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Research paper

Genetic diversity patterns of *Haemonchus contortus* isolated from sheep and goats in Bangladesh

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ABSTRACT

Haemonchus contortus is the most prevalent parasitic nematode among the Trichostrongylids causing severe health hazards leading to production losses in small ruminants around the world. This study was conducted to explore genetic variation within and among *H. contortus* populations from seven topographic zones of Bangladesh in small ruminants using second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA and the mitochondrial nicotinamide dehydrogenase subunit 4 (*nad4*) genes. To do this, a total of 95 adult *H. contortus* were collected from abomasa of slaughtered sheep and goats from seven different geographic zones of Bangladesh. After the extraction of DNA, ITS-2 of nuclear ribosomal DNA and partial region of the mitochondrial *nad4* genes were amplified and sequenced for 95 and 85 worms, respectively. After editing and alignment, sequences were employed for analysis to determine sequence variation, genetic diversity and population genetic structure. Genetic analysis defined 19 distinct ITS-2 genotypes and 77 unique *nad4* haplotypes among the *H. contortus* isolates. The nucleotide diversities were 0.0098 and 0.025 for ITS-2 and *nad4* gene, respectively. Phylogenetic analysis (neighbor joining, maximum likelihood and maximum parsimony) of haplotypes indicated the existence of two populations without marked specification of host and locations within *H. contortus* populations in Bangladesh. By population genetic analysis, 93.67% of genetic variance was partitioned within the population. Very low genetic differentiation but high gene flow was observed among different populations of *H. contortus* in Bangladesh. This is the first study on genetic variability of *H. contortus* isolates of small ruminants in Bangladesh. Our study could be the basis for further molecular epidemiological studies, using more discriminative markers and tracing possible changes in the population structure of *H. contortus*.

1. Introduction

H. contortus is the greatest concern among trichostrongylid nematodes of small ruminants around the world (Gibbs and Herd, 1986; O'Connor et al., 2006). It inhabits in the abomasum and it is an extremely fecund parasite laying 5000–10,000 eggs per day (Abutarbush, 2010). It causes blood loss (0.05 ml/parasite/day) leading to serious health effects such as anemia, edema (bottle jaw) and even death in severely affected animals (Taylor et al., 2007). The parasite is responsible for serious production and economic loss in terms of reduced body weight, cost and labor for anthelmintic treatment and mortality of animals. The estimated economic loss by this parasitic infection is to be $26 million, $46 million and $103 million per annum for Kenya, South Africa and India, respectively (Gasser et al., 2008; Waller and Chandrawathani, 2005). Bangladesh is a subtropical country, and the monsoon climate provides adequate moisture and temperature, which encourages parasitic development and survival (Tariq, 2015). Among GI nematodes, *H. contortus* is hyper-endemic in different parts of Bangladesh (Nahar et al., 2015; Nuruzzaman et al., 2012; Shahiduzzaman et al., 2003).

H. contortus is characterized by a large population size and high genetic variability (Anderson et al., 1998; Blouin, 2002). The genetic structure of population is determined by several factors such as environmental and geographical barriers, population size and the life pattern of populations (Troell et al., 2006). The population genetic structure of the parasite is promoted by a high rate of gene flow among populations (Anderson et al., 1998; Braisher et al., 2004; Yin et al., 2013) due to frequent movement of the host indicating an opportunity for the spread of alleles that confer resistance to anthelmintics (Blouin et al., 1995). Genetic information such as genetic diversity portrayed on

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the phylogenetic tree is essential for a better understanding of ecology, epidemiology and evolution of parasitic nematodes (Jacquiet et al., 1995).

ITS-2 region of nuclear ribosomal DNA is designated as an important marker because of its ease of amplification, availability of conserved regions, sufficient number of rRNA clusters, fast evolution of variable nuclear loci and adequate amount of variation to distinguish closely related species (Avramenko et al., 2015; Cerutti et al., 2010; Gasser and Newton, 2000). Commonly, sequences of ITS-2 are employed for species identification of helminthes, such as nematodes (Stevenson et al., 1995), trematodes (Luton et al., 1992) and cestodes (Králová-Hromadová et al., 2012). Fixed nucleotide differences (1.3%) were observed between *H. contortus* and *H. placei* within the genus *Haemonchus* (Stevenson et al., 1995) and low (< 1%) intra-specific genetic variation were detected (Gasser and Newton, 2000) from the sequence data of ITS-2.

Mitochondrial DNA (mtDNA) has been used as an important tool to study population genetics, taxonomy and evolutionary development of different bursate nematodes due to its high mutation rate and maternal inheritance (Cerutti et al., 2010; Hu and Gasser, 2006; Jex et al., 2008). The *nicotinamide dehydrogenase subunit 4* (*nad4*) of the mitochondria is a good candidate for studying genetic diversity and population structure (Jex et al., 2008) and has been commonly used in various molecular studies for *Haemonchus* populations (Blouin et al., 1995; Cerutti et al., 2010; Troell et al., 2006).

