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Differentiation of amphistome species of cattle in Kerala by polymerase chain reaction - restriction fragment length polymorphism#

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Abstract

The present study evaluated the PCR-RFLP of ITS-2+genes for species differentiation of three major amphistomes namely Gastrothylax crumenifer, Fischoederius cobboldi and Paramphistomum spp. Molecular analysis using PCR yielded amplicons of 515 bp for each species. The nucleotide differences among the sequences of the three species of amphistomes at different positions were further used for designing suitable RFLP. The amphistome parasites were distinguished taking into account the differences in the recognition sequences of TspRI on the ITS-2+ region by PCR-RFLP. The enzyme cleaved F.cobboldi at two recognition sites, CAGTG and CACTG and yielded 331, 93 and 91 bp products. Gastrothylax crumenifer had one recognition site CAGTG and resulted in 331 bp and 184 bp fragments. Since no recognition sequence was found in Paramphistomum spp., there was no cleavage. It is concluded that PCR-RFLP was a promising molecular tool for species identification of amphistomes.

Keywords: *Amphistomes, ITS -2+, PCR-RFLP, cattle*

Amphistomosis is a snail borne trematode disease of livestock widely distributed in the tropics and subtropics. Although the disease is diagnosed by coprological examination for parasite ova, identification of the causative species is difficult based on the morphological characters of the

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ova. Majority of the amphistome species are morphologically similar and the pathogenesis is caused by sexually immature flukes. Hence, the molecular techniques need to be employed in species identification for region-wise and season-wise epidemiological studies. The second internal transcribed spacer (ITS-2) region of the ribosomal DNA (rDNA) in amphistomes is of great value and is one of the frequently used regions for phylogenetic analysis at the genus and species levels (Itagaki *et al*., 2003). Hussain *et al.* (2014) analysed different genera of amphistomes based on rDNA ITS-2 + region; while Shameem *et al.* (2018) suggested that it could be a useful genetic marker for species identification based on nucleotide sequence analysis and *in silico* restriction map analysis. It was also opined by Ichikawa *et al*. (2013) that Polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) technique will provide a good tool for identification of bovine amphistomes for further epidemiological studies.

The present paper describes the amplification of ITS-2+ region of the three major amphistome species prevalent in Thrissur and their differentiation using PCR–RFLP.

Materials and methods

Collection and morphological identification of amphistomes

Amphistomes collected from naturally infected cattle slaughtered at local abattoirs of Thrissur were washed thoroughly in Phosphate Buffered Saline (PBS), pH 7.4, and separated as pouched and unpouched flukes. A few of these flues were processed and stained in carmine for morphological identification and the remaining were stored in 1X PBS at -20° C for molecular analysis.

Extraction of genomic DNA and assessment of integrity and purity of DNA

A single adult fluke from each species was used for the extraction of genomic DNA by QIAGEN blood and tissue kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions. Each isolated genomic DNA sample was stored at -20° C for PCR.

The integrity of the extracted DNA was assessed by electrophoresis in one per cent agarose gel in TBE buffer. Electrophoresis was performed at 70 V for 40 minutes and the gel was photographed in a Gel Documentation system (Bio-Rad, USA). The DNA concentration at 260 nm and purity at the ratio of 260/280 nm was determined using nanospectrophotometer (Nano drop 200C, Thermo Scientific, USA).

PCR analysis of the Internal Transcribed Spacer (ITS-2+)

Polymerase chain reaction was carried out in 25 µL of reaction mixture using DNA extracted from the three most predominant amphistomes namely, *Fischoederius cobboldi, Gastrothylax crumenifer* and *Paramphistomum spp*. using the forward and reverse primers consisting of 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′ and 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′ respectively, by targeting the ITS-2+ region of rDNA.

The PCR reaction mixture consisted of forward and reverse primers 1 µL each (concentration of forward and reverse primer was 25pmol); 12.5 µL master mix (Taq PCR smart mix with dye, Origin); 2 µL template DNA and 8.5 µL nuclease free water. The PCR conditions employed were as follows: initial denaturation at 94°C for 5 min, followed by 35 repeated cycles of 94°C for 1 min, 75°C for 30 sec, 72°C for 1 min and final extension at 72°C for 7 min.

The PCR product along with the 100 bp DNA ladder were resolved by 1.5% agarose gel electrophoresis in TBE buffer and the ethidium bromide stained gel was visualized in a gel documentation system.

The amplicons obtained consequent to PCR were column purified and sequenced using Sanger's dideoxy nucleotide chain termination method (AgriGenome Labs Private Limited, Cochin). Multiple sequence alignment of the ITS-2 rDNA sequences of the three amphistomes was carried out using CLUSTAL OMEGA online software (www.ebi.ac.uk/Tools/msa/clustalo).

