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Research Article

ITS2 TARGETED MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF CAPRINE AMPHISTOMES FROM UTTAR PRADESH (NORTH INDIA)

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ABSTRACT

The present study aimed for molecular characterization and phylogenetic analysis of caprine amphistomes in the northern part of India. A total of 92 rumens from slaughtered goats naturally infected with amphistome species were collected. Based on the morphological characters, three major species of amphistomes *viz*. *Paramphistomum epiclitum, Gastrothylax crumenifer* and *Fischoederius* spp. were identified. Randomly selected amphistomes were pressed in between glass slides and examined microscopically and having similar morphology with identified amphistomes were subjected to molecular characterization by amplification of ITS2 DNA region. Genes of Mathura isolates were aligned with previously published sequences of amphistome species from different parts of globe. Multiple sequence alignment reports and phylogenetic analysis with previously published sequences throughout the globe suggested, the minimum evolutionary divergence between sequences of *P. epiclitum* Mathura isolates (KX657873; KX657875; KX657874) and Indian isolates (KF564870) without showing any difference of goat or buffalo origin. Amplified nucleotide sequence of *Fischoederius* spp. of Mathura isolate showed 86.5% phylogeny similarity with *G. crumenifer* India isolate of cattle. The *G. crumenifer* Mathura isolate showed 96.1% phylogeny similarity with *Calcicophoron microbothrium* of Egypt The percent identity matrix revealed the *Fischoederius* spp. of Mathura isolate showed 87.2% identity with *F. elongates* India isolate from *Bos indicus* (JQ688410). The *P. epiclitum* of present study showed 74.0% idedity with *P. epiclitum* India isolate from *Bubalus bubalis*. The Percent identity of *G. crumenifer* of present study showed 95.6% with *G. crumenifer* india isolate of *Bos indicus*.

Keywords: Goat amphistomes, ITS-2, Molecular characterization, Phylogenetic analysis

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Among the various parasite of goats, amphistomosis, caused by digenetic trematode (fluke) of the superfamily Paramphistomoidea is one of the least explored trematodal infectious diseases Yamaguti (1971) listed 62 species of paramphistomes of domestic ruminants from various countries. Paramphistomosis is caused by specific species of the parasite in a particular region (Anuracpreeda *et al.*, 2008) with different pathogenicity (Mehlhorn, 2008). Among them, most prevalent amphistomes in India are *P. epiclitum*, *G. crumenifer*, *Fischoederius elongatus*, *F. cobboldi* and *Gigantocotyle explanatum* with the prevalence ranging 50-70% (Prasad and Varma, 1999). In Indian subcontinent, immature paramphistomosis of domestic ruminant ranks next to fasciolosis and the mortality can reach up to 88% in sheep and goats (Agrawal, 2003). The conventional method for identification of these parasites is only based upon morphology. But it is stated that various genera of family Paramphistomatidae are difficult to identify on a morphological basis alone (Horak, 1971). Second internal transcribed spacer (ITS2) of ribosomal DNA sequence is a highly conserved region due to high degree of an inter-specific and low level of intra-specific genetic variations among populations from different hosts and geographical localities at generic and species level (Choudhary *et al.*, 2015). PCR-based techniques providing ITS2 sequences have proven to be a reliable tool to identify digenean species and their phylogenetic relationships (Mohanta *et al.*, 2017). The present study explores the molecular characterization and phylogenetic analysis of caprine amphistome of north India origin.

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MATERIALS AND METHODS

Collection, Processing, Preservation and morphological identification of parasites

The 92 rumen of goats (infected with amphistome species) were collected in clean polythene bags from a slaughter house in Mathura, Uttar Pradesh, India, and were transported with ice packs immediately to Department of Parasitology, College of Veterinary Science & Animal Husbandry, Mathura (Uttar Pradesh), India for further processing. The slaughter house at Mathura has a catchment of goats from various parts of northern India. The adult amphistomes are localized in the rumen and generally mono species of amphistome is localized in a specific part of rumen at a time. Amphistomes were

removed gently from each rumen with the help of forceps and transferred to a beaker containing Phosphate Buffered Saline (PBS) and washed several times with PBS solution to remove ruminal contents. Two cleaned parasites with similar in gross morphological characters from the localized area of rumen were selected. They were gently pressed in between glass slides to confirm the similarity in morphology. Among them one glass slide pressed parasite was stored at -20 ºC for DNA isolation and molecular characterization while rest one parasites were processed for morphological identification by staining procedure. The preservation and morphological identification of amphistomes were done as per protocol (Say, 1990).