Molecular characterization is mainly used as an essential tool for the validation of nematode species and phylogenetic analysis. Knowledge about genetic variation of *H. contortus* within and among populations can help understand transmission patterns, the spread of drug resistance alleles, and eventually supports the formulation of an effective control strategy (Gasser et al., 2008). Scientist from different countries in different geological zones of the world, including neighboring countries of Bangladesh have described the genetic variability of *H. contortus* (Blouin et al., 1998; Brasil et al., 2012; Gharamah et al., 2012; Hunt et al., 2008; Hussain et al., 2014; Troell et al., 2006; Yin et al., 2013). *H. contortus* is the most endemic and economically important parasite of livestock in Bangladesh (Nahar et al., 2015; Nuruzzaman et al., 2012). Also, the diversified genetic features of this parasite have an impact on the development and spread of anthelmintic resistance (Silvestre et al., 2009). Indeed information on genetic variability within and among *H. contortus* populations is yet to be determined in Bangladesh. In this context, our study was conducted to explore genetic variation within and among *H. contortus* populations from seven topographic zones of Bangladesh in small ruminants using ITS-2 of nuclear ribosomal DNA and the mitochondrial *nad4* genes as genetic markers.

2. Materials and methods

2.1. Study area

A total of 95 adult male *H. contortus* were collected from abomasa of slaughtered sheep (36) and goats (59) from seven different topographic zones of Bangladesh (Tangail of Madhupur Tract, Rajshahi of Barind Tract, Rangpur of Tista Silt, Mymensingh of Brahmaputra Alluvium, Jhenaidah of Gangetic Alluvium, Bhola of Coastal Saline Tract and Rangamati of Hill Tract) as shown in Table 1 and Fig. 1.

2.2. Collection of adult parasites

Recovery of adult worm was carried out according to standard procedures as described by (MAFF, 1986). Briefly, after slaughtering, the abomasum was removed from the other stomach parts and ligated at both ends. The abomasum was then taken directly to the laboratory, and the contents were poured into a glass beaker. Both the abomasum and its contents were carefully examined and individual adult male worms were collected. The parasites were washed extensively in

Table 1
ITS-2 genotypes and *nad4* haplotypes of *H. contortus* isolates representing seven topographic zones of Bangladesh.

Topographic zones (Longitude, latitude)	ITS-2				*nad4*			
	No. of sequences	No. of genotypes	Genotype diversity (Gd)	Nucleotide diversity	No. of sequences	No. of haplotypes	Haplotype diversity	Nucleotide diversity
Mymensingh (90.3560°E, 24.7851°N)	14	6	0.824	0.0088	14	13	0.989	0.027
Tangail (86.6480°E, 24.2654°N)	12	8	0.924	0.0129	8	7	0.964	0.030
Rangamati (92.2985°E, 22.7324° N)	13	8	0.885	0.0099	12	11	0.985	0.20
Rangpur (89.2301°E, 25.7435°N)	13	6	0.833	0.0088	12	11	0.985	0.025
Rajshahi (88.6241°E, 24.3635°N)	16	9	0.900	0.0097	15	14	0.990	0.027
Jhenaidah (89.2420°E 23.6841° N)	14	8	0.923	0.0115	11	10	0.982	0.022
Bhola (90.7153°E, 22.0938°N)	13	6	0.872	0.0083	13	12	0.987	0.018
Overall	95	19	0.867	0.0097	85	77	0.998	0.025

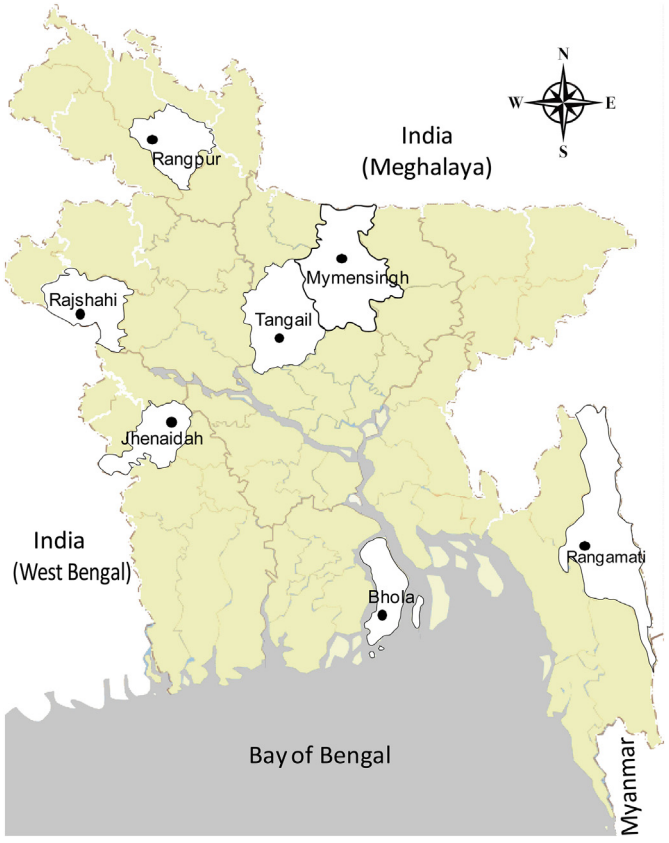

Fig. 1. Study areas. Seven topographic zones of Bangladesh (longitude and latitude shown in Table 1) from where samples were collected from sheep and goats.

physiological saline. Male worms were identified by microscopic examination according to the procedures outlined by Soulsby (1982) and preserved in 70% ethanol and stored at −20 °C, until DNA extraction was performed.