In silico restriction enzyme mapping of the ITS-2+ rDNA sequences was performed to identify the restriction enzyme (RE) cutting sites using the online software NEB cutter (BioLabs) and suitable enzymes were designed.

Restriction fragment length polymorphism (RFLP)

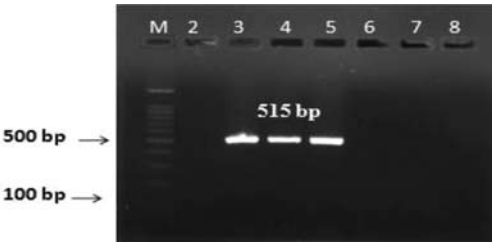
The RFLP was performed with 10 µL reaction volume comprising of PCR products of the three major amphistomes (3 µL), 10X buffer Tango (1 µL) and *TspRI* enzyme (0.5 µL, Thermo Scientific, USA) by spinning. The tubes were placed in a water bath (65°C) and then the thermal cycler was run at 65° C for 4 h. The RFLP product along with the 50 bp DNA ladder were resolved by 3% agarose gel electrophoresis in TBE buffer for visualisation of the RFLP pattern of each species.

Results and discussion

Based on the position and shape of genital suckers, oesophagus, intestinal caeca, testes and uterus, *Paramphistomum cervi, Gastrothylax crumenifer, Fischoederius elongatus, F. Cobboldi* and *Ceylonocotyle spp.* were identified and *G. crumenifer* was the most predominant species. According to Nath (1987) and Kandasamy and Devada (2011), *G. crumenifer* was the predominant species of amphistomes recorded from the local abattoirs of Kerala, while Shameem (2016) recorded *F.cobboldi* as the most prevalent amphistome species.

For molecular analysis, only the three major species of identified amphistomes *viz., F. cobboldi, G. crumenifer* and *Paramphistomum* spp. were studied. Polymerase chain reaction was performed using primers amplifying second internal transcribed spacer sequence (ITS-2) flanking 5.8S and 28S partial ribosomal gene (ITS-2+) at an annealing temperature of 75°C. The amplicon size of three amphistomes obtained after PCR with ITS-2+ primers was 515 bp (Fig.1).

Ghatani *et al.* (2012) amplified ITS-2+ region of *Carmyerius spatiosus, F. elongatus, Velasquezotrema trematripurensis* and *G. crumenifer,* and observed products of sizes 463



Lane M - 100 bp ladder

L2 - No Template Control
L3 - *Fischoederius cobboldi* (515 bp)
L4 - *Gastrothylax crumenifer* (515 bp)
L5 - *Paramphistomum* **spp. (515 bp)**

Fig. 1. PCR products from three different amphistomes using ITS-2+ primer

bp, 477 bp, 482 bp and 520 bp , respectively. Shameem *et al.* (2018) obtained 503 bp, 494 bp, 514 bp and 494 bp product sizes for ITS-2+ sequence of *F. elongatus, F. cobboldi, G. crumenifer* and *Paramphistomum* spp, respectively. Goswami *et al.* (2009), amplified ITS-1 and 2 regions of *Gastrodiscoides hominis* and observed 841 bp and 494 bp product sizes, respectively. Ferreras *et al*. (2014) also confirmed the use of ITS-2 for molecular characterisation of *Calicophoron daubneyi.* The ITS-2+ region was thus found particularly valuable and could be utilised for phylogenetic analysis at the generic and specific levels as reported by Itagaki *et al.* (2003).

Multiple sequence alignment (MSA) of the ITS-2 rDNA sequences of the three amphistomes under study was carried out using CLUSTAL OMEGA online software generated the nucleotide variations in various positions. Chromatograms were used for the identification of the nucleotide differences between the forward and reverse sequence of the various species of amphistomes (Fig. 2).

The CAGTG sequence was found in 327th position in both *F. cobboldi* and *G. crumenifer* and were cleaved by *TspRI*. But in *Paramphistomum* spp., it was CAATG sequence that was found at the 327th position. In *G. crumenifer* instead of the nucleotide T, C was seen at the 421st position and in *Paramphistomum* spp., instead of C, .the