Molecular characterization of amphistomes

Previously published primers for the partial sequence of ITS2 region of amphistomes were custom synthesized using nucleotide pattern from a highly conserved region (GenBank Accession Number JF834888). Details position of nucleotides, nucleotide sequences, and expected PCR products of the primer is mentioned in Table 1. Genomic DNA was extracted from four amphistomes using phenol-chloroform protocol (Sambrook and Russel, 2006) with minor modifications. The purity and concentration were estimated by Nanodrop (Eppendorf, Biophotometer, Germany). The optical density ratio 260/280 was observed for estimation of purity of double-stranded DNA. A concentration of DNA was measured by taking A260 value. After quantitative and qualitative analysis, the working concentration of isolated DNA was adjusted to 100ng/µl with nuclease-free water (NFW).

The PCR reaction was set up in 25 µl volume containing 12.5 µl PCR Master Mix Thermofisher Scientific (0.05/ µlTaq DNA polymerase in reaction buffer, 4 mM MgCl2, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µl of 10 pmol each primer (ITS 2 F/R), 2 µl of the DNA template and total volume was made up to 25µl using NFW. The thermocycling conditions (BioRad) used for amplification of the ITS 2 gene was initial denaturation at 95 ºC for 5 minutes, denaturation at 94 ºC at 5 minute, annealing at 54 ºC for 45 seconds, extension and final extension at 72 ºC for 1 and 10 minutes, respectively. The total of 35 cycles were used for denaturation, annealing and extension.

The amplified amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel (Invitrogen). After that PCR products were purified using the Gene Jet PCR purification kit (Thermo Fisher Scientific, US) as per the manufacturer's protocol.

Gene sequencing and Phylogenetic analysis

Purified PCR products of four parasite samples were sequenced by outsource sequencing (Thermo Fisher Scientific, US). Nucleotide sequencing was done in one side direction using the same set of primers that were used for DNA amplification. The sequences, hence obtained, were submitted into the NCBI, and corresponding accession numbers were obtained. Thereafter, the sequences were analyzed by online tool Mega 6. A multiple sequence alignment was generated with the Clustal W program within Mega 6 software (Tamura *et al.*, 2013). The phylogenetic relationship of amphistomes with other isolates of across the globe was commutated based on nucleotide sequences of the ITS using Mega 6 software. A phylogenetic tree for ITS2 was reconstructed using maximum likelihood. Haemonchus contortus and Fasciola gigantica was used as out-group species to root the tree. The phylogeny was analysed by Maximum Likelihood (ML) with bootstrap method using 1000 bootstrap replications. Model adopted for this analysis was Kimura 2-parameter with nucleotide substitution model. The rates among sites selected gamma distributed with 5 number of discrete gamma categories. Here was complete deletion of gaps/missing data treatment. The selected ML Heuristic Method was Nearest-Neighbor-Interchange (NNI) with strong Branch Swap Filter (Tamura *et al.*, 2013).

RESULTS AND DISCUSSION

The collected amphistomes from the rumen were identified as *Paramphistomum epiclitum*, *Gastrothylax crumenifer* and *Fischoederius* spp (Agrawal, 2003). Among 92 rumen samples, 88 times (95.6%) *Paramphistomum epiclitum* was identified, while *Gastrothylax crumenifer* and *Fischoederius* spp were identified in 3 (3.2%) and 1 (1.08%) rumen, respectively. The stored amphistomes at -20 ºC from these three groups were subjected to DNA isolation and molecular characterization using the partial sequence of the 28S rRNA, internal transcribed spacer 2 (ITS2) for confirmation of morphology and the amplicons of about 400 bp were obtained by thermocycling (Fig. 1). The sequence analysis of these amolicons revealed with *Paramphistomum epiclitum*, *Gastrothylax crumenifer* and *Fischoederius* spp.