2.3. Isolation of DNA

Total genomic DNA was isolated from 95 individual male worms using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, single adult parasite was taken into an eppendorf tube, tissues were disintegrated, lysed and proteins were digested by proteinase K, and washed extensively. Trapped DNA was eluted by elution buffer. Extracted DNA was measured in a Nanodrop spectrophotometer (ThermoFisher Scientifica, Germany) to ascertain the concentration and quality. DNA samples were stored at −20 °C until use.

2.4. PCR amplification and sequencing

ITS-2 (~350 bp) was amplified from genomic DNA by using the conserved oligo-nucleotide primer pair: NC1 (forward: 5-ACGTCTGGT TCAGGGTTGTT-3) and NC2 (reverse: 5-TTAGTTTCTTTTCCTCCGCT-3) (Stevenson et al., 1995). For analysis of genetic diversity, amplification of the *nad4* gene (~800) was performed using Primer 1-F (5-GGATTT GGTCAGCAAATTGAA-3) and Primer 2-R (5-GCCTGCAAATGAATTA ACA-3) (Yin et al., 2013). The PCR amplification reaction contained 5 µl of 10XPCR buffer (10 mM of Tris–HCl (pH 8.3), 50 mM of KCl and 4 mM of MgCl2), 1 µl of 10 mM each of dNTP, 2 µl of 10 pmol of each primer, 5 µl of DNA template and 1 µl of 5 U Taq DNA polymerase (TaKaRaTaq™, TaKaRa Clontech, China) in a total volume of 50 µl. The thermocycling conditions used were at 94 °C for 5 min for the initial denaturation followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

The PCR products were visualized through agarose gels (1.5%) to verify that they represented single bands. The PCR products were column purified (Wizard PCR-Preps, Promega) and then subjected to sequencing directly (BigDye Terminator v.3.1 cycle sequencing kit, Applied Biosystems) in an automated sequencer (PRISM3730, ABI) using respective forward and reverse primers (in separate reactions). Forward and reverse sequences were aligned and edited using the BioEdit software (Hall, 1999). The sequences were aligned using MEGA6 software (Tamura et al., 2013) and deposited in GenBank under the accession numbers: ITS-2 sequences (LC360145- LC360163); *nad4* sequences (LC361049- LC361102, LC376827- LC376849).

2.5. Data analysis

The sequences of ITS-2 and *nad4* were aligned over a consensus length of 231 bp and 730 bp, respectively, using the program Clustal W within MEGA v.6.0 (Tamura et al., 2013). Intra-population diversity parameters such as nucleotide diversity, haplotype diversity, average number of nucleotide differences were calculated using DnaSP version 5.1 (Rozas, 2009). Pairwise comparisons were performed with previously published sequences, and identities (%) were calculated using the program BioEdit (Hall, 1999). Phylogenetic analysis was performed using neighbor joining, maximum likelihood and minimum parsimony methods, respectively, based on the Tamura-Nei model (Tamura et al., 2013). Confidence limits were assessed using the bootstrap procedure (1000 replicates) for neighbor joining, maximum likelihood and minimum parsimony trees, and other settings were obtained using the default values in MEGA v.6.0 (Tamura et al., 2013). A 50% cut-off value was implemented for the consensus tree. Haplotype sequences were defined by using the online Collapse program (http://sing.ei.uvigo.es/ALTER/). The diversity indices (Fst and Nst) were calculated using the program DnaSP 5.1 (Rozas, 2009) to evaluate the degree of gene flow among populations. The median joining (MJ) network was constructed to study haplotype relationships, using the program Network v.4.6.1.1 (Bandelt et al., 1999). A hierarchical analysis of molecular variance (AMOVA) was performed to estimate genetic diversity within and among populations (isolates) using the Arlequin 3.1 package (Excoffier et al., 1992). For this analysis, the data set was divided into two groups. Groups 1 and 2 contained samples from northern part (Mymensingh, Tangail, Rangpur and Rajshahi) and southern part (Jhenaidah, Rangamati and Bhola) of Bangladesh, respectively. In addition, a total of 324 sequences (Blouin et al., 1995; Cerutti et al., 2010; Gharamah et al., 2012; Hussain et al., 2014; Yin et al., 2013) retrieved from GenBank were used for comparisons.

3. Results

3.1. Species identification and genotyping

We analyzed ITS-2 gene to identify the species of *H. contortus*. To do this, *H. contortus* specific ITS-2 gene isolated from 95 samples from different topographic zones of Bangladesh were amplified and sequenced. After the editing and alignment of sequences, a 231 bp consensus length was obtained for all the samples. From 95 ITS-2 sequences, 19 distinct genotypes were identified. The sequence identities ranged from 96.9% to 100%, when compared with each other, or with two ITS-2 reference sequences of *H. contortus* (GenBank accession nos. X78803.1 and EU084691.1) (Table 2). The contents of GC nucleotides in ITS-2 sequences of *H. contortus* were ranged from 32.5–33.8%.

