factors, MNP, LNP and KNP fell into the low disturbance category, whereas NBG, CU, DU and K were classified as high disturbance areas.

We captured frogs using hand-held nets and the sampled frogs were identified by morphological comparison with the best field guide available for the country (Hasan et al. 2014). We also noted GPS coordinates and land cover categories from where the frogs had been collected. Before swabbing, we inspected common clinical signs of chytridiomycosis, for example, shedding of skin, redness or discoloration of skin, abnormal posture (abduction of hind legs), ulceration of skin especially ventral part of the body, etc. (Van Rooij et al. 2015). We collected three frogs from MNP (two *Fejervarya pierrei*, one *F. asmati*) (that showed very slightly discolored skin and stored them in 70% alcohol. We suspected that these frogs might be affected by the *Bd* fungus. Swabs were obtained by using sterile cotton swab sticks manufactured by the local pharmaceutical company. Each individual was handled with a fresh pair of sterile gloves. Swabs were taken by following the techniques adopted by Skerratt et al. (2007) and Dahanukar et al. (2013). In brief, the swabbing for each specimen was done by applying a minimum of 60 strokes (5 strokes each on both thighs, shanks, toes and fingers and 10 strokes on the drink patch, ventrum and the region between groin and armpit). All individuals were released back to their respective collection sites. A drop of ethanol was applied to each swab stick and then allowed to air dry. Each swab stick was kept in a sterile vial placed within an icebox until the vials were transported to the laboratory. The vials were then stored in − 20°C until required for DNA extraction. To avoid cross-contamination and spreading to other locations, equipment, shoes and hands were sanitized with a mixture of 70% ethanol and Dettol antiseptic liquid. Used gloves were collected in a bag for incineration at the end of the trip.

DNA Extraction and Nested PCR Sequencing

Bd DNA was extracted from each swab sample by using Thermo genomic DNA purification kit following the instruction provided with it. According to the protocol, each swab was placed in an eppendorf tube containing 180 µl of digestion solution. 20 µl of Proteinase K was added, and the sample was shaken at 56°C in an incubator. After incubation, 200 µl Lysis Solution and 400 µl 50% ethanol were added and mixed thoroughly by pipetting. The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column, inserted in a collection tube and centrifuged for 1 min at 6000 × g. Discarding the flow-through, Wash Buffer was added and centrifuged for 3 min at 13,000 × g. Finally, to elute the genomic DNA, 100 µl Elution Buffer was added to the extract and the diluted sample was stored at -20°C for further use as DNA template in PCR assay.

According to the procedure described by Goka et al. (2009), a PCR-based assay was used to detect *Bd*. We used *Bd*-specific primers to amplify a region of the 5.8S ribosomal RNA gene, and ITS1 and ITS2 regions from extracted DNA. For the first step of nested PCR, *Bd*18SF1 (5'-TTTGTACACACCGCCCGTCGC-3') and *Bd*28SR1 (5'-ATATGCTTAAGTTCAGCGGG-3') primers were used to amplify the target DNA that was located at the end region of 18S rRNA gene and at the start region of 28S rRNA gene of the fungus. In the second amplification step, we then amplified the first-round PCR products using *Bd*1a (5'-CAGTGTGCCATATGTCACG-3') and *Bd*2a (5'-CATGGTTCATATCTGTCCAG-3') primers, resulting in a PCR product of approximately 300 bp.

