



International Journal of Veterinary Sciences and Animal Husbandry



ISSN: 2456-2912

NAAS Rating: 4.61

VET 2025; 10(4): 92-97

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www.veterinarypaper.com

Received: 13-02-2025

Accepted: 17-03-2025

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Morpho-molecular detection and characterization of rumen fluke in cattle at northern area of Bangladesh

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DOI: <https://www.doi.org/10.22271/veterinary.2025.v10.i4b.2184>

Abstract

Rumen fluke infection is a common disease of ruminants caused by different species of paramphistomes and causes heavy economic loss worldwide. This study was conducted to morphological identification, molecular detection and characterization with genotyping of rumen fluke in cattle of Bangladesh. Adult fluke specimens were collected from rumen, reticulum as well as bile duct of slaughtered cattle then washed extensively with physiological saline and flukes were identified morphologically as paramphistomes depending on family and genus specific structural characteristics for molecular study. In our research, paramphistomes were identified through morphological and molecular analysis, subsequently confirmed through sequence analysis and the sequences were submitted to GenBank and assigned as accession numbers of OP751135. PCR was standardized with extracted DNA using published primers and annealing temperatures amplifying ITS2 ribosomal gene sequences with 520bp amplicon sizes. The results of sequence alignment indicated that, the study sequences shared 98-100% similarity with sequences of different species of paramphistomes from India, China and other countries and the collected isolate was considered as paramphistomes. The collected fluke was also confirmed as paramphistomes on the basis of ITS2 gene partial fragment with 53% G+C and 47% A+T composition. Phylogenetic tree reconstruction demonstrated that they all clustered together with high homogeneity within their respective groups. PCR method, based on ITS2 gene sequences, proved as specific and essential approach for detection, characterization and classification of various rumen fluke like. The findings from baseline molecular research could serve as cornerstone for future applied molecular research and control of this group of trematodes.

Keywords: Cattle, rumen fluke, DNA, PCR, ITS2 gene

1. Introduction

Cattle are the most important livestock species which are the major animal protein source as they give milk, meat, hides and also draught power etc. (Banglapedia). Gastrointestinal helminthes are the major constraint to livestock production throughout the tropics and elsewhere (Githiori *et al.*, 2014) [8]. Paramphistomes are one of the most common and pathogenic among different gastrointestinal helminthes (Paul *et al.* 2011) [24]. Paramphistomes are digenetic trematodes belong to the family paramphistomatidae, are characterized by-conical or elongated in shape, pink colored, smooth cuticle with thick fleshy body and having two suckers, rounded or lobbed testis at the middle third of the body, ovary is post testicular and uterus long fold occupying the intercaecal region, genital pore is present at the anterior third of the body behind the intestinal bifurcation (Choudhary *et al.* 2015) [5].

Various species under the family paramphistomatidae causes a disease called, paramphistomiasis which distributed all over the world (Sanabria and Romero, 2008) [27]. Low level of infection does not cause significant damage to the animals, but heavy infection with huge number of immature rumen fluke migrating through the intestinal tract cause acute parasitic gastroenteritis with high morbidity and death rates, especially in young animals (Azam *et al.*, 2020) [1]. Pathology caused by paramphistomes includes destruction and denudation of rumen papillae, hemorrhage, thickening as well as corrugation in duodenum (Mondal *et al.* 2002) [23]. Death rates in domesticated ruminants due to immature paramphistomes can be high (upto 80-90%) in some content (IIha *et al.* 2005; Khan *et al.* 2008) [14, 19].