We aligned 19 ITS-2 genotypes with the reference sequence of *H. contortus* (X78803.1) and detected ten single nucleotide polymorphism (SNPs), which were the result of substitutions in nucleotide positions 18, 21, 22, 28, 55, 59, 63, 93, 123 and 196. There were five

Table 2
Pairwise identities (%) among 19 ITS-2 genotypes of *H. contortus* representing 95 samples from Bangladesh using selected sequence of *H. contortus* from GenBank.

Sample ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1.BDMYHC06 (LC360145)	–																				
2.BDRPHC74 (LC360146)	97.4	–																			
3.BDBHHC118 (LC360147)	98.2	99.1	–																		
4.BDBHHC111b (LC360148)	98.7	98.7	99.5	–																	
5.BDMYHC17 (LC360149)	99.1	98.2	99.1	99.5	–																
6.BDTAHC25 (LC360150)	98.2	97.4	98.2	98.7	99.1	–															
7.BDTAHC26 (LC360151)	97.8	96.9	97.8	98.2	98.7	99.5	–														
8.BDTAHC35 (LC360152)	98.2	97.4	98.2	98.7	99.1	99.1	99.5	–													
9.BDRMHC37 (LC360153)	98.7	98.7	99.5	99.1	99.5	98.7	98.2	98.7	–												
10.BDRMHC46 (LC360154)	98.2	98.2	99.1	99.5	99.1	98.2	98.7	99.1	98.7	–											
11.BDRPHC59 (LC360155)	98.7	97.8	98.7	99.1	99.5	99.5	99.1	99.5	99.1	98.7	–										
12.BDRSHC78b (LC360156)	98.2	99.1	99.1	99.5	99.1	98.2	97.8	98.7	98.2	99.1	98.7	–									
13.BDRSHC82 (LC360157)	98.2	98.2	99.1	98.7	99.1	99.1	98.7	99.1	99.5	98.2	99.5	98.2	–								
14.BDMYHC13b (LC360158)	97.4	99.1	99.1	98.7	98.2	97.4	96.9	97.4	98.7	98.2	97.8	99.1	98.2	–							
15.BDTAHC21b (LC360159)	98.2	99.1	99.1	98.7	99.1	98.2	97.8	98.2	99.5	98.2	98.7	99.1	99.1	99.1	–						
16.BDRPHC64b (LC360160)	97.8	99.5	99.5	99.1	98.7	97.8	97.4	97.8	99.1	98.7	98.2	99.5	98.7	99.5	99.5	–					
17.BDRSHC79b (LC360161)	97.4	99.1	98.2	98.7	98.2	98.2	97.8	98.2	97.8	98.2	98.7	99.1	98.2	98.2	98.2	98.7	–				
18.BDJHHC104b (LC360162)	98.2	98.2	99.1	99.5	99.1	99.1	98.7	99.1	98.7	99.1	99.5	99.1	99.1	98.2	98.2	98.7	99.1	–			
19.BDJHHC109b (LC360163)	98.7	97.8	98.7	99.1	99.5	98.7	99.1	99.5	99.1	99.5	99.1	98.7	98.7	97.8	98.7	98.2	97.8	98.7	–		
20.*H. contortus* (X78803)	97.4	99.1	98.2	98.7	98.2	97.4	97.8	98.2	97.8	99.1	97.8	99.1	97.4	98.2	98.2	98.7	99.1	98.2	98.7	–	
21. *H. contortus* (EU084691.1)	97.4	100	99.1	98.7	98.2	97.4	96.9	97.4	98.7	98.2	97.8	99.1	98.2	99.1	99.1	99.5	99.1	98.2	97.8	99.1	–

transversions (three A < - > T, one G < - > C and one A < - > C) and five transitions (T < - > C) in those substitutions (Table 3). The sequence data of *H. contortus* (present study) were also aligned with *H. placei* (X78812). The differences in the sequences between *H. contortus* and *H. placei* were in three nucleotide positions (24, 205 and 219) indicating 1.3% variation between sequences of our isolates and *H. placei*. Also, the variations in three places were represented by purines (G < - > A) (Supplementary file 1).

The overall nucleotide diversity and genotype diversity were 0.0098 and 0.8695, respectively, among the ITS-2 sequences of *H. contortus* from different geographical zones of Bangladesh (Table 1).

The phylogenetic tree (UPGM tree) built by 19 genotypes was divided into two clusters except two genotypes where two *H. placei* were used as out groups. However, there was no distinct boundary in relation to host and geographic locations among the two clusters of *H. contortus* isolates (Supplementary Fig. 1).

3.2. Genetic diversity and phylogeny of nad4

To determine genetic diversity, we amplified and sequenced the *nad4* gene. Sequences (85) were aligned and a 730 bp length was generated for all samples. All the sequences were employed for nucleotide BLAST search and harmonized with another *nad4* gene of *H. contortus* with high sequence identities (98%–99%). From 85 amplicons, 77 unique haplotypes were detected. Among 115 polymorphic sites (at different nucleotide positions), 68 parsimony informative sites were recognized. A high degree of gene diversity of *nad4* was observed among *H. contortus* isolates of Bangladesh and the average nucleotide diversity and haplotype diversity were 0.025 and 0.998, respectively (Table 1). High level of genetic variability provided an opportunity to test the existence of host specific sub-populations of different *Haemonchus* species.

The *H. contortus* populations from seven topographic zones were also analyzed for selective neutrality by using Tajima's D, Fu and Li's D

Table 3
Nucleotide details and distribution of 19 genotypes from 95*H. contortus* worms from sheep and goats.