The PCR assays were conducted using Promega PCR Master Mix where the total reaction volume was 25 µl (12.5 µl of Promega 2X PCR Master Mix, 2 µl of each primer, 1.5 µl of DNA template and 7 µl of Nuclease-Free water). The conditions for the first amplification were an initial denaturation for 5 min at 95°C; 30 cycles in 30 s at

94°C, 30 s at 50°C and 30 s at 72°C and a final extension for 7 min at 72°C. The conditions for the second amplification were an initial denaturation for 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 65°C and 30 s at 72°C and a final extension for 7 min at 72°C. Each sample was tested at least twice however, *Bd*-positive samples were tested up to four times in a few cases. We included a positive and negative control for each amplification. PCR products were separated on 1.5% agarose gels, and bands of DNA fragments were observed in ethidium bromide staining under UV light (Fig. 2). Two randomly selected positive samples were purified by Thermo GeneJET PCR purification kit (ThermoFisher Scientific) for sequencing. The purified products were sequenced by standard sequencing technique using ABI prism 3730XL sequencer (Macrogen Inc., South Korea). Blast tool was used to analyze the sequences (Altschul et al. 1990). The two sequences have been submitted to GenBank under the accession numbers MN527242 and MN527246. A phylogenetic tree was developed using *Bd* ITS1-5.8S-ITS2 sequences available at GenBank (see details in Supplementary Table 2), where *Kappamyces laurelensis* was used as outgroup. Clustal W (Thompson et al. 1994) was used in MEGA X (version 10.0.1, Kumar et al. 2018) to align all the sequences. The evolutionary history was inferred from the tree using Neighbor-Joining method (Saitou and Nei 1987) where the confidence probability for branch length was estimated using the bootstrap test (100 replicates) (Dopazo 1994; Rzhetsky and Nei 1992). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the number of differences method (Nei and Kumar 2000) and are in the units of the number of base differences per sequence.

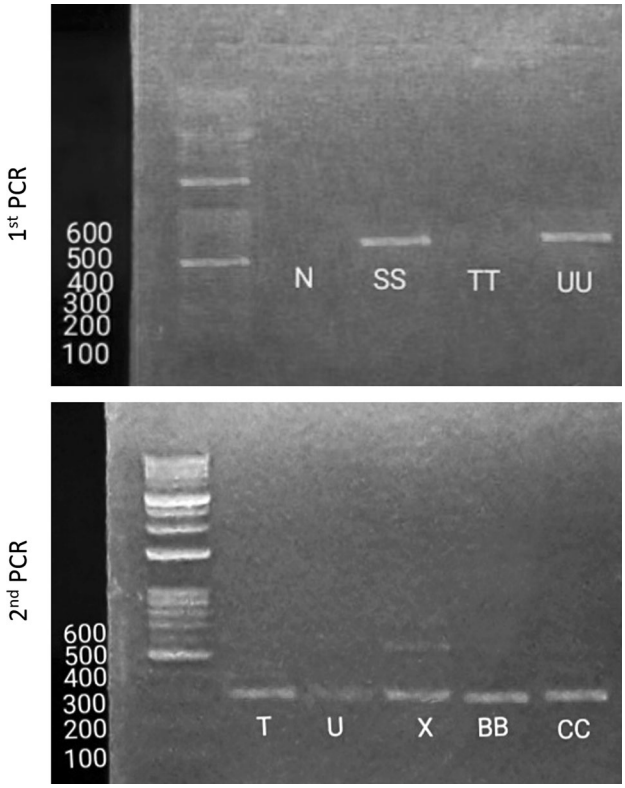

Figure 2. *Batrachochytrium dendrobatidis* isolates amplified with the primers Bd18SF1 and Bd28SR1 in the first step of the nested PCR assay and with the primers Bd1a and Bd2a in the second step of the nested PCR assay. 'N' represents the negative control containing only the amplification reaction mix with deionized water instead of the DNA template. Each letter/s indicate frog species; SS = *Microhyla ornata*, TT = *Fejervarya pierrei*, UU = *F. asmati*, T = *F. asmati*, U = *F. pierrei*, X = *Euphlyctis cyanophlyctis*, BB = *F. pierrei*, CC = *F. teraiensis*.