Detection of paramphistomes is mainly based on faecal examination but early detection of rumen fluke is hard due to absence of egg output in faeces until the parasite becomes mature (Hafeez *et al.* 2006) [11]. Identification of rumen flukes is difficult due to their thick vigorous bodies resulting very difficult to observe the internal organs. The identification of paramphistomes is usually done on the basis of morphological and histological examinations but accurate detection of paramphistomes requires more precise methods like molecular approaches due to morphological similarities (Reinitz *et al.* 2007) [25]. The appropriate tool to identify digenetic trematode species and recover their phylogenetic relationships is PCR-based techniques like ITS-2 rDNA sequences (Van Van *et al.* 2009; Lotfy *et al.* 2010) [28, 21]. The mitochondrial genome is an important molecular marker which is used for species identification and phylogenetic analysis of rumen fluke (Cheng *et al.* 2016; Gasser *et al.* 2012) [4, 7]. There is no morphological and molecular research on identification and characterization of paramphistome in cattle of Bangladesh. This study was designed to contribute a morpho-molecular and phylogenetic framework for identification and characterization of rumen fluke in cattle of Bangladesh. This will be facilitate for the detection genetic polymorphism in different paramphistome and helpful for controlling of this parasite.

2. Materials and Methods

2.1 Specimens

Adult flukes (paramphistome) were collected from infected rumen, reticulum and bile duct of slaughtered cattle from different slaughter house of research area during January, 2021 to December, 2021. The sample size was 150 cattle and randomly 20 adult flukes were collected from each cattle. The flukes were transported to the parasitology laboratory of Hajee Mohammad Danesh Science and Technology University, Bangladesh and preserved in 70% ethanol.

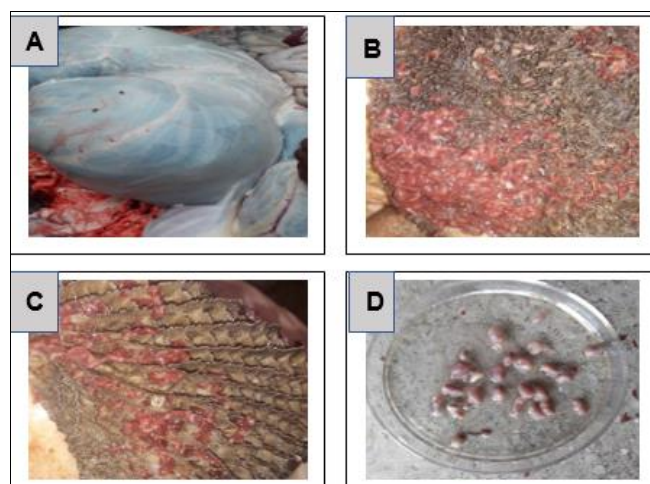


Fig 1: Examination of viscera; (A) Collection of viscera, (B) Examination of rumen and collection of adult fluke (C) Examination of reticulum (D) Adult fluke (Paramphistomes)

2.2 Morphological identification

The flukes were washed in normal saline extensively and identified the genus & species on the basis of morphological characteristics. The identification of rumen flukes were preliminary done by observing the flukes under microscope with low power objectives and then finally by following the standard key (Yamaguti, 1958) [31] after preparation of

permanent slides of fluke as described by Cable (1967) [3].

2.3 DNA extraction

Genomic DNA was extracted from 10 adult flukes of morphologically identified paramphistomes using the Biospin Tissue Genomic DNA Extraction Kit (BioFlux, Beijing, China) following the instructions of manufacturer. The obtained DNA was preserved at -20°C until use. The quality and quantity of genomic DNA was analyzed with a NanoDrop spectrophotometer.

2.4 PCR and Molecular analysis

The ITS2 region for *Paramphistomum* sp. was amplified using the primers Pg.ITS2.F