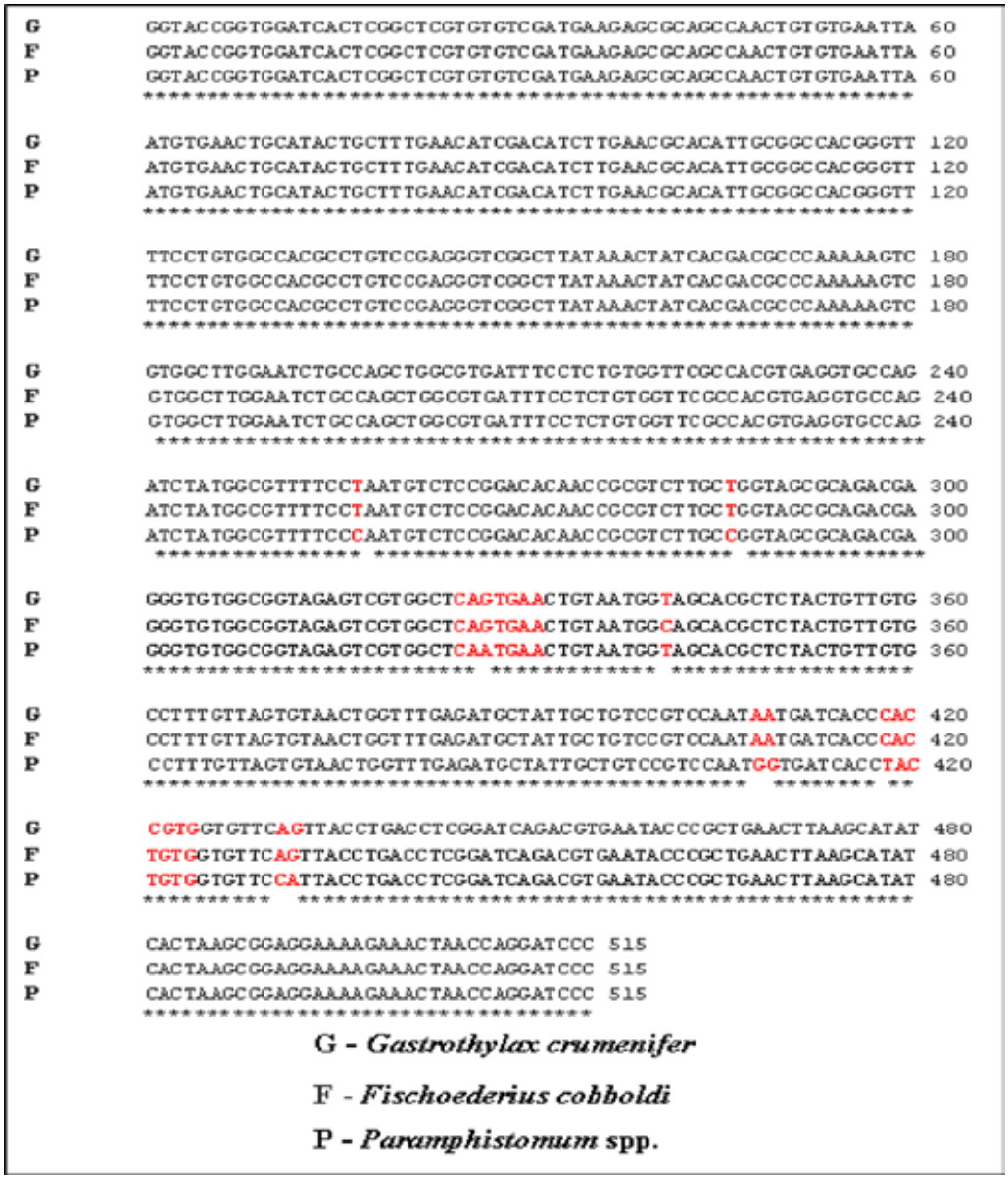


Fig. 2. Multiple sequence alignment (MSA) of ITS-2+rDNA

nucleotide T was observed at the 418th position. In *F. cobboldi,* the variations in nucleotide were seen at the 341st position, whereas in *G. crumenifer,* they were seen at the 421st position. At the same time, the variations were seen at the positions of 432, 431, 418, 409, 408, 327, 286 and 257 in *Paramphistomum* spp.

The above nucleotide differences among the sequences of the three species of amphistomes at different positions (Fig. 2) were used for designing the RFLP. It was reported by Ichikawa *et al.* (2013) that *Gigantocotyle explanatum* separated from *P. leydeni* at seven nucleotide sites, and that the study on the epidemiology of *Explanatum explanatum* was made easy with the use of these variations as molecular markers. Ghatani *et al.* (2012) also reported that difference of even one nucleotide in the ITS-2+ region could effectively be used as a genetic marker to distinguish the closely related trematode species.