Model Formulation

We used Maximum Entropy Modelling (Maxent version 3.4.1) to forecast possible *Bd* distribution in Bangladesh. Maxent was downloaded (accessed on 8 May 2019) from the American Museum of Natural History (https://biodiversityinformatics.amnh.org/open_source/maxent) in jar format. The model has been prepared based on the *Bd* occurrence data (csv format) from our 20 different specific point locations, and 19 bioclimatic variables (in ASCII format). We downloaded 30 arc seconds ($\sim$ 1 km^2) resolution bioclimatic variables as environmental variables dataset in GeoTIFF raster format from WorldClim version2 (http://worldclim.org/version2) (Fick and Hijmans 2017). Using SDM toolbox (downloaded from http://sdmtoolbox.org (Brown 2014)), the downloaded raster dataset was primarily processed in ArcGIS (version 10.4). World bioclimatic variables were clipped by masking from the study area shapefile (Bangladesh), and then all the bioclimatic variables were converted in ASCII format as a readable format for Maxent. The district-level shapefile (downloaded from Bangladesh Agricultural Research Council, http://maps.barcapps.gov.bd/index.php) was used to clip the bioclimatic variables. We converted the csv format occurrence dataset to shapefile, which was spatially rarefied, to reduce the effects of spatial clusters of localities by using a spatially rarefied occurrence data tool from SDM toolbox. The projection of all environmental variables and the positive occurrence dataset were set in WGS1984. The

occurrence dataset was then converted again in csv format from the shapefile. Finally, a 30 arc seconds ($\sim$ 1 km^2) resolution ESRI coordinate bias (downloaded from http://sdmtoolbox.org, (Brown 2014)) was used to fit the model.

Data Analysis

We performed a Kruskal–Wallis test to see whether *Bd* prevalence varies in different land cover categories. We also employed the unpaired Wilcoxon rank-sum test to see the effects of disturbance on *Bd* prevalence rate. All statistical tests and the confidence intervals (95%) for *Bd* prevalence among species and sites were calculated by using R version 3.5.1 (R Development Core Team, 2018).

Results

A total of 20 samples tested positive for *Bd* among 133 samples, with an average prevalence of 15.04% (Table 1). Among the 18 species sampled, 50% of taxa (9 species belonging to 6 genera and 4 families) were *Bd*-positive. We found the highest prevalence of *Bd* in *Polypedates leucomystax* (100%, n = 2) (Table 1). Prevalence of the nine *Bd*-positive frog species also varied significantly (χ^2 = 224.95, df = 8, $p < 0.001$). Samples from all seven study sites tested *Bd*-positive, with the highest prevalence occurring in Kaptai National Park (37.5%) and the lowest in Lawachara National Park (3.23%), although our sample number was the highest in Lawachara National Park (n = 31) and the lowest in Kaptai National Park (n = 8) (Supplementary Table 1). The prevalence among the seven study sites differed significantly (χ^2 = 58.125, df = 6, $p < 0.001$).

Considering the affected site in terms of *Bd*-positive-affected species, Madhupur National Park was the highest and Lawachara National Park was the lowest affected site; 71.43% and 12.50% of tested species found were *Bd*-positive, respectively (Supplementary Table 1). In Madhupur National Park, 30% samples from five species (*Euphlyctis cyanophlyctis*, *F. asmati*, *F. pierrei*, *F. teraiensis* and *P. leucomystax*) found were *Bd*-positive and 3.23% samples from one species (*Hylarana nigrovittata*) found were *Bd*-positive in Lawachara National Park (Supplementary Table 1). We did not find common clinical signs of chytridiomycosis, severely affected, or dead frogs. All of our collected amphibians tested *Bd*-negative.

Our analysis of *Bd*-positive locations showed that *Bd* prevalence does not significantly vary among different land cover categories (Kruskal–Wallis test, χ^2 = 3.06, df = 3, p = 0.3). The post hoc pair-wise comparison also showed that prevalence does not significantly differ between any two land cover categories. However, the prevalence range was higher in the forest category than in the other categories (Fig. 3a). The prevalence rate in the high and low disturbed areas did not significantly vary (unpaired Wilcoxon rank-sum test, W = 14.5, p = 0.13), but in low disturbed areas, the range of prevalence was higher (Fig. 3b).