[5-GGTACCGGTGGATCACTCGGCTCGTG-3] and Pg.ITS2.R

[5-GGGATCCTGGTTAGTTTTCCTCCGC-3] (Yamaguti 1939) [31]. The reaction volume of PCR was 25 μL containing 12 μL of PCR Master Mix (2x), 1 μL of primer, 1 μL of DNA template & rest of the volume of PCR water. PCR cycles were performed on Eppendorf Master-cycler epigradient machine by following the methods described previously by Littlewood *et al.* (2006) [22] with slight modifications. The reaction condition was programmed as initial denaturation in 95°C for 2 min, denaturation in 94°C for 30s, elongation in 72°C for 1min, final elongation in 72°C for 8min and follow the 35 cycle of amplification with annealing temperature was 55.5°C . The PCR product of each amplicon was visualized in 1.5% (w/v) agarose gel, stained with ethidium bromide (Jia *et al.* 2010) [16] and molecular sizes of each rDNA amplicons were estimated with a standard DNA ladder (BP100) in GeneTools software (Syngene, USA). Favor Prep PCR Purification kit (FAVORGEN, Biotech Corp, Taiwan) were used to purify the PCR products. Unique bands isolated from each genus of paramphistomes for each primer were reamplified and purified using the FavorPrep GEL Purification kit (FAVORGEN, Biotech Corp, Taiwan) as per the manufacturer's instructions.

2.5 Sequencing, alignment and phylogenetic analysis

Big-Dye Terminator v3.1 Cycle Sequencing Kit was used to sequenced the purified PCR

products directly on the ABI PRISM 310 Genetic Analyzer (Invitrogen, USA) at a molecular biology unit of National Institute of Biotechnology (NIB, Dhaka, Bangladesh) according to manufacturer instructions. The obtained sequences were assembled using DNA BASER V2.6.6 and trimmed to eliminate any primer contamination. The purified sequence was subjected to BLAST analysis in NCBI to identify similar sequences deposited from various regions for the same gene fragment. The retrieved sequences from different genera of paramphistomes were downloaded and aligned using the BioEdit Sequence Alignment Editor v7.0.

Phylogenetic analysis was conducted using the DAMBE online database software (<http://dambe.bio.uottawa.ca/dambe.asp>) with corresponding ITS-2 + rDNA gene sequences of related paramphistomes available in GenBank (www.ncbi.nlm.nih.gov). The maximum likelihood method was used to construct a phylogenetic tree with 1,000 bootstrap replicates for branch support in MEGA X software. The phylogenetic relationship of paramphistomes was reconstructed based on ITS-2 gene sequences.

3. Results

3.1 Gross pathological findings of paramphistomiasis

Adult and young rumen flukes were attached with the walls of rumen and reticulum. A high burden of parasites was observed in the esophageal and/or reticular end of rumen. The gross pathological findings of mature rumen flukes were found mostly in the rumen and reticular fold of cattle causing denudation and destruction (about 30% to 40%) of rumen papillae and sloughing off reticular mucosa (Figure 2) whereas severe hemorrhagic enteritis mainly in the pyloric region of duodenum was caused by immature rumen fluke. Thickened, corrugated and congested mucosa was found in the upper part of the duodenum and mucosa was covered with blood stained mucus.

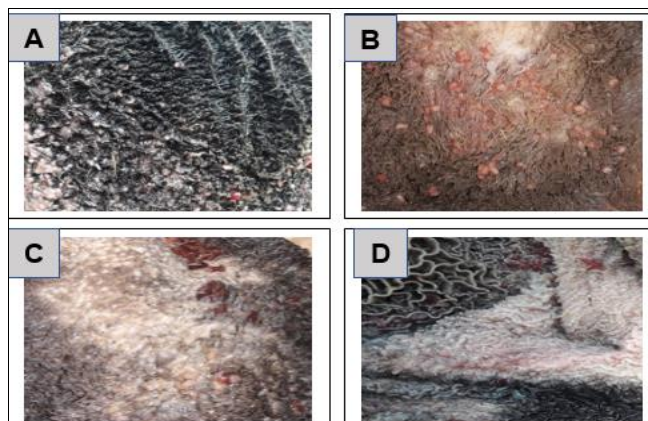


Fig 2: Pathological Changes in GIT - (A) Normal rumen papillae (B) Denudation of rumen papillae, (C) Destruction rumen papillae, (D) Sloughing off mucosa from reticular fold.