In silico restriction enzyme

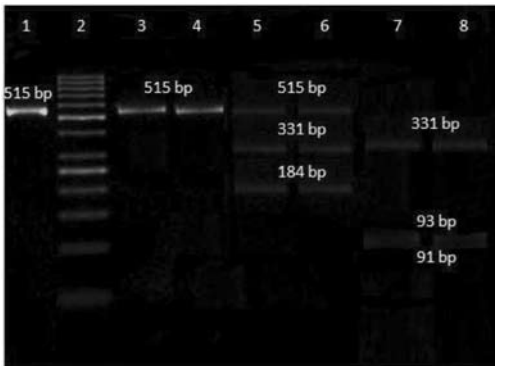
mapping of the ITS-2+ rDNA sequences of the amphistome spp., performed using the online software NEB cutter, revealed that the pouched amphistomes shared several restriction enzyme recognition sites for the enzyme, *TspRI.* Hence, RFLP with *TspRI* (*TscAI)* enzyme was designed for the fragmentation of the amphistomes to study the cleavage pattern and restriction sites in these species (Fig. 3). The 515 bp PCR product of *F. cobboldi* of ITS-2+ region had two recognition sequences CAGTG and CACTG for *TspRI* at positions 327 and 420 from the 5' end. The cleaving sites were 331 bp and 424 bp of 515 bp fragment. *TspRI* restriction digestion of 515 bp PCR product resulted in a unique three band pattern in agarose gel electrophoresis with 91 bp, 93 bp and 331 bp. The smaller fragments 93 and 91 had a small difference in the base pair length and were viewed as a single thick band in agarose gel.

The 515 bp PCR product of *G crumenifer* had only one restriction site CAGTG for the *TspRI* at position 327 from the 5' end. Since, the screened samples were heterozygous the recognition sequence was present only in one allele, which after restriction digestion resulted in 331 bp and 184 bp fragments. The other allele did not have the recognition sequence hence, it was uncut. Hence, *TspRI* restriction digestion of 515 bp PCR product resulted in a three band pattern in agarose gel electrophoresis with uncut 515 bp, 331 bp and 184 bp.

Paramphistomum spp. was found to be uncleaved with *TspRI*, as there were no recognition sequences for the enzyme in the amplicon. The sequence had Adenine (A) at 327th position, instead of S (G or C). As the recognition sequence of *TspRI* was CASTG (S = G or C), the 515 bp fragment had no cleavage site. Hence, it resulted in an uncut 515 bp fragment after restriction enzyme digestion in agarose gel electrophoresis.

The enzyme *TspRI* worked effectively at 65° C for 1 to 16 h in water bath, dry bath and thermal cycler and the results were good in water bath and thermal cycler. And for better visualisation, the digested products were electrophoresed for one hour 15 min in three per cent agarose gel

Lofty *et al.* (2010) also agreed that ITS-2+ was a good marker for the identification of species along with Ichikawa *et al.* (2013) who reported that ITS was a useful marker in the molecular characterisation of *G.explanatum.* This was in agreement with Shameem *et al*. (2018) who concluded that ITS-2+ region could be used for delineation of the different amphistome species.



L1 - PCR product (515bp)
L2 - 50 bp ladder
L3 & L4 - *Paramphistomum* spp. (515 bp)
L5 & L6 - *Gastrothylax crumenifer* (515 bp, 331 bp, 184 bp)
L7 & L8 - *Fischoederius cobboldi* **(331 bp, 93 bp, 91bp)**

Fig. 3. RFLP pattern of three species of amphistomes

Sibula *et al.* (2014) carried out the restriction enzyme digestion using *HaeIII* enzyme in *Calicophoron microbothrium* and *Calicophoron clavula* in an amplified rDNA restriction analysis. Identification of amphistomes such as, *Calicophoron. calicophorum, Orthocoelium streptocoelium* and *Haemalogaster paloniae* with *AccII* restriction enzymes on the ITS sequence by PCR-RFLP recorded restriction pattern at 250 bp, 125 bp and 105 bp for *C. calicophorum* and 250 bp and 230 bp for *O. streptocoelium. Homalogaster paloniae* was undigested using this enzyme.

Conclusion

The second internal transcribed spacer sequence flanking 5.8S and 28S partial ribosomal gene sequences (ITS-2+) region of

three species (*G. crumenifer*, *F. cobboldi* and *Paramphistomum* spp.) were amplified in the present study and revealed a 515 bp PCR product. Sequencing of the products from each species revealed ten single nucleotide variations. Digestion with *TspRI* resulted in unique banding pattern for each species. PCR-RFLP was found to be a good tool for the identification of the prevalent species of amphistomes. This forms the first study of differentiation of *F. cobboldi, G. crumenifer* and *Paramphistomum* spp. using *TspRI* restriction enzyme by PCR-RFLP in Kerala state.

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Conflict of interest

The authors declare that they have no conflict of interest.

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