The maximum entropy distribution model showed the highest probability of *Bd* infection in the southeast and the central-north parts of Bangladesh. Among the seven sites sampled, four (K, MNP, NBG, DU) fell under medium probability areas and the rest three (CU, KNP, LNP) in moderate to high probability areas. The coastal region showed a moderate probability of *Bd* infection. The mid-western region along with some parts of the north-western had the lowest probability of *Bd* (Fig. 1).

Bd-specific ITS1-5.8S-ITS2 ribosomal gene sequences obtained from two different frog species (sampled in MNP); MN527242.1 and MN527246.1 for *F. teraiensis* and *F. asmati* respectively, were 100% identical. In the Neighbor-Joining tree, haplotypes from Bangladesh grouped with the haplotypes from India, China, Japan, Italy, Africa, Texas and Ecuador. On the other hand, haplotypes from Brazil, Japan and Korea formed a separate clade (Fig. 4, Supplementary Fig. 1).

Discussion

This study has demonstrated that the amphibian chytrid fungus *Bd* is present in Bangladesh and is the first report of *Bd* in the country despite the fact that there is plenty of research on *Bd* prevalence in other Asian countries. A more detailed investigation is needed including the effects of temperature, elevation and seasonality to accurately represent the status of *Bd* in Bangladesh. Skerratt et al. (2007) showed that *Bd* becomes virulent and pathogenic when temperature exists between 12 and 27°C, although small fluctuations of temperature may significantly affect *Bd* occurrence (Whitfield et al. 2012). There is distinctive evidence that *Bd* infection increases in winter compared to the other seasons (Berger et al. 2004). While taking behavioral ecology into consideration, in the winter season

Table 1. Prevalence (with 95% Confidence Intervals) of *Bd* among Different Species

Family	Species name	English name	Sampling sites	Samples tested (*Bd*-positive)	Prevalence (95% CI)
Dicroglossidae	*Euphlyctis cyanophlyctis*[#]	Skipper frog	MNP, NBG, K, LNP	11 (2)	18.18 (2.28–51.78)
	Euphlyctis hexadactylus	Green frog	NBG	12 (0)	0 (0–26.46)
	Fejervarya asmati	Asmat's cricket frog	MNP, NBG, CU, DU, LNP	20 (4)	20 (5.73–43.66)
	Fejervarya pierrei	Pierre's cricket frog	MNP	11 (2)	18.18 (2.28–51.78)
	Fejervarya syhadrensis	Bombay wart frog	MNP, CU	5 (0)	0 (0–52.18)
	Fejervarya teraiensis	Terai wart frog	MNP, K, CU, DU, KNP, LNP	21 (3)	14.29 (3.04–36.34)
	Fejervarya cancrivora	Crab-eating frog	KNP, MNP	2 (0)	0 (0–84.18)
	Occidozyga lima	Puddle frog	KNP	1 (0)	0 (0–97.5)
	Hoplobatrachus tigerinus	Indian bullfrog	CU, K, NBG	12 (1)	8.33 (0.21–38.47)
Ranidae	*Hylarana nigrovittata*	Dark-sided frog	KNP, LNP	11 (2)	18.18 (2.28–51.78)
	Clinotarsus alticola	Point-nosed frog	LNP	1 (0)	0 (0–97.5)
Microhylidae	*Kaloula pulchra*	Painted bullfrog	CU	1 (0)	0 (0–97.5)
	Microhyla berdmorei	Berdmore's narrow-mouthed frog	KNP, LNP	5 (1)	20 (0.51–71.64)
	Microhyla ornata	Ornate microhylid frog	CU, DU	10 (3)	30 (6.67–65.24)
	Microhyla rubra	Red microhylid frog	CU	1 (0)	0 (0–97.5)
Rhacophoridae	*Polypedates leucomystax*	Common tree frog	MNP, KNP	2 (2)	100(15.81–100)
	Polypedates maculatus	Maculated tree frog	KNP, NBG	2 (0)	0 (0–84.18)
Megophryidae	*Leptobrachium smithi*	Smith's litter frog	LNP	5 (0)	0 (0–52.18)
Total				133 (20)	15.04 (9.43–22.26)

[#]Tested previously and found *Bd*-negative.

