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Genetic characterization of paramphistomes of buffalo by HAT-RAPD analysis

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Abstract: Paramphistomosis is widely prevalent in domestic farm animals, incurring heavy economic losses in several countries across the world. As the identification of these parasites based on morphological features is very difficult, molecular characterization is necessary to discriminate among different species. We performed polymorphic DNA fingerprint analysis of 3 different species of amphistomes collected from the rumen and bile ducts of buffaloes slaughtered at a local abattoir in Anand, Gujarat, India. The ruminal amphistomes were identified as *Paramphistomum cervi* and *Gastrothylax indicus*, while the hepatic amphistomes were identified as *Gigantocotyle bathycotyle*. High annealing temperature-random amplified polymorphic DNA (HAT-RAPD) analysis with 4 primers, OPA2, OPA4, OPA8, and OPA9, identified species-specific banding patterns for each species. The unique bands specific for species were sequenced, resulting in identification of 15 sequences, which included 6 sequences for *G. bathycotyle*, 3 sequences for *G. indicus*, and 6 sequences for *P. cervi*. The dendrogram analysis of RAPD bands revealed genetic distance of 0.5798 between *G. bathycotyle* and G. *indicus*, compared to a distance of 1.0986 and 0.9163 between *G. bathycotyle* and *P. cervi* and between *G. indicus* and *P. cervi*, respectively. The primers derived from sequenced RAPD amplicons generated one species-specific sequence characterized amplified region marker for the identification of *G. indicus.*

Key words: Paramphistomes, DNA finger prints, dendrogram, random amplified polymorphic DNA, sequence characterized amplified region, buffalo

1. Introduction

Paramphistomosis of livestock is regarded as a disease of great economic importance. Paramphistomosis is widely prevalent in domestic ruminants in India and several other countries, resulting in heavy losses in terms of mortality, morbidity, and reduced wool, meat, and milk production (1,2). Approximately 40 species of amphistomes have been reported (3), but the predominant species are *Paramphistomum cervi*, *Gigantocotyle explanatum*, *Gastrothylax crumenifer*, and *Fischoederius elongatus*. Horak (4) stated that various genera of family Paramphistomatidae are difficult to identify on a morphological basis only. However, Nasmark (5) discussed an alternative to overcome this difficulty by histological examination of median sagittal sections.

The random amplification of polymorphic DNA (RAPD) is relatively simple way of creating genomic DNA 'fingerprints' and can be used successfully to identify various parasites (6). RAPD markers can be useful for the identification and differentiation of species and strains of a range of parasite groups including protozoa and helminths. Furthermore, high annealing temperature (HAT)-RAPD has been shown to increase the reproducibility of the technique and is used successfully for genetic characterization of numerous parasites, including paramphistomes (7–9). Studies on detection of genetic polymorphism in different paramphistome species in buffalo have not been reported from India. Hence, the present study was undertaken to differentiate amphistomes from rumen and bile ducts by using 4 different random primers.

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2. Materials and methods

2.1. Collection of amphistomes

Adult amphistomes from the rumen and bile duct were collected from naturally infected buffaloes sacrificed at a local abattoir in Anand, Gujarat, India. The flukes from the rumen were identified as *Gastrothylax indicus* and *P. cervi*, whereas *Gigantocotyle bathycotyle* was identified from bile ducts. The flukes were preserved in 70% alcohol for DNA extraction and stored at –20 °C.

2.2. DNA extraction

Genomic DNA extraction from adult amphistomes was prepared according to Yu et al. (10), with slight modifications. Two species of adult amphistomes from rumen (G. *indicus* and *P. cervi*) and 1 species from bile ducts (*G. bathycotyle*) were pulverized by mortar and pestle in the presence of liquid nitrogen. Grinding was continued with addition of lysis buffer containing Tris-HCL (10 mM, pH 8.0), EDTA (100 mM), and SDS (5%). The pulverized tissue was placed into a 1.5-mL tubes, incubated with proteinase K (100 µg/mL) overnight at 37 °C, and subsequently purified by phenol chloroform extraction and ethanol precipitation as per standard protocols. The quality and quantity of genomic DNA was analyzed with a NanoDrop spectrophotometer and agarose gel electrophoresis.