Genotypes	Nucleotide position										No. of worms
	18	21	22	28	55	59	63	93	123	196	
X78803	T	C	T	C	C	T	C	A	C	A	
1. BDMYHC06 (LC360145)	A	G	C	T	T	.	.	.	T	.	1
2. BDRPHC74 (LC360146)	.	.	.	.	.	.	.	.	T	T	23
3. BDBHHC118 (LC360147)	.	G	C	.	.	.	.	.	T	T	19
4. BDBHHC111b (LC360148)	.	G	C	.	.	.	.	.	T	.	12
5. BDMYHC17 (LC360149)	A	G	C	.	.	.	.	.	T	.	12
6. BDTAHC25 (LC360150)	A	G	C	.	.	A	A	.	T	.	3
7. BDTAHC26 (LC360151)	A	G	C	.	.	A	A	.	.	.	1
8. BDTAHC35 (LC360152)	A	G	C	.	.	.	.	.	.	.	2
9. BDRMHC37 (LC360153)	A	G	C	.	.	.	.	.	T	T	1
10. BDRMHC46 (LC360154)	.	G	C	.	.	.	.	.	T	.	1
11. BDRPHC59 (LC360155)	A	G	C	.	.	.	.	.	T	.	4
12. BDRSHC78b (LC360156)	.	G	.	.	.	.	.	.	T	.	2
13. BDRSHC82 (LC360157)	A	G	C	.	.	.	.	.	T	T	1
14. BDMYHC13b (LC360158)	.	G	.	.	.	.	.	C	T	T	1
15. BDTAHC21b (LC360159)	A	G	.	.	.	.	.	.	T	T	1
16. BDRPHC64b (LC360160)	.	G	.	.	.	.	.	.	T	T	8
17. BDRSHC79b (LC360161)	.	.	.	.	.	.	.	.	T	.	1
18. BDJHHC104b (LC360162)	.	G	C	.	.	.	.	.	T	.	1
19. BDJHHC109b (LC360163)	A	G	C	.	.	.	.	.	,	.	1
Total											95

Dot (.) represents similar position with X78803.

Table 4
Test for selective neutrality among *nad4* sequences from *H. contortus* isolates from different topographic zones of Bangladesh.

Locations	Tajima's D	p value	Fu & Li's D	p value	Fu & Li's F	p value
Mymensingh	−0.22249	p > 0.10	−0. 72,535	p > 0.10	−0.67476	p > 0.10
Tangail	0.53181	p > 0.10	0.49722	p > 0.10	−0.56373	p > 0.10
Rangamati	−1.19013	p > 0.10	−1.08911	p > 0.10	−1.27226	p > 0.10
Rangpur	−0.14762	p > 0.10	−0.39657	p > 0.10	−0.37750	p > 0.10
Rajshahi	−0.38662	p > 0.10	−0.76581	p > 0.10	−0.76045	p > 0.10
Jhenaidah	−0.75869	p > 0.10	−0.88730	p > 0.10	−0.96981	p > 0.10
Bhola	−0.85614	p > 0.10	−0.84666	p > 0.10	−0.97203	p > 0.10
Overall	−1.29410	p > 0.10	−2.32586	0.10 > p > 0.05	−2.27399	0.10 > p > 0.05

and Fu and Li's F statistics. Among the *H. contortus* isolates of Bangladesh, no significant (p > 0.05) deviation from neutrality was observed in all three tests; indicating absence of any selective forces operating on these populations (Table 4).

The phylogenetic tree was constructed using haplotype data set of seven different *H. contortus* populations of Bangladesh (Supplementary Fig. 2). Trees produced by three methods (NJ, ML and MP) revealed similar results. However, for convenience, only the NJ tree has been presented and described. The NJ dendogram generated with 1000 replicates showed two distinct clades supported by strong bootstrap value (84%). However, haplotypes formed two distinct clades but isolates were randomly distributed within the two clades without any relation of host and geographical origin of isolates. Both clades also consisted of some groups where the bootstrap values were > 50%. Additionally, the median joining network was also constructed to discern the relationship between 77 haplotypes (Fig. 2). The network profile also supported the clustering of two groups among the haplotypes of different geographical regions except a few isolates from different geographical location.

To compare population genetics, 50 *nad4* sequences of *H. contortus* were selected randomly from Bangladesh (Accession nos. LC361049-