MNP = Madhupur National Park, CU = Chittagong University campus, K = Khatkhatia, DU = Dhaka University campus, KNP = Kaptai National Park, NBG = National Botanical Garden, LNP = Lawachara National Park.

amphibians usually decrease their activity (Wei et al. 2010) which could potentially decrease the chance of infection. Therefore, presumably, there are strong seasonal effects on *Bd* occurrences. In Asian countries, *Bd* infection occurs between 330 to 1949 m (Swei et al. 2011) and 663 to 1231 m (Dahanukar et al. 2013) above sea level. Rahman et al. (2020) found 70% of *Bd*-positive locations (among the 209 *Bd*-positive locations in Asia) fall under 500 m above sea level, suggesting that *Bd* is more widespread in lower altitudes. The study also found 83% of *Bd*-positive locations are located in regions with rainfall between 1001 to 3000 mm. In Bangladesh, the range of temperature, rainfall and altitude for *Bd* infections are widely present. Species-specific responses to *Bd* infection, including the ability to carry the fungus without showing outward symptoms, and lower levels of susceptibility to clinical

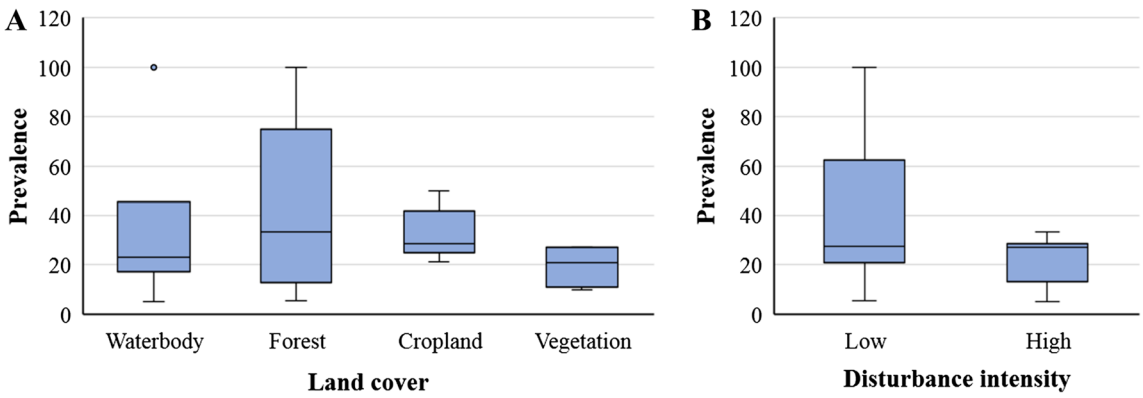

Figure 3. *Bd* prevalence in different land cover categories (**a**) and disturbance intensities (**b**). Quartile 2 and 3 are shaded with the dividing line as median, whiskers indicate quartile 1 and 4, outlier is indicated by circle.

