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# First Report of Morphological and Molecular Identification of *Explanatum explanatum* from Domestic Water Buffalo, *Bubalus bubalis* from Meerut Region, India

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**Abstract:** Present communication deals with the morphological and molecular studies of *Explanatum explanatum* collected from the infected liver of abattoirs of domestic water buffalo (*Bubalus bubalis*). *Explanatum explanatum* is a digenetic gastrointestinal trematode majorly found in the bile duct and gall bladder of buffaloes in India .The parasite produces productivity loss and occasionally turns out as severe clinical disease leading to pocked in the bile duct mucosa, glandular hyperplasia and thickening of the blood vessels .This is the first report which analyzed the morphological and molecular studies on *Explanatum explanatum* from Meerut region, India. The 5.8S sequences of rDNA usually distinguish species clearly, including combinations for which morphology gives ambiguous results. This study also suggests that morphological characters along with molecular identification are essential for validation and identification of species and this will serve as a resource for comparative genomics and systematic studies of parasitic trematodes.

**Keywords:** *Explanatum explanatum*, *Bubalus bubalis*, Molecular identification, Fasciolidae, rDNA

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## Introduction

Livestock plays a key role in Indian economy constituting 16% of the income of the poor farmers and people living in rural areas. About 20.5 million people are dependent upon livestock for their livelihood and buffaloes have been of great economic importance as they are closely associated with the life activities of resource-poor rural farmer as it provides milk, meat, skin, manure and traction. Domesticated buffalo (*Bubalus bubalis*) usually graze in the open natural

external environment, and face regular natural calamities such as draught or flood and are very susceptible to many diseases. The environments in which water buffalo are kept are more prone to the water and mud snails, and these further act as intermediate hosts for a wide range of Fasciolidae, Paramphistomatidae, Dicrocoeliidae and Schistosomatidae trematode parasites infecting the liver, forestomach, intestinal tract and blood, causing disease and production loss. Immature and adult parasites of the Fasciolidae (Amor *et al.*, 2011; Shahzad *et al.*, 2012; Afshan *et al.*, 2014; Shoriki *et al.*, 2014) and Paramphistomatidae families (Hanna *et al.*, 1988; Khan *et al.*, 1990; Mazahery *et al.*, 1994; Ichikawa *et al.*, 2013) are common in the liver parenchyma and bile ducts of slaughtered buffalo throughout subtropical regions. Adult worms of *Explanatum* spp. infect the bile ducts, where they feed on blood across the luminal epithelium. Massive infection of intra-hepatic ductules and larger bile ducts lead to the formation of multifocal granulomatous nodules throughout the luminal surfaces with grossly visible fibrosis and thickening. Till date there are three validated species in the genus *Explanatum* which are considered to be present in buffalo throughout Asia: *Explanatum explanatum, Explanatum bathycotyle* and *Explanatum anisocotylea* (Eduardo, 1984; Mazahery *et al.*, 1994; Ichikawa *et al.*, 2013). These species are morphologically similar to rumen flukes of the genus *Gigantocotyle* (Rojo-Vazquez *et al.*, 2012), of which *Explanatum* was formerly considered to be a subgenus (Eduardo, 1984). *Explanatum explanatum* is a digenetic gastrointestinal trematode majorly found in the bile duct and gall bladder of buffaloes in India .The parasite produces productivity loss and occasionally turns out as severe clinical disease leading to pocked in the bile duct mucosa, glandular hyperplasia and thickening of the blood vessels. However, till date few attempts have been made to confirm the true species identity of Paramphistomatidae flukes by morphological examination, while molecular study methods are seldomly used. Molecular approaches are the most effective and accurate means for identification and taxonomic validation of the parasites.

In Meerut region, scanty studies have been done to study the species of Paramphistomatidae and transmission of these pathogens and forecasting systems for outbreaks of the trematode diseases. The present study describes, for the first time, the molecular confirmation of *E. explanatum* infection in domesticated water buffalo, *Bubalus bubalis* using rDNA ITS-2 marker, and further discuss the significance of its phylogenetic relationship and its sequence alignment results with worldwide submitted genome of *E. explanatum*.

## Materials and Methods

*Collection of Flukes*:

Infected livers were collected from domestic buffalo, slaughtered in city abattoirs, and transported to the laboratory on ice. The livers were then dissected to reveal flukes in the biliary ducts and 21 adult flukes were collected from it.

*Light Microscopy*:

For light microscopy, parasites were fixed in 70% ethanol for 24 h and then washed several times with water. For staining, Borax carmine was used. The prepared permanent mounts were then observed and studied under Trinocular Stereozoom microscope and Motic digital microscope.