A total of 04 representative amplicons (two from *Paramphistomum epiclitum*, one from each *Gastrothylax crumenifer* and *Fischoederius* spp.) were underwent BLAST to confirm these with morphological identification. Then after amplicons were submitted to the National Centre for Biotechnology Information (NCBI) and accession numbers were obtained (KX657873, KX657875, KX639720 and MN535799) Multiple sequence alignment report suggested that there was few nucleotide variations in Mathura isolate with other Indian isolates (JX678261;

Table 1. Primer sequence of ITS2 region of amphistome

Gene	Primer Sequence		Size of amplicon (bp)
ITS 2	5'GAATTAATGTGAACTGC ATACTGC3'	FORWARD	Approx 400 bp
	5'TGATATGCTTAAGTTCA GCGGG3'	REVERSE	

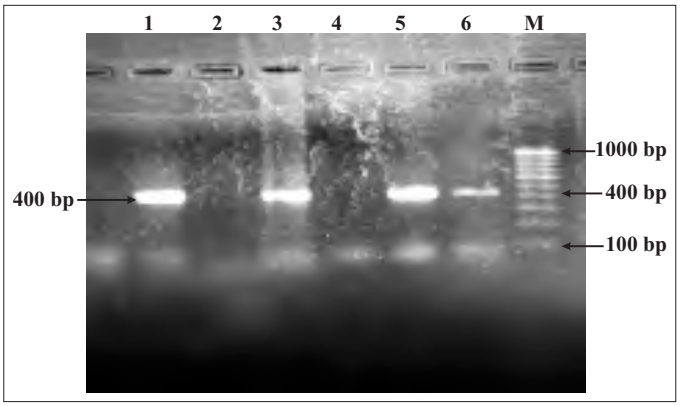


Fig. 1. PCR amplification of ITS 2 region of amphistomes (approx. 400bp product); Lane M: 100bp plus DNA ladder; Lane 1, 3, 5, 6: amplification of paramphistomes DNA

KF564870 and JQ688412) and higher nucleotide substitution with isolates outside from India (KP341659; KX274233 and AB743577). The constructed phylogenetic tree and identity matrix are given in Figs. 2 and 3, respectively.

The minimum evolutionary divergence between sequences were reported in *P. epiclitum* Mathura isolates (KX657873; KX657875; KX657874) and Indian isolates (KF564870) without showing any difference of goat or buffalo origin. The phylogenetic analysis revealed that *P. epiclitum* of Mathura isolates related in the same clad without showing any difference of goat or buffalo origin. The phylogenetic analysis and percent identity matrix of amplified nucleotide sequence of *Fischoederius* spp. of Mathura isolate showed 86.5% phylogeny similarity with *G. crumenifer* India isolate of cattle. The *G. crumenifer* Mathura isolate showed 96.1% phylogeny similarity with *Calcicophoron microbothrium* of Egypt. The percent identity matrix revealed the *Fischoederius* spp. of Mathura isolate showed 87.2% idedity with *F. elongates* India isolate from *Bos indicus* (JQ688410). The *P. epiclitum* of present study showed 74.0% idedity with *P. epiclitum* India isolate from *Bubalus bubalis*. The Percent identity of *G. crumenifer* of present study showed 95.6% with *G. crumenifer* india isolate of *Bos indicus*.

Primer based on ITS2 region of amphistomes is now considered as a reliable tool for identification of species of amphistomes. In the present study, ITS2 region was amplified from the templates of genomic DNA of all the