3.1 Morphological Identification of Paramphistomes

Paramphistomum sp. was identified based on its morphological features including shape of fluke and position of anterior and posterior sucker. The morphological features of *Paramphistomum* sp. found in the present study were light pink in color, pear-shaped body, slightly concave ventrally and convex dorsally, terminal and funnel-shaped mouth with a sucker and another sucker ventrally at the posterior end. Caeca were wide, pursued a serpentine course and reached anterior level of acetabulum with blind ends more dorsal than lateral. Genital pore was situated behind intestinal bifurcation without genital sucker. Clusters of vitelline glands were extended from the pharynx to the posterior sucker and lie between the caeca and the lateral margins of the body. The uterus was wavy and runs dorsally to testes and also pretesticular ovary.

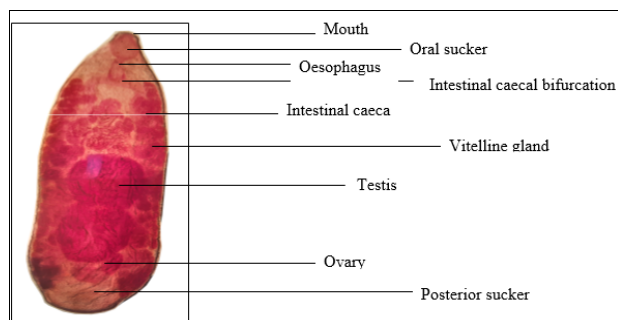


Fig 3: Morphology of *Paramphistomum* sp.

3.3 Molecular detection and characterization of Paramphistomes

The standardized protocol of PCR using family specific

Pg.ITS-2 primers amplified an approximately 520bp product for all paramphistomes under the family Paramphistomatidae (Figure 4). Successful amplification of target region of the ITS-2 gene by PCR confirmed the Paramphistomatidae family with 47% A+T and 53% G+C composition. The interspecific variations in ITS2 among the members of Paramphistomatidae family were 1.3-5.4%. The BLAST analysis of the ITS-2 rDNA sequence from the present study revealed a 98%-100% similarity with *Paramphistomum* spp. found in cattle from India (JX678271) and China (KJ459934, KJ459935, KJ459936, KJ459937, KJ459938); 98%-99% similarity with *Gastrothylax* spp. from cattle of India and neighboring countries (KU530204, MG183681, KY786332, JQ688413); 98%-99% similarity with *Fiscoederius* spp. from cattle of India and neighboring country (KU530204, MG183681, KY786332, JQ688413); 96%-97% similarity with members Paramphistomatidae from cattle of India and neighboring country (MT268130, MT268118, MT268116, MT268103). Based on 96-100% sequence similarity with different genus of Paramphistomatidae from Indian sub-continent, the collected isolate was considered as member of family Paramphistomatidae which inhibit in cattle of Bangladesh. The sequences were analyzed using the NCBI BLASTn tool for further confirmation and submitted to the GenBank via BankIt which registered as the accession number of OP751135.

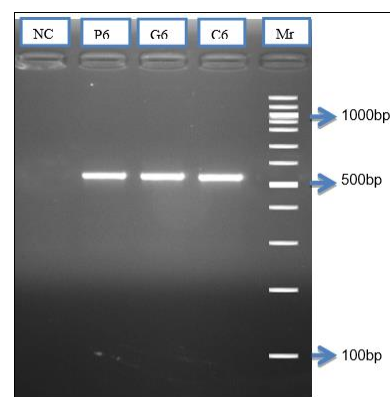


Fig 4: Amplified PCR Product of Paramphistomes specific ITS2 rDNA gene (Approx. 520bp), P6-G6-C6: PCR product from different genus, Mr: 100bp marker. NC: Negative Control. Primer: Pg. ITS2

>Paramphistomatidae.