*Molecular Genomic DNA isolation, PCR amplification and sequence analysis of rDNA ITS-2*:

Individual flukes recovered were washed repeatedly in phosphate buffered saline (PBS) and preserved in 70% ethanol at −80 °C. For DNA extraction, about 2 mg of tissue was removed from each fluke and rinsed for 5 min in $dH_2O$, twice. Tissue sections were lysed in lysis buffer (50 mM KCL, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.045% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin and $dH_2O$ in 50 ml) and Protinease K (10 mg/ml, New England BioLabs). Samples were lysed in 50 µl for 98 min at 60 °C, followed by 15 min at 94 °C, and then stored at −80 °C until required. A fragment of

the rDNA 5.8S region was amplified from individual adult fluke lysates using primer (fwd_seq: GTACCGGTGGATCACTCGGCTCGTG and rev_seq: GGGATCCTGGTTAGTTTCTTTTCCTCCGC). Thermo cycling conditions were 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s, with a final extension of 72 °C for 5 min. PCR products were cleaned using Omega BioTek Micro Elute Cycle Pure Kit (D6293-02) and the same amplification primers were used to sequence both DNA strands using an Applied Biosystems 3730Xl genetic analyzer. Amplified PCR products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and examined under ultraviolet light (Fig. 1). The amplified products were then purified with the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and sequenced with Big Dye Terminator vr. 3.1 cycle sequencing kit in ABI 3130 Genetic Analyzer, Applied Biosystems by using above-mentioned primers.

*Phylogenetic analysis*:

ITS gene sequence was used to carry out Basic Local Alignment Search Tool (BLAST) with database of NCBI GenBank database. Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between protein or nucleotide sequences. Based on maximum identity score, first ten sequences were selected and aligned.

## Results and Discussion

*Morphological studies*:

Morphological measurements of the fluke sample (Figs. 1 A, B) were done and for further taxonomic validity of the worm molecular and phylogenetic analysis were performed. Table 1 depicts the morphometric measurements of the trematode parasite.

*Confirmation of liver Paramphistomatidae fluke species identity by phylogenetic analysis of rDNA ITS-2 sequences*:

Sequence length of 539 bp was obtained after PCR. Gel electrophoresis pictures of the same were taken (Fig. 2). The obtained 5.8S rDNA partial sequence was then submitted to GenBank under the accession number MZ068119. Fragments of amplified DNA from *Explanatum explanatum* had 539 bp and were compared with sequences of other top ten *Explanatum* species obtained from GenBank. *Explanatum explanatum* under accession number AB743577 and LC592712 showed 100% similarity with the sequence obtained by the authors in the present study. Moreover, this parasite sequence showed 99.77% similarity with sequence of *E. explanatum* under accession number LC101683 and KF564869. The BLAST hit results has shown that our query sequences were much more similar to the sequences of various geographical isolates of *Explanatum* spp. These sequences were aligned with the 5.8S ITS-2rDNA genes and revealed clear differences in nucleotide sequences among *Explanatum* species (Fig. 3). The phylogenetic tree was constructed by doing BLAST with the sequences of GenBank by Neighbour Joining method (Fig. 3). Clustal Omega was also used to construct a phylogenetic tree for the same (Fig. 4). Upon construction of the tree of the submitted sequence of interest, it showed similarity with *E. explanatum* and formed a separate clade with other *Explanatum explanatum* sequences from different geographic regions. This confirmed the taxonomic validity of the worm. This study for the first time describes the molecular studies on *Explanatum explanatum* .We also recommend that the criteria to validate and identify species of *Explanatum* must be based on morphological characteristics, genetic identification and sequence comparison of genes having taxonomic importance.

## Conclusion

The present study describes the morphological and molecular studies of *Explanatum explanatum* for the first time from Meerut regions. Though the loss of livestock caused by the subgenus of Paramphistomadiae is very hazardous but still less attempts has been made from India on it. The sequence alignment of the 5.8S rDNA of *Explanatum explanatum* from different geographic

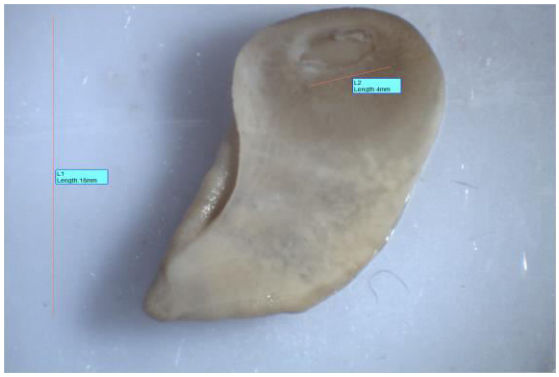

Fig. 1A: Horizontal and vertical measurements of the Fluke sample.