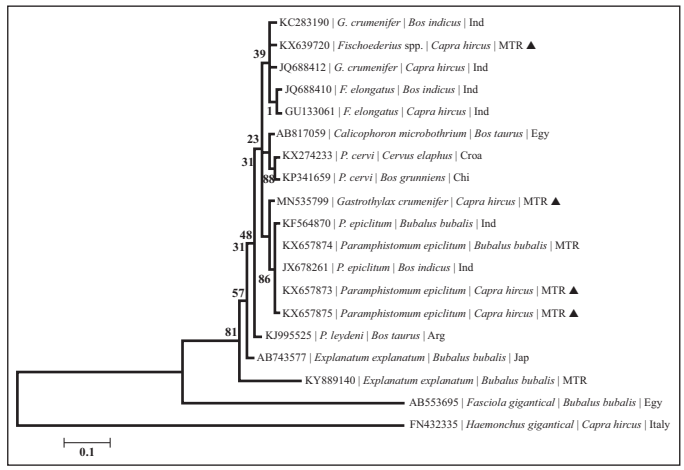
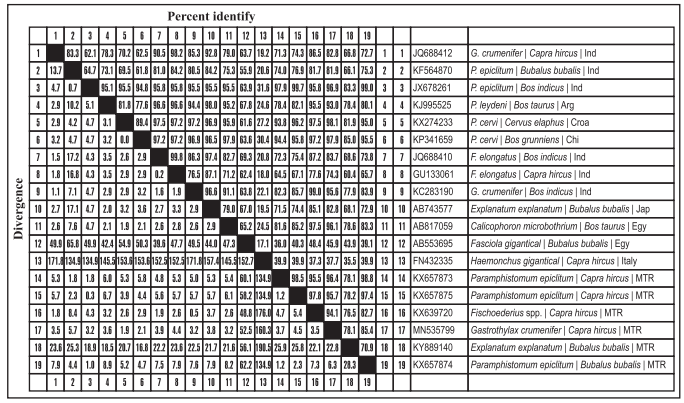


Fig. 2. Phylogenetic tree for selected species of the *P. epiclitum*, *G. crumenifer* and *Fischoederius* sp. of amphistomes inferred using the maximum parsimony method based on partial sequences of ITS 2. The percentage of replicate trees (bootstrap value) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The sequences generated in the present study are marked as triangle



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19			
1		83.3	82.1	78.3	78.2	82.5	90.5	88.2	85.3	92.8	78.0	83.7	18.2	71.3	74.3	86.5	82.8	86.8	72.7	1	1	JQ688412	*G. crumenifer* | *Capra hircus* | Ind
2	13.7		84.7	73.1	69.5	81.8	91.8	84.2	86.5	84.2	75.3	55.8	20.9	74.0	76.8	91.7	81.3	88.1	75.3	2	2	KF564870	*P. epicilitum* | *Bubalus bubalis* | Ind
3	4.7	0.7		95.1	95.3	94.8	95.8	95.8	95.5	95.5	95.5	63.8	31.8	97.9	99.7	95.8	96.9	63.2	99.8	3	3	JX678261	*P. epicilitum* | *Bos indicus* | Ind
4	2.9	18.2	5.1		81.8	77.6	86.6	86.6	94.4	98.0	95.2	67.8	24.8	78.4	82.1	95.5	93.0	78.4	98.1	4	4	KJ995525	*P. leydeni* | *Bos taurus* | Arg
5	2.9	4.2	4.7	3.1		88.4	97.5	97.2	97.2	96.9	95.9	61.6	27.2	93.8	96.2	97.5	98.1	81.9	95.0	5	5	KX274233	*P. cervi* | *Cervus elaphus* | Croa
6	3.2	4.7	4.7	3.2	0.0		97.2	97.2	96.9	96.5	97.9	63.6	30.4	94.4	95.8	97.2	97.9	65.0	95.5	6	6	KP341659	*P. cervi* | *Bos grunniens* | Chi
7	1.5	17.2	4.3	3.5	2.6	2.9		89.0	96.3	97.4	97.7	69.3	28.8	72.3	75.4	97.2	93.7	88.6	73.8	7	7	JQ688410	*F. elongatus* | *Bos indicus* | Ind
8	1.8	16.8	4.3	3.5	2.9	2.9	0.2		76.5	97.1	71.2	62.4	18.0	64.5	67.1	77.8	74.3	68.4	65.7	8	8	GU133061	*F. elongatus* | *Capra hircus* | Ind
9	1.1	7.1	4.7	2.8	2.9	3.2	1.8	1.8		96.6	91.1	63.8	22.1	92.3	85.7	98.0	95.9	77.8	93.9	9	9	KC283190	*G. crumenifer* | *Bos indicus* | Ind
10	2.7	17.1	4.7	2.8	3.2	3.8	2.7	3.3	2.9		78.0	67.8	19.5	71.5	74.4	95.1	82.3	68.1	72.9	10	10	AB743577	*Explanatum explanatum* | *Bubalus bubalis* | Jap
11	2.6	7.6	4.7	2.1	1.9	2.1	2.6	2.8	2.9	2.9		65.2	24.5	91.6	85.2	97.5	96.1	78.6	93.3	11	11	AB817059	*Calicophoron microbothrium* | *Bos taurus* | Egy
12	49.9	65.8	49.9	42.4	54.9	50.3	39.8	47.7	49.5	44.0	47.3		17.1	38.0	69.3	48.4	45.9	43.9	39.1	12	12	AB553695	*Fasciola gigantical* | *Bubalus bubalis* | Egy
13	171.8	134.9	134.9	145.5	153.0	153.8	152.5	152.5	171.8	157.4	145.3	152.7		38.9	39.8	37.3	37.7	35.5	39.8	13	13	FN432335	*Haemonchus gigantical* | *Capra hircus* | Italy
14	5.3	1.8	1.8	0.8	5.3	5.8	4.8	5.3	5.9	5.3	5.4	88.1	134.9		99.5	95.5	98.4	78.1	98.8	14	14	KX657873	*Paramphistomum epicilitum* | *Capra hircus* | MTR
15	5.7	2.3	0.3	0.7	3.9	4.4	5.8	5.7	5.7	5.7	6.1	56.2	134.9	1.2		97.8	95.7	78.2	97.4	15	15	KX657875	*Paramphistomum epicilitum* | *Capra hircus* | MTR
16	1.8	8.4	4.3	3.2	2.6	2.9	1.9	2.6	0.5	3.7	2.9	60.8	176.6	4.7	5.4		94.1	76.5	92.7	16	16	KX639720	*Fischoederius* spp. | *Capra hircus* | MTR
17	3.5	5.7	3.2	3.6	1.9	2.1	3.8	4.4	3.2	3.8	3.2	57.5	168.3	3.7	4.5	3.5		78.1	95.4	17	17	MN535799	*Gastrothylax crumenifer* | *Capra hircus* | MTR
18	23.8	25.3	18.9	18.5	29.7	18.8	22.2	23.8	22.5	21.7	21.8	56.1	198.5	25.9	25.8	22.1	22.9		76.9	18	18	KY889140	*Explanatum explanatum* | *Bubalus bubalis* | MTR
19	7.9	4.4	1.8	0.9	5.2	4.7	7.5	7.9	7.8	7.9	8.2	92.2	134.9	1.2	2.3	7.3	6.3	28.3		19	19	KX657874	*Paramphistomum epicilitum* | *Bubalus bubalis* | MTR
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19				