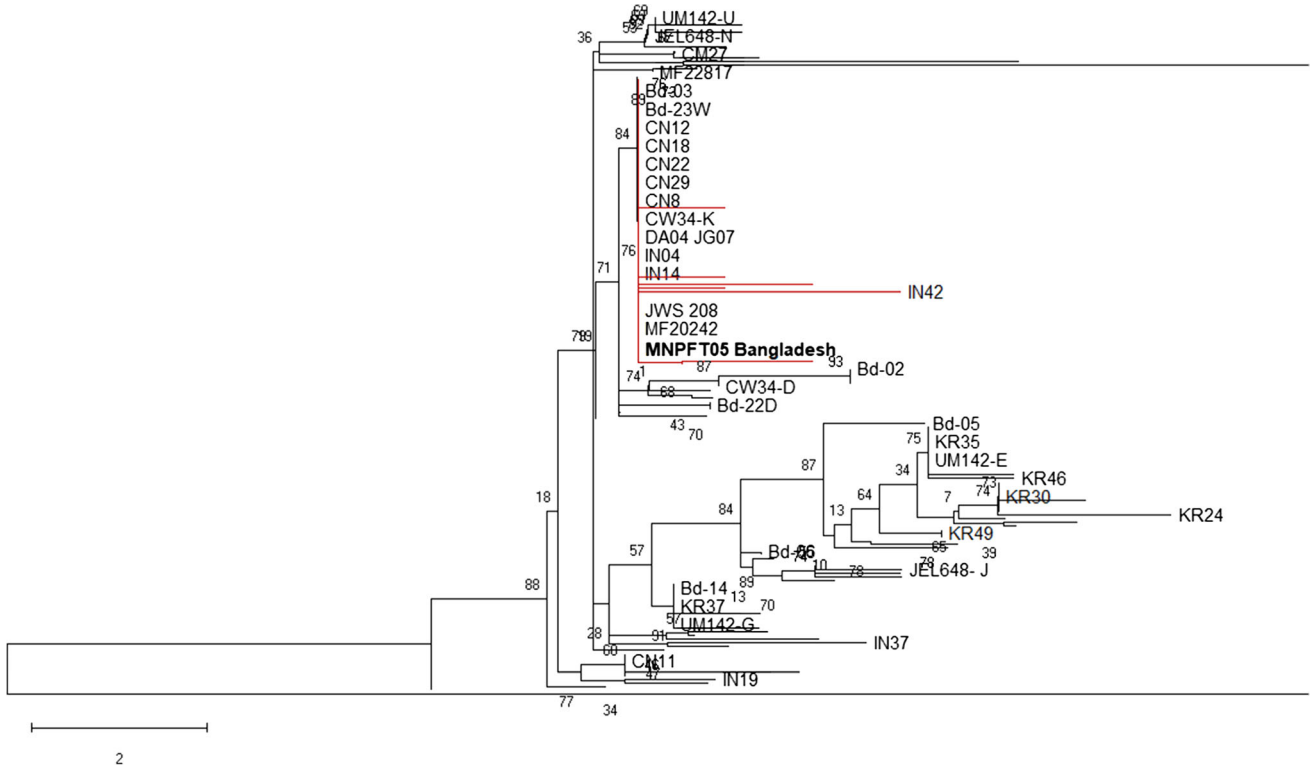

Figure 4. Phylogenetic tree for *Bd* ITS haplotypes using Neighbor-Joining method. Number shown next to the branches represent the confidence probability estimated using the bootstrap test (100 replicates). *Kappamyces laurelensis* was used as outgroup. Clade in red color showing the haplotypes grouped with the haplotype from Bangladesh. Prefix of haplotype representing the country: Bd = Japan, KR = Korea, IN = India, CN = China, CW = South Africa, UM and JEL = Brazil, MF = Ecuador, MIB = Italy, DA = Texas, JWS = Massachusetts, CM/ND/EF/LB = Central Africa.

chytridiomycosis (Retallick et al. 2004), can make the duration of infection longer and prevalence rate higher (Nelson and Williams 2014). These lines of study in Bangladesh are crucial because the amphibian populations are being wiped out at such a rapid rate across Asia that many would go extinct before scientists have a chance to discover them.