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TGGGTACCGCGGGTATCACTCGG
CTCGTGTGCCGAGAAGGCGCAG
CCACTGTGTGATTAATGTGAACG
CATACTGCTTTGAACATCGACATC
TTGAACGCACATTCCGGCCATGG
GTTTTCTGTGGCCACGCCTGTC
CGAGGGTCGGCTTATAAACTATC
ACGACGCCCCAAAAGTCGTGGCT
TGGAATCTGCCAGCTGGCGTGAT
TTCCTCTGTGGTTTCGCCACGTGA
GGTGCCAGATCTATGGCGTTTTTC
CTAATGTCTCCGGACACAACCGC
GTCTTGCTGGTAGCGCAGACGAG
GGTGTGGCGGTAGAGTCGTGGCT
CAGTGAACGTAATGGTAGCAGC
CTCTGCTGTTGTGCCTTTGTTAGT
GTAACGTTTGAGATGCTATTGC
TGTTTCGTCCAATAATGACACCCA
CTGTGGTGTTCAATTACCTGACCT
CGGATCAGACGTGAAGACCCGCG
AACTAAGCATATCACTAAGCGGAA
AACTACCCAAGAATCCCCA
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Fig 5: ITS2 gene Sequence of family Paramphistomatidae

The phylogenetic relationship of the study sequence was analyzed in comparison with similar sequences of trematode species. The ITS2 sequences were input into the MEGA X software for phylogenetic tree construction using the unweighted pair group method with arithmetic mean, while evolutionary distances were analyzed using the Maximum Likelihood method and the Tamura-Nei model. Phylogenetic analysis of the generated sequence showed that, the tree contains two large clades with different pouched and unpouched paramphistomes where the study isolates (OP 751135) remain in upper clade (Figure 6). The study isolates

had the closest relationship to *Fischoederius elongatus* (GU133061) with high bootstrap value (65) and the clade was extended to *Paramphistomum* sp. (KY786334) and *Gastrothylax* sp. (KY786332) with bootstrap value of 41 and 83 respectively, which reveals their relationship in taxonomy. There was a species complex is formed with different members of family paramphistomatidae and various sub-clades in phylogenetic tree in which pouched and unpouched paramphistomes were segregate in separate sub-clade which reveals their genetic difference.