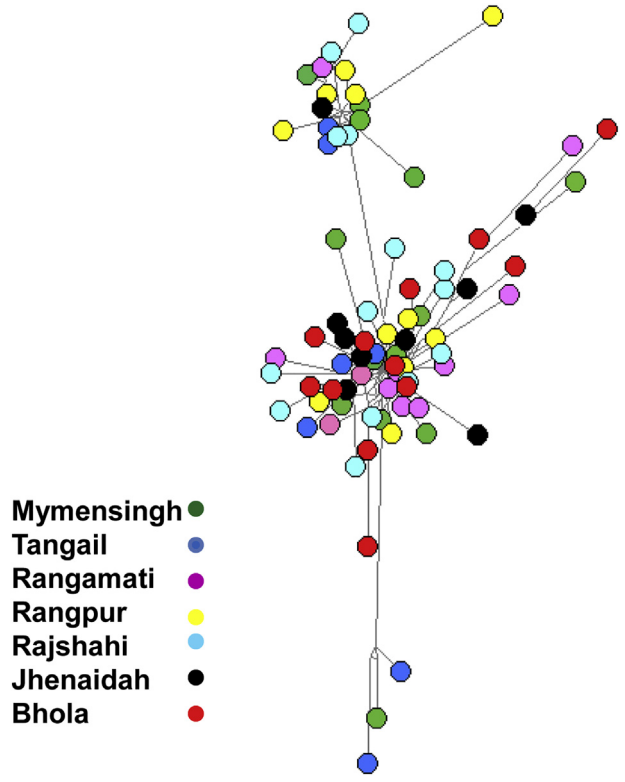

Fig. 2. The median joining network of 77 *nad4* haplotypes of *H. contortus* isolates from different topographic zones of Bangladesh. The different colored dots represent different *H. contortus* populations/locations.

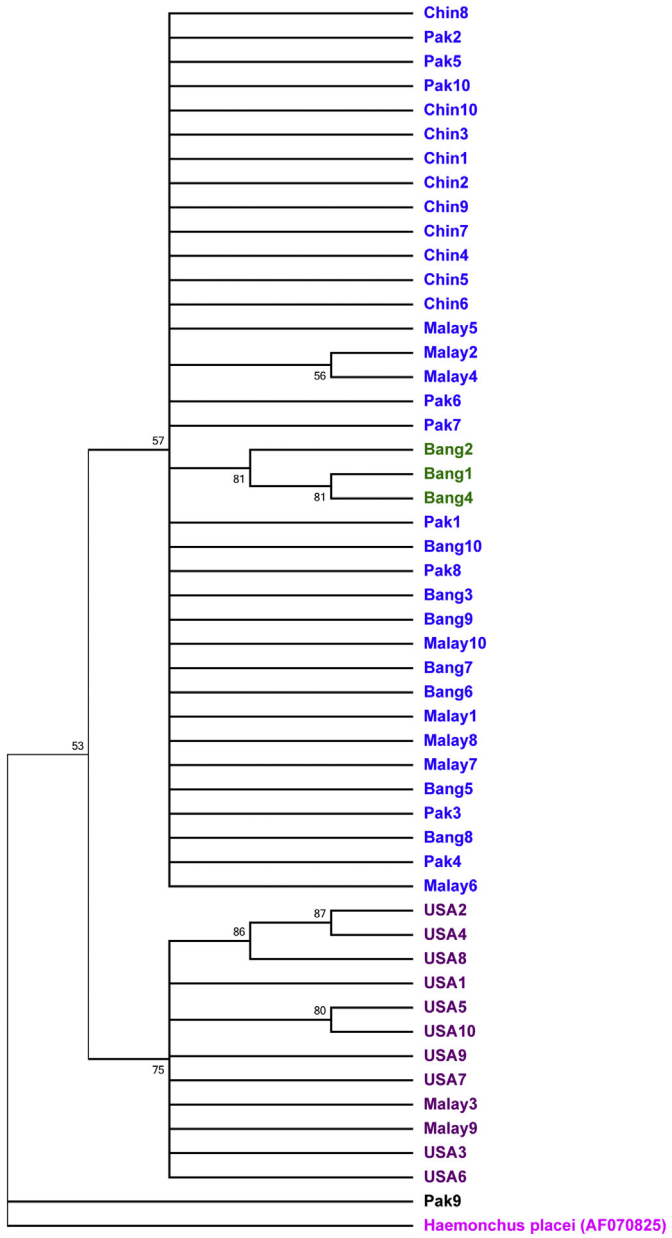

Fig. 3. Phylogenetic tree constructed using 50 *nad4* sequences from *H. contortus* from five different countries. The bootstrap value of > 50% was displayed in the tree. Forty *nad4* sequences of *H. contortus* were retrieved from GenBank databases and used for comparison; ten sequences from each country; USA (Accession nos. AF070776-AF070785), Malaysia (Accession nos.HQ660291-HQ660300), China (Accession nos.KC429951-KC429960) and Pakistan (Accession nos.KJ724500-KJ724509) *H. placei* (Accession no. AF070825) was used as outgroup. Abbreviations: Bang: Bangladesh; Chin: China; Pak: Pakistan; Malay: Malaysia; USA; United States of America.

LC361058) and previously published sequences of four other countries, including the USA (Accession nos. AF070776- AF070785), Malaysia (Accession nos. HQ660291- HQ660300), China (Accession nos. KC429951- KC429960) and Pakistan (Accession nos. KJ724500- KJ724509). In NJ method, two major clusters were obtained by bootstrap analysis (˃50%) with 1000 replicates where *H. placei* (AF0707825) was used as the outgroup (Fig. 3). Most of the isolates from the same continent formed clusters, particularly isolates from America and Asia. The same clustering pattern was also found in ML and MP method. In the American cluster, two Malaysian isolates were found to merge. But Asian cluster was unique from the isolates of Bangladesh, Pakistan, Malaysia and China. However, three Bangladeshi isolates formed a group within the Asian cluster with strong nodal support (82%).