Out of nine *Bd*-positive host species, one species is endemic to Bangladesh (*F. asmati*) (Howlader 2011), three species (*F. pierrei, F. teraiensis, M. ornata*) are endemic to

South Asia, four species (*H. tigerinus, H. nigrovittata, M. berdmorei, P. leucomystax*) to South and South-East Asia and one species (*E. cyanophlyctis*) to South and Middle-East Asia (IUCN 2019). Only *P. leucomystax* was previously found in infected frogs from Cambodia (Mendoza et al. 2011) and Singapore (Gilbert et al. 2012), while the other eight species discovered are newly infected hosts. However, *E. cyanophlyctis* tested for *Bd* found negative in India (Dahanukar et al. 2013; Thorpe et al. 2018a). Samples for these studies collected from forests (Dahanukar et al. 2013) and streams (Thorpe et al. 2018a) where the infection intensity was low (Thorpe et al. 2018a). Two of our *Bd*-positive samples of *E. cyanophlyctis* were collected from forest and waterbodies where the prevalence rate is high. This inconsistency among the studies might be due to the temporal variation in species susceptible rate, variation in habitat structure and distribution in *Bd* among the study habitats. We also found *Euphlyctis* as a newly infected genus, while the other five genera have previous records of infected individuals in other South-East Asian countries (Mendoza et al. 2011; Savage et al. 2011; Gilbert et al. 2012; Molur et al. 2015). Most of our sampling frogs did not have the most common clinical signs of infection such as discoloration, ulceration, shedding of skin, abnormal postures (Van Rooij et al. 2015), or find dead frogs in the population from where the samples were collected. This finding is very consistent with other *Bd* studies in China (Bai et al. 2012; Kolby et al. 2014), South Korea (Yang et al. 2009), Indonesia (Kusrini et al. 2008), and Thailand (Vörös et al. 2012). Though we observed slightly discolored skin in three frogs, all tested *Bd*-negative. We suspect that the discoloration was due to cutaneous injuries, bacterial infections or excessive exposure to chemical fertilizers applied in paddy fields. Swei et al. (2011) found only 2.35% prevalence over three thousand samples from 15 Asian countries. Similarly, the prevalence for some Asian countries ranges from around 1% to (Gilbert et al. 2012; Molur et al. 2015) zero (Soorae et al. 2012; Chaber et al. 2016). In contrast, Bataille et al. (2013) found 17.7% *Bd*-positive samples from nearly two thousand samples in South Korea, 25% samples were *Bd*-positive in India (Dahanukar et al. 2013), 41% in Cambodia (Mendoza et al. 2011), and 48% in Iran (Sharifi et al. 2014). The overall *Bd* prevalence in Asia is low according to currently available scientific research (Swei et al. 2011; Rahman et al. 2020). The absence of clinical signs of chytridiomycosis and the overall low prevalence of *Bd* infection in this region suggest that *Bd* is endemic to Asia and has a strong natural selection power, allowing sufficient time for amphibians to evolve mechanisms of resistance to or tolerance of it (O'Hanlon et al. 2018).

Croplands, vegetation areas and water bodies that are predictably close to human settlement, have a lower prevalence rate than forest areas, which are more isolated from human settlements. Our results are consistent with some previous studies; *Bd* prevalence is lower in highly exposed habitats than forested habitats (Saenz et al. 2015; Van Sluys and Hero 2009). The exact reasons for the differences in the prevalence of *Bd* are still unknown, but we suspect that our high disturbance sites are attributed to rapidly heating surfaces such as small ponds, ditches, short grasses and paddy fields which are unlikely in low disturbance forested areas. Forest shade may play an important role in *Bd* infection by lowering the temperature (Raffel et al. 2010), as forest canopy cover, natural vegetation and daily temperature are the predictors of *Bd* occurrence, suggested by Becker et al. (2012). However, our study was not designed to account for the effects of forest density and daily temperature on the *Bd*; hence we cannot rule these out as contributing factors on *Bd* prevalence.