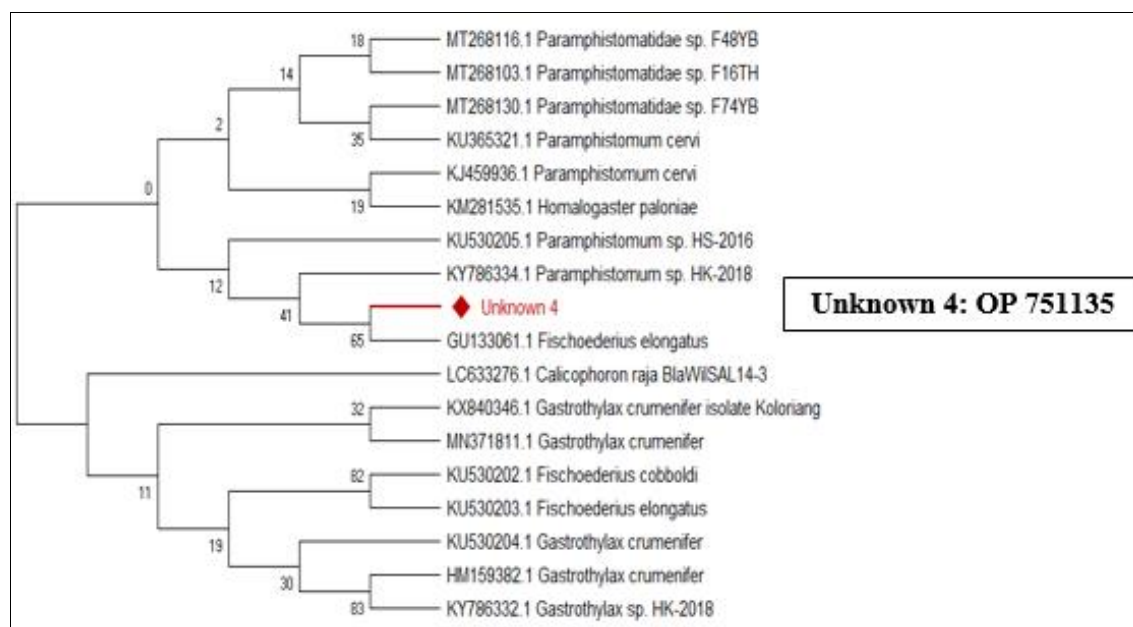


Fig 6: Phylogenetic tree of family Paramphistomatidae

4. Discussion

Gross pathological changes caused by mature flukes were primarily observed in the rumen and reticular fold. Immature flukes primarily cause severe hemorrhagic enteritis, predominantly affecting the pyloric region of the duodenum. At the sites where the worms attach, the rumen papillae exhibit severe denudation, leading to extensive tissue damage and sloughing off reticular mucosa (Figure 2). Approximately 30% to 40% of the rumen papillae were completely destroyed due to severe denudation. The present findings are in agreement with the previous report of Hossain and Baki (1987) [12] and Mondal *et al.* (2002) [23] who observed pathological changes associated with paramphistomiasis primarily in rumen, reticulum and duodenum including denudation and destruction of rumen papillae, hemorrhagic lesions, and mild thickening with mucosal corrugation in the duodenum. The fluke exhibits distinct morphological characteristics include color, shape and size of body, position of both sucker, presence of genital pore and vitelline glands were similar to the findings of Azam *et al.* (2020) [2], Choudhary *et al.* (2015) [5].

The complete gene sequence of ITS-2 from paramphistomes, was amplified and yielding a sequenced product of 520 bp (Figure 4). Hussain *et al.* (2014) conducted similar studies and reported the amplicon size of 500 bp of ITS-2+ region for various paramphistome species, including *P. epiclitum*, *G. crumenifer*, and *G. explanatum*. The study findings align with those of Ghatani *et al.* (2012) [10], who reported amplification of the ITS-2+ region in various paramphistome species (*F. cobboldi*, *F. elongatus*, *G. crumenifer*, *C. spatiosus*, and *V.*

tripurensis), yielding product sizes of 477 bp, 520 bp, 463 bp, and 482 bp. A similar study was performed by Yusuf *et al.* (2020) [32] in Turkey and obtained 399bp of ITS2 rDNA gene sequences in electrophoresis. This variation might be due to variation in primer sequence, possibility in deletion of a part of gene and geographic location of parasite (Goswami *et al.* 2009) [9]. The ITS-2 rDNA sequence analyzed in this study using BLAST revealed a 98%-100% similarity with *Paramphistomum* spp., 98%-99% similarity with *Gastrothylax* spp., 98%-99% similarity with *Fischoederius* spp. and 96%-97% similarity with other paramphistomes from cattle of India and neighboring country; this indicating the common phylogenetic history of that parasite. This observation is similar with previous study of Rinaldi L. *et al.* (2005) [26]. This similarity may be attributed to the proximity of these areas to Bangladesh, as they share similar geographical and environmental conditions, allowing the free movement of infective materials. The nucleotide content of the collected sample, based on complete ITS2 rDNA gene fragment was 47% A+T and 53% G+C composition. Earlier studies have amplified the full-length ITS2 rDNA gene of *Paramphistomum* spp., which includes 48.25% A+T and 51.75% C+G nucleotide content (Zheng *et al.* 2014) [30]. In phylogenetic analysis, the study isolate (OP 751135) formed two large clades with different pouched and unpouched paramphistomes where the isolates remain in upper clade (Figure 6). The study isolates (OP 751135) has the closest relationship to *Fischoederius elongatus* (GU133061) and the clade was extended to *Paramphistomum* sp. (KY786334) and *Gastrothylax* sp. (KY786332) with