2.3. HAT-RAPD analysis

Four arbitrary primers (10 nucleotides), OPA2, OPA4, OPA8, and OPA9, were used to examine the DNA banding from different species of adult flukes. The sequences and GC content of the 4 primers are listed in Table 1. HAT-RAPD polymerase chain reaction (PCR) was carried out as per the method described by Sripalwit et al. (7). Amplification was carried out in 20 µL of reaction volume, which contained 200 µM dNTPs, 200 nM primer, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, and 60 ng of DNA template. Amplification was carried out in a thermal cycler (Veriti, 96-well thermal cycler, Applied Biosystems) with initial denaturation at 94 °C for 5 min, followed by 40 cycles each with denaturation at 94 °C for 0.45 min, annealing at 42 °C for 2 min, and extension at 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. The PCR product was electrophoresed using 2.0% w/v agarose gels at 80 V and visualized by UV transilluminator. Molecular sizes of the distinct bands were estimated by their mobility with that of a standard molecular size marker in GeneTools software (Syngene, USA).

2.4. RAPD data analysis

Data were scored for computer analysis on the basis of the presence or absence of the PCR products. The polymorphism percentage was calculated as per the following formula (11):

Polymorphism (%) = (total number of bands – number of monomorphic bands) / total number of bands × 100.

A dendrogram was drawn based on Nei's (12) genetic distances using UPGMA. This program is an adoption of the program NEIGHBOR of PHYLIP version 3.5c by Felsenstein (13). The drawing was executed for multiple populations.

2.5. Sequencing

Unique bands isolated from each species of amphistomes, indicated by arrows in Figure 1, from each primer were reamplified and purified using the QIAquick Gel Extraction Kit (QIAGEN, USA) as per the manufacturer's instructions. The purified products of RAPD bands unique for each species comprising a total of 31 bands were cloned using the QIAGEN PCR Cloning Kit (QIAGEN) as per the manufacturer's instructions and were sequenced by vector-specific M13 primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, USA) following the manufacturer's instructions on the ABI PRISM 310 Genetic Analyzer. Raw data also contained some DNA sequence of the pTZ57R/T vector and so the vector DNA sequence was removed by the VecScreen program available from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). After vector screening, a sequence similarity search with the DNA public database available from GenBank, maintained by the NCBI, was carried out with the help of BLAST (http://www.ncbi.nlm.nih.gov/blast/). Finally, sequence submission to the NCBI database was done using the BankIt online sequence submission tool in GSS format.

2.6. SCAR marker development

Sequence characterized amplified region (SCAR) marker analysis was performed by designing primers from RAPD derived sequences. Two pairs of primers amphi1 (F: AGCCGAGCTGAATGAGAAACA; R: AACCCCACCGAACATATACAC) and primer pair amphi2 (F: ACTTCGCAAGCGGAAACAAC; R: AGTGACGTAGGAAGGCATCTC) amplifying sequences of *G. indicus* were used for PCR analysis. The DNA was isolated from cattle, buffalo, sheep, goat, rumen microflora, *G. bathycotyle*, *G. indicus*, and *P. cervi* following standard protocols and used for PCR analysis by SCAR primers. The PCR amplification was performed as described earlier with an annealing temperature of 53 °C and 55 °C for the amphi1 and amphi2 primers, respectively, and analyzed by agarose gel electrophoresis.

3. Results

3.1. RAPD results

Four arbitrary oligonucleotide primers, OPA2, OPA4, OPA8, and OPA9, were used for RAPD-PCR to generate polymorphic DNA fingerprints in 3 species of amphistomes, *G. bathycotyle*, *G. indicus*, and *P. cervi.* Information regarding monomorphic, polymorphic, and unique bands and percentage of polymorphism generated by all 4 primers is given in Table 1.

Table 1. Details of monomorphic, polymorphic, and unique bands as well as percentage of polymorphism generated by 4 RAPD primers.