The discovery of *Bd* in Bangladesh is a major conservation concern, because the infection may increase the vulnerability of amphibians. To determine the potential distribution of *Bd* in the country, we performed an SDM by using bioclimatic variables that are considered as important parameters of *Bd* distribution (Puschendorf et al. 2009; Rödder et al. 2010; Olson et al. 2013; James et al. 2015; Xie et al. 2016). The SDM shows *Bd* can be found throughout Bangladesh which supports the statement from different researchers, that the environment in Asia is suitable for *Bd* (Swei et al. 2011; Rahman et al. 2020). According to probability statistics (derived by maximum entropy modeling), the highest probability of infection is found in the southeastern part, i.e., the hilly region, and the central south. The central northern part of the country also shows a moderate to high probability of having *Bd* infection. However, the probability statistic does not mean that the higher level of probability will confirm *Bd*-presence; rather it symbolizes a strong possibility of *Bd* infection, and vice versa. The southeastern parts of the country are primarily evergreen forest patches. There are different types of small and large wetlands within and around these forests, creating ideal habitats for various species of amphibians (IUCN Bangladesh 2015). The high rate of annual rainfall ensures the availability of water and humidity even in the dry season (IUCN Bangladesh 2015). These areas fall within the Indo-Burma and the Himalaya biodiversity hotspots

(Mittermeier et al. 2011) and support more than half of the country's amphibian fauna in terms of both diversity and abundance (Reza 2014). Murphy et al. (2011) suggested that wetter habitats increase the occurrence of *Bd* and decrease the survival of infected amphibians. There is also evidence that suggests species richness in forested habitats increases the likelihood of *Bd* infection and transmission (Olson et al. 2013). Therefore, we suggest that wetter habitats and species richness could likely be potential drivers of *Bd* infection and transmission in these areas of Bangladesh. The recent record of the globally threatened (and endemic to India) species Khare's stream frog (*Pterorana khare*) from the southeastern part of Bangladesh revealed the importance of the area as a potential site for amphibian study (Khan 2012). Thus, an outbreak of *Bd* in this area may severely affect the amphibian diversity in the country.

The sequence data obtained from the present study suggests the existence of a single *Bd*-strain in Bangladesh that shows a close resemblance with the haplotypes identified from other Asian countries. Our Neighbor-Joining tree showed a similar pattern in clade formation with that of a Bayesian tree reported by Mutnale et al. (2018) where Asian haplotypes, excluding Korea, grouped with Pandemic *Bd*GPL (*Bd* Global Panzootic Lineage) haplotype. There is no record of exotic amphibians in the country and it is highly unlikely that the haplotypes invaded the country through the food or pet trade, because the country has no record of importing amphibians (personal communication with Prof. Noor Jahan Sarkar). Therefore, we assume that it is the native infectious fungal strain, although an extensive molecular study involving more species from unstudied areas may alter the current hypothesis. The complex ancestral relationship of the *Bd*-strain (observed in the phylogenetic tree) demands a more thorough individual and population level of study. This should include different amphibians in Bangladesh in order to fully investigate the ancestral pathway of the particular disease-causing fungus.

The results concluded in this study are still preliminary and our study does not take into account the proximate environmental and climatic effects on the *Bd* occurrence, although, it clearly addresses the knowledge gap in *Bd* distribution in Asia. This study also significantly contributes to the list of infected host species that warrant further intensive investigation. We recommend further detailed studies focusing on the effects of temperature, moisture, seasonality, and elevation on *Bd* occurrence. This research, along with the inclusion of quantitative PCR, is required to determine the infection intensity in various habitats and the susceptibility of different species to chytrid infections.

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Authors Contribution

MMR designed and managed funds for the study. MMR and MFR collected samples, HJ and MC completed laboratory analysis, and MS made the distribution model. MMR, HJ and MFR wrote the manuscript. All authors revised the manuscript and gave their consent to publish.

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Declarations

Conflict of interest Authors declare no conflict of interest for this work.

Ethical Approval and Permission Animal handling was carried out by following the guidelines for the use of live amphibians and reptiles in field research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR). All animals were handled in strict accordance and

good animal practice as approved by the Ethical Clearance Committee (live wild animal involving) of the Faculty of Biological Science, University of Dhaka (reference no. 62/Biol.Scs./2017–2018).

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