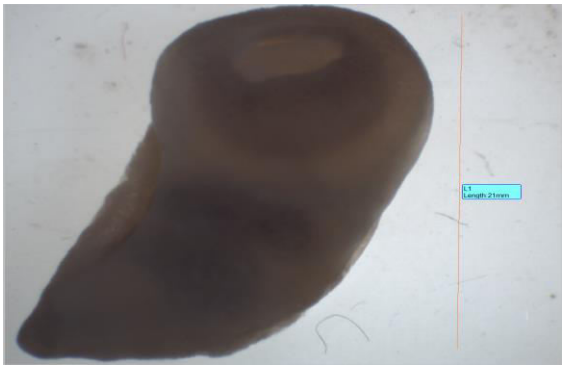

Fig. 1B: Morphometric picture of the fluke with prominent oral sucker.

Table 1: Morphometric measurements of *Explanatum explanatum*

| **Parameters (mm)** | ***Explanatum explanatum*** |
|---|---|
| Body length (mm) | 19.95 ± 1.95 (18.00 – 21.9) |
| Body width (mm) | 5.05 ± 0.75 (4.3 – 5.8) |
| Acetabulum (mm) | 4.1 ± 0.3 (3.8 – 4.4) |
| Anterior testes (mm) n=4 | |
| Width | 3.31 ± 0.46 (2.85 – 3.78) |
| Length | 1.8 ± 0.51 (1.29 – 2.31) |
| Ovary (mm) n = 2 | |
| Width | 1.16 ± 0.2 (0.96 – 1.37) |
| Length | 2.05 ± 0.2 (1.85- 2.25) |

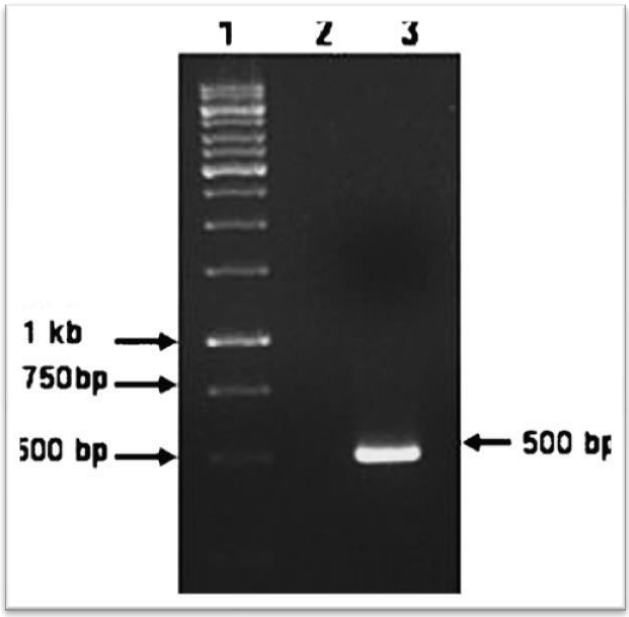


Fig. 2: Gel electrophoresis picture of genomic DNA.