bootstrap value of 41 and 83 respectively, which reveals their relationship in taxonomy because all are the members of paramphistomatidae. Similar reports were stated by Harpreet K *et al.* (2018) ^[13], Dube S *et al.* (2016) ^[6] and Laura C *et al.* (2016) ^[20]; Harpreet K *et al.* (2018) ^[13] reported that, *Gastrothylax* sp. (KU530204 and HM159382) formed two major clades with the members of paramphistomatidae where highest bootstrap value was 60. Dube S *et al.* (2016) ^[6] also showed that, *Carmyerius* sp. formed a species complex with various paramphistomes where bootstrap value was 58. The association between genotype of our study isolate and genotype from other countries were revealed similarity by the constructed tree and the studied flukes showed a close association with geographically linked countries. Considering the geographical proximity and similar topography of the two countries, this relationship is expected, which implies that their inhabitant species are not reproductively isolated (Worsham *et al.*, 2017) ^[29]. In most cases, the findings of this study compares with the findings of India and China because Bangladesh, India and China are geographically linked countries, environmental condition and livestock practice near about same; so, there is a chance to exhibit and shared evolutionary relationship within various inhabitant of thus countries.

This study validates the use of ITS2 rDNA as an effective molecular marker for genus and species identification of different paramphistomes and other digenetic trematodes. This statement is supported by Lotfy *et al.* (2010) ^[21], who stated that rDNA ITS2 sequence-based PCR diagnosis is a proven method for identifying digenetic species and determining their phylogenetic relationships. This statement is also supported by Itagaki *et al.* (2003) ^[15], Kumar V. (1999) ^[17] who reported that ITS2 region of rDNA is an important molecular marker that can be effectively used for phylogenetic studies and for identifying species and genera. Ghatani *et al.* (2014) ^[10], Van Van *et al.* (2009) ^[28] and Goswami *et al.* (2009) ^[9]; also stated that ITS2 rDNA have been used effectively to identify various trematode species.

5. Conclusion

In conclusion, this study characterized complete rDNA sequence of ITS2 ribosomal gene from different paramphistomes, which can be used to determine the affiliation within the family Paramphistomatidae. These data provided useful and innovative genetic markers that facilitate the study of both intra- and interspecific variation in different Paramphistomoida. The data are essential for the study of systematics and phylogenetics in different digenetic trematodes, including paramphistomes. The results of the present study may contribute to the development of a necessary framework for the identification and eventual control of this often overlooked cosmopolitan group of trematodes. The findings of this base line molecular research will be helpful for future applied research and to connect definitive host with intermediate host and also help to select specific anthelmintic; through which we can controlled of thus melody. This study demonstrates that ITS2 rDNA is an effective molecular marker for the identification of different paramphistomes and other digenetic trematodes. The sequence data obtained also provides a foundational resource for future epidemiological and molecular research on paramphistomes in Bangladesh and other regions.

6. Acknowledgments

The authors would like to thank the Department of Pathology

and Parasitology and Department of Microbiology, Hajee Mohammad Danesh Science and Technology University; Department of Veterinary and Animal Science, University of Rajshahi and National Institute of Biotechnology (NIB), Bangladesh, for supporting this research. The authors express their sincere gratitude to the National Science and Technology (NST) program, Ministry of Science and Technology, Bangladesh for their financial support.

Conflict of Interest

There is no conflict of interest

Author contributions

Moizur Rahman and Lovely Nahar conceptualized the study. Md. Golam Azam designed and performed all experiments and also wrote the draft of manuscript. Md. Golam Azam and Md. Salauddin designed and analyzed the sequence. Md. Golam Azam, Moizur Rahman, Md. Jamal Uddin and Md. Gausur Rahman responsible for writing, critical revisions and data analysis. Md. Salauddin and Md. Golam Azam managed the sequence submission to NCBI. All authors have reviewed and approved the final version of the manuscript for submission.

Financial Support

This research work was financially supported by National Science and Technology (NST) program, Ministry of Science and Technology, Bangladesh.

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How to Cite This Article

Azam MG, Rahman M, Nahar L, Salauddin M, Rahman MG, Uddin MJ. Morpho-molecular detection and characterization of rumen fluke in cattle at northern area of Bangladesh. *International Journal of Veterinary Sciences and Animal Husbandry.* 2025;10(4):92-97

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