3.3. Population genetic structure

To determine genetic differentiation among the studied populations, we estimated Fst and Nst value of *H. contortus* populations in different topographic zones of Bangladesh. We observed very low genetic differentiation that ranged from −0.02538 to 0.12558 for Fst and −0.0.02570 to 0.12786 for Nst. Relatively high genetic differentiation was observed in *H. contortus* population of Bhola showing highest level of Fst (0.12558) and Nst (0.12786) value than compared with other populations of different topographic zones in Bangladesh (Table 5). The pairwise Fst value was calculated to determine the genetic relationship between *H. contortus* populations of Bangladesh and the other four countries including the USA, Malaysia, China and Pakistan. From the results, the highest level of genetic differentiation was observed in the USA (Fst = 0.53) and the lowest level in Pakistan (Fst = 0.09) compared to Bangladesh (Table 6).

A hierarchical Analysis of Molecular Variance (AMOVA) was computed to assess the possible factors that affect gene flow within and among populations. The calculated result showed that the majority of genetic variance was distributed within population at 93.67% (Fst = 0.063, $p = 0.04$) while only 6.84% were distributed among groups (Fct = 0.068, $p = 0.00$) (Table 7).

4. Discussion

H. contortus, an important blood feeding nematode, is found throughout the world with a high rate of infection (Choubisa and Jaroli, 2013; Ntonifor et al., 2013; Raza et al., 2014; Tsotetsi and Mbati, 2003; Vassilev, 1995; Zeryehun, 2012). In our study, the population genetic structure and diversity of *H. contortus* was investigated. The results derived from the isolates of different topographic zones of Bangladesh, as well as other countries of the world were appraised and their relationship was determined.

According to Jacquiet et al. (1995), in the genus *Haemonchus*, *H. contortus* and *H. placei* are closely related to each other. The intra-specific variation is important to confirm the species of a parasite. The results of our study confirm the published data of Stevenson et al. (1995) which revealed differences in the structure of ITS-2 spacers between *H. contortus* and *H. placei* at the level of 1.3% on the three nucleotides. Thus, the studied ITS-2 sequences from these worms confirmed their specific identity as *H. contortus*. Further analysis for the variation of sequence identities in our study was 3.1% in ITS-2 sequences among *H. contortus* isolates. The extent of variation is consistent (2.6%) with the variation of *H. contortus* isolates of other countries; Germany, Sweden and Kenya (Heise et al., 1999; Troell et al., 2003). However, disparity (5.2%) was also recorded in France, Australia, New Zealand and the UK (Gasser et al., 1998). Nineteen (19) distinct ITS-2 genotypes were defined among *H. contortus* isolates in our study. But the number of ITS-2 genotypes of *H. contortus* isolates diverged in previously published articles, eighteen among Chinese isolates (Yin et al., 2013), seven among Pakistani isolates (Hussain et al., 2014) and six among Yemeni and Malaysian isolates (Gharamah et al., 2012). The number of polymorphic loci also differed between countries but the variation in loci 123 and 196 was common in all cases. According to Stevenson et al. (1995), heterozygous nucleotides at the position of 18, 123, 174, 196 and 202 were considered as *H. contortus* isolates. Similar nucleotide heterozygosity was viewed in all loci except at nucleotide position 174 and 202 in our study.

In present study, to determine the presence and extent of genetic diversity, the *nad4* gene of mtDNA was used from *H. contortus* isolates of seven topographic zones of Bangladesh where the nucleotide diversity was found to be 0.025. The recorded value is in agreement with the previously published values of nucleotide diversity from *H. contortus* isolates in the USA (0.025), Malaysia (0.038), China (0.029) and Pakistan (0.030) (Gharamah et al., 2012; Hussain et al., 2014; Yin et al., 2013). This high degree of intra-population diversity is an important character of the Trichostrongylid, especially *Haemonchus* (Archie and Ezenwa, 2011; Blouin et al., 1998). It is a parasite of high biotic potential, together with high infection rate in small ruminants and a direct lifecycle which results in a large effective population with marked genetic variability (Blouin et al., 1998; Brasil et al., 2012; Prichard, 2001).

We found 77 haplotypes from 85 individual which is typical for Trichostrongylids (Braisher et al., 2004). In a similar study using *nad4* on *H. contortus*, 113 haplotypes were identified among the 120 individuals sequences (Blouin et al., 1995). Blouin et al. (1997) found 42 haplotypes from 50*H. contortus* individuals sequenced for *nad4* gene while Troell et al. (2006) found 94 haplotypes from 150 *H. contortus* worms.

Table 6
Fst value between *H. contortus* populations of Bangladesh and four other different countries.

Countries	GenBank accession no.	No. of sequences	Fst value	Nucleotide diversity
USA	AF070736-AF070785	50	0.53	0.026
Malaysia	HQ660255-HQ660308	54	0.13	0.039
China	KC429944-KC430085	142	0.16	0.029
Pakistan	KJ724439-KJ724511	73	0.09	0.030
Bangladesh	LC361049-LC361102, LC376827-LC376849	77		0.026

Table 5
Pairwise Fst and Nst value between *H. contortus* populations from seven topographic zones of Bangladesh.