Fig. 3. Percent identity of Inter transcriber gene of *P. epiclitum*, *G. crumenifer* and *Fischoederius* sp. originate from different goat with other amphistome species originate from other species on the basis of nucleotide sequences estimated using DNASTAR (above diagonal)

amphistomes. It is suggested that primer based on ITS2 is a good marker for identification of species of amphistomes after using too BLAST It is also confirmed that 28S ribosomal DNA region is a useful molecular marker for species identification and Platyhelminthes phylogeny (Kumar *et al.*, 2014). Moreover, ITS2 has been described as a useful marker for species identification of amphistomes (Goswami *et al.*, 2009). The phylogenetic analysis also revealed that the Mathura isolates were closer to the published sequence of different isolates of amphistomes species of Indian origin without showing difference of goat and buffalo origin. The study also indicated the strain of amphistomes particularly *P. epiclitum* was not host species specific. Despite quite an expensiveness of molecular method, it could be helpful for identification of amphistomes with high accuracy as well as to know the phylogenetic relationship with other isolates. In

conclusion, the present study revealed that the amplification of ITS2 region of amphistomes genomic DNA is a reliable tool to know the phylogenetic relationship among *P. epiclitum*, *G. crumenifer* and *Fischoederius* sp. using tool BLAST The species of *P. epiclitum* of present study showed a close phylogenetic relationship in between and other Indian isolates without showing difference of goat or buffalo origin. The study indicated that the strain of amphistomes particularly *P. epiclitum* was not a host species specific (Sanguankiat *et al.*, 2016 and Jadhav *et al.*, 2018). Although further investigation is required with large number of samples.

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