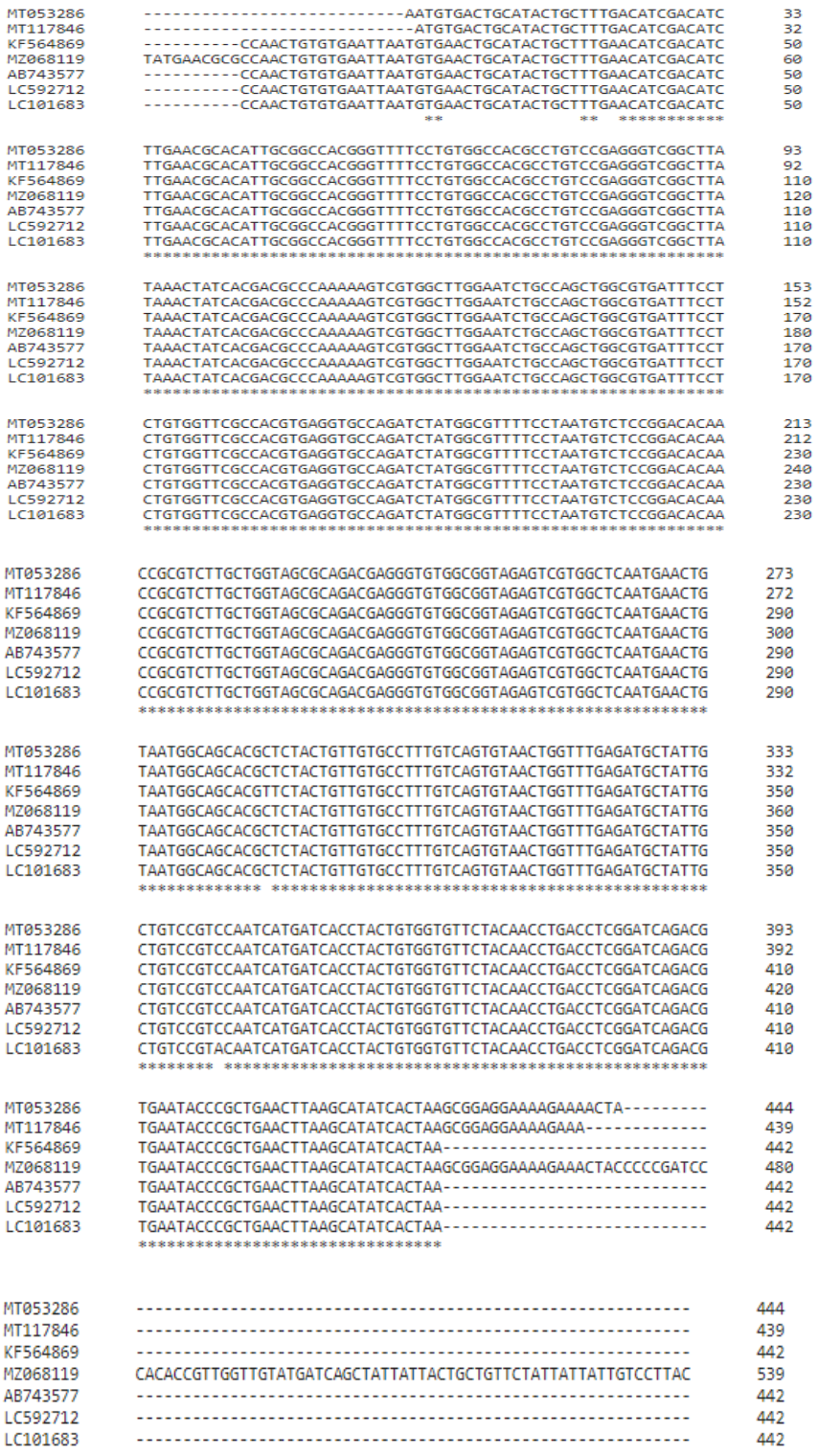


Fig. 3: Alignment of 5.8S sequences for comparative purposes of *Explanatum* spp. from different geographical locations shows nucleotide identical to *Explanatum explanatum*. Dots indicate identity with the first sequence and dashes are inferred insertion deletion events.

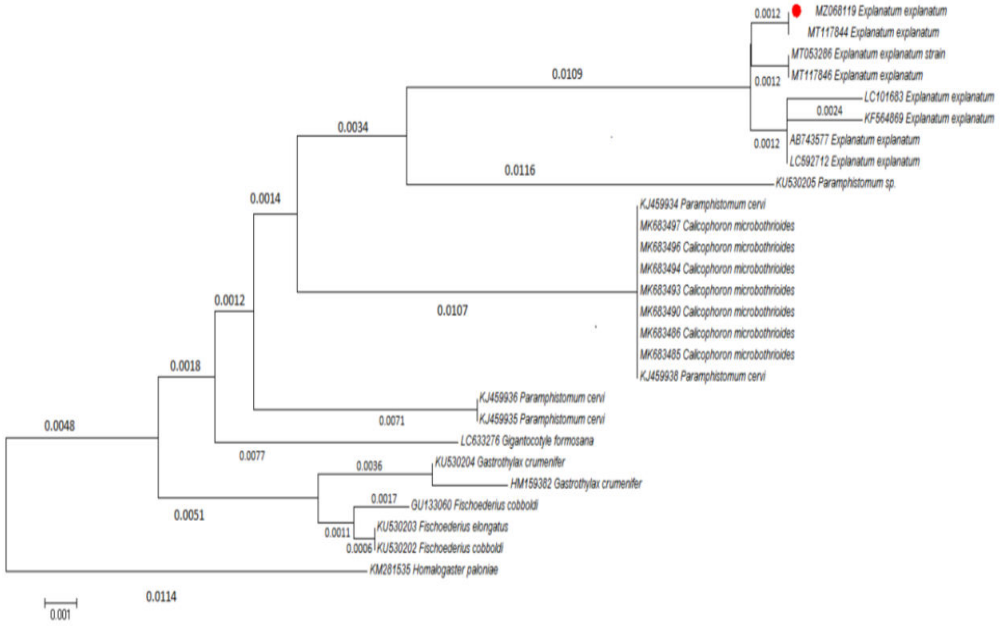


Fig. 4: Phylogenetic tree constructed by Neighbour- Joining method.

regions showed less divergence in the gene. The aim of this study was to study the rDNA gene of the worm so that further genomics study could be conducted that would throw light on its gene functionality. Moreover, the authors also believe that the molecular studies are the best way for identification and confirming the taxonomic validity of the worm.

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