Locations	Mymensingh	Tangail	Rangamati	Rangpur	Rajshahi	Jhenaidah	Bhola
Mymensingh	–	−0.02570	0.04654	−0.02321	−0.00834	0.02115	0.06713
Tangail	−0.02538	–	0.11066	−0.01399	−0.00606	0.05195	0.11302
Rangamati	0.04595	0.11007	–	0.08650	0.05201	0.04173	−0.00520
Rangpur	−0.02308	−0.0146	0.08500	–	−0.02473	0.07092	0.12786
Rajshahi	−0.00859	−0.00616	0.05173	−0.02464	–	0.001858	0.05827
Jhenaidah	0.02042	0.05098	0.04174	0.06959	0.01838	–	0.00485
Bhola	0.06571	0.11181	−0.00476	0.12558	0.05756	0.00475	–

Fst and Nst are below and above the diagonal, respectively. Negative values signify more nucleotide substitutions within than between populations.

Table 7
Analysis of Molecular Variance (AMOVA) and F-statistics of partial *nad4* gene for different populations of *H. contortus* in Bangladesh.

Source of variation	Percentage of variation	F-statistics	*p*-Value
Among group	6.84	Fct = 0.068	0.00*
Among group within population	−0.51	Fsc = −0.005	0.56
Within population	93.67	Fst = 0.063	0.04*

Seven populations were divided into two groups including northern part (Mymensingh, Tangail Rajshahi and Rangpur) and southern part (Jhenaidah, Rangamati and Bhola) in Bangladesh.
Negative AMOVA value might indicate more nucleotide differences between isolates within the same group than between group.
* $p < 0.05$.

The phylogenetic tree of *H. contortus* populations from seven topographic zones revealed two main clusters, indicating two populations of *H. contortus* isolates in Bangladesh. Notably, the clusters were neither formed with the isolates of defined geographical origin nor the host species of the data set. Here, we hypothesized that Bangladesh is a small country, and therefore, a strong geographical barrier does not exist. Furthermore, different host species such as goats, sheep, cattle and buffaloes share common grazing fields under the extensive management system available in Bangladesh; thereby transmission of infection from one host to another is a common event. Therefore, it could be assumed that possibly two distinct clusters were prevalent during the establishment of *H. contortus* in this country in ruminants. And, due to sharing of common grazing land they have been distributed in different host species, this breaks the host barrier, and the cluster obtained during the study is possibly due to ancestral polymorphisms between two parasitic populations prevalent at that time point (Cerutti et al., 2010). In global view, two clusters were distinct according to their geographical pattern such as continental demarcation. There is considerable genetic differentiation between continental areas in different populations of *H. contortus*. The explanation is the limited dispersal ability of parasites between the continents, due to restricted opportunity of host movement with this genetic subdivision (Troell et al., 2006). The genetic subdivision of parasite population was also observed between France and Morocco where the ocean acts as a strong geographical barrier (Leignel and Humbert, 2001). However, high gene flow was observed within the same geographical region such as China (Yin et al., 2013), Pakistan (Hussain et al., 2014), Malaysia and Yemen (Gharamah et al., 2012). In our study, an unexpected result was observed in the NJ dendogram (Fig. 2) where a close association between two Malaysian isolates with American isolates. The similar results were also recorded by Troell et al., (2006) where isolates from Greece overlapped with the isolates obtained from Australia. The explanation in such case is the introduction of parasite population through imported animals of the same origin because there is no evidence of direct movement of animals between two continents.

On the other hand, in population genetic structures, the host is a key element between the parasite populations for determining genetic variability and gene flow (McCoy et al., 2003). The majority (93.67%) of genetic variation was distributed within the population, indicating high gene flow within the population. The high level of gene flow has an important implication for spreading resistant parasites within the population. High within-population variation, followed by variation of among groups and least variation among population within group are in accordance with the previous study and attributed to a possible faster mtDNA evolutionary rate in nematodes than in other taxa (Blouin et al., 1995). On a large scale, high genetic differentiation was recorded between the populations from different continents where it was low within same continent (Blouin et al., 1995), due to random access to hosts within continent or among the neighboring countries for trading purpose.

In conclusion, this study showed that two populations of *H. contortus* exist in Bangladesh. Isolates of *H. contortus* shared a gene pool irrespective of host (goats and sheep) and geographic locations imply high gene flow within the population. The results also revealed high within-population variation and very low population genetic differentiation of *H. contortus* in Bangladesh. Globally, low genetic differentiation among *H. contortus* isolates was observed between same continent and high between different continents. The findings of our study provide a preliminary insight on genetic diversity of *H. contortus* isolates in Bangladesh. These results will be useful for further molecular epidemiology survey applying hypervariable microsatellite markers and for recognition and following up on epidemiological changes, as well as for control measure.

Ethics approval and consent to participate

During carrying out this research, no animals were harmed or unethically injured or killed. Also the authors tried to maintain highest possible ethical standards in their works. The study was approved by Animal welfare and ethical committee of Bangladesh Agricultural University (06/AWEC/2017).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The data were submitted in the GenBank and publicly available after acceptance of this article.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MZA and NB conceived the project. ARD, MZA and MAA were involved in study design and coordination of data collection. ARD, MZA and ZZ conducted laboratory work and performed data analyses. ARD, MZA, ZZ and MH interpreted the data. ARD, MZA, MH, NB and MAA drafted the manuscript. All authors read and approved the final manuscript.

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