Sequence of oligo 5'-3'	Primer	% GC	Range of fragment size (bp)	Total no. of bands	Polymorphic bands	Monomorphic bands	Unique bands	% of polymorphism
TGCCGAGCTG	OPA2	70.00	270–1500	16	4	0	12	100
AATCGGGCTG	OPA4	60.00	277–1024	13	1	1	11	92.3
GTGACGTAGG	OPA8	60.00	154–1079	14	5	1	8	92.8
GGGTAACGCC	OPA9	70.00	265–1526	24	6	1	17	95.8

The primers OPA2, OPA4, OPA8, and OPA9 produced amplicons of 270–1500 bp, 277–1024 bp, 154–1079 bp and 265–1526 bp, respectively, from 3 different species of amphistomes (Figures 1A–1D). The unique bands for each species (indicated by arrows) were eluted from the gel and subsequently used for cloning and sequencing.

3.2. Dendrogram analysis

The RAPD data generated with all primers from 3 species of amphistomes were pooled to construct dendrogram. The distance between *G. bathycotyle* and *G. indicus* was less (0.5798) when compared to the distances between *G. bathycotyle* and *P. cervi* (1.0986) and *G. indicus* and *P. cervi* (0.9163) (Figure 2).

3.3. Submission of sequences

The unique bands specific for species were purified and sequenced. A total of 15 sequences were obtained from all the species, which included 6 sequences for *G. bathycotyle*, 3 sequences for *G. indicus*, and 6 sequences for *P. cervi*. The sequences obtained were submitted to GenBank. Sequence length of different species of amphistomes obtained with different RAPD primers along with their accession numbers (HM148437–HM148451) are summarized in Table 2.

3.4. Marker specificity

Two new primer pairs were designed from RAPD-derived sequences. They were amphi1 (278 bp) and amphi2 (175 bp) for *G. indicus*. The amphi1 primer designed from an OPA2-derived sequence worked as a species-specific marker against *G. indicus* (Figure 3A). There was no amplification with the amphi1 primer when DNA from cattle, buffalo, sheep, goat, and rumen microflora was used in PCR (Figure 3B). There was also no amplification when the DNA from *G. bathycotyle* and *P. cervi* was used in PCR. The results of the present study show that primer amphi1 can be used as a species-specific marker for *G. indicus*. The other primer, amphi2, did not produce any amplification even though a species-specific template was used for PCR.

4. Discussion

Morphological differences found in stained and mounted adult specimens were commonly used to differentiate between platyhelminths (14). RAPD has been widely used as a genetic screening method (15) because it is rapid, is relatively simple to perform, and requires only a small amount of genomic DNA without genome sequence information prior to analysis (6).

Low annealing temperatures of 25–30 °C have been generally used in RAPD-PCR; however, in the present study, the increased annealing temperature of 40 °C resulted in good banding patterns to differentiate *G. bathycotyle*, *G. indicus*, and *P. cervi*. HAT-RAPD was carried out using 4 primers of the OPA series (OPA2, OPA4, OPA8, and OPA9) to differentiate *P. cervi*, *G. indicus*, and *G. bathycotyle*. Annealing at 42 °C for 2 min of 40 cycles resulted in good amplification and polymorphism among all 3 species. This holds true even with separate components instead of using master mix for the PCR. Our results are in accordance with those of Anuntalabhochai et al. (16), who reported that a high annealing temperature of 46 °C gave greater polymorphism, reproducibility, and resolution in RAPD. They concluded that RAPD is a useful procedure to differentiate between closely related and morphologically indistinct species.

Sripalwit et al. (7) performed HAT-RAPD using 5 random 10-mer oligonucleotide primers, OPA2, OPA4, OPB18, OPC9, and OPH11, to differentiate 3 species of amphistomes, *Paramphistomum epiclitum*, *Orthocoelium streptocoelium*, and *Fischoederius elongatus*. Primer OPA2 generated different species-specific markers of 350 and 400 bp for *P. epiclitum*; 135, 240, and 435 bp for *O. streptocoelium*; and 760 bp for *F. elongatus*, whereas primer OPA4 produced fragments of 435 and 530 bp for *P. epiclitum* and 180, 500, and 600 bp for *O. streptocoelium*. The primer OPB18 resulted in fragments of approximately 710, 880, 980, and 1300 bp for *P. epiclitum*; 220, 540, and 1450 bp for *O. streptocoelium*; and 670 and 1159 bp for *F. elongatus*. Primer OPC9 generated species-specific bands of 790, 890, and 2000 bp for *P. epiclitum* and 670 and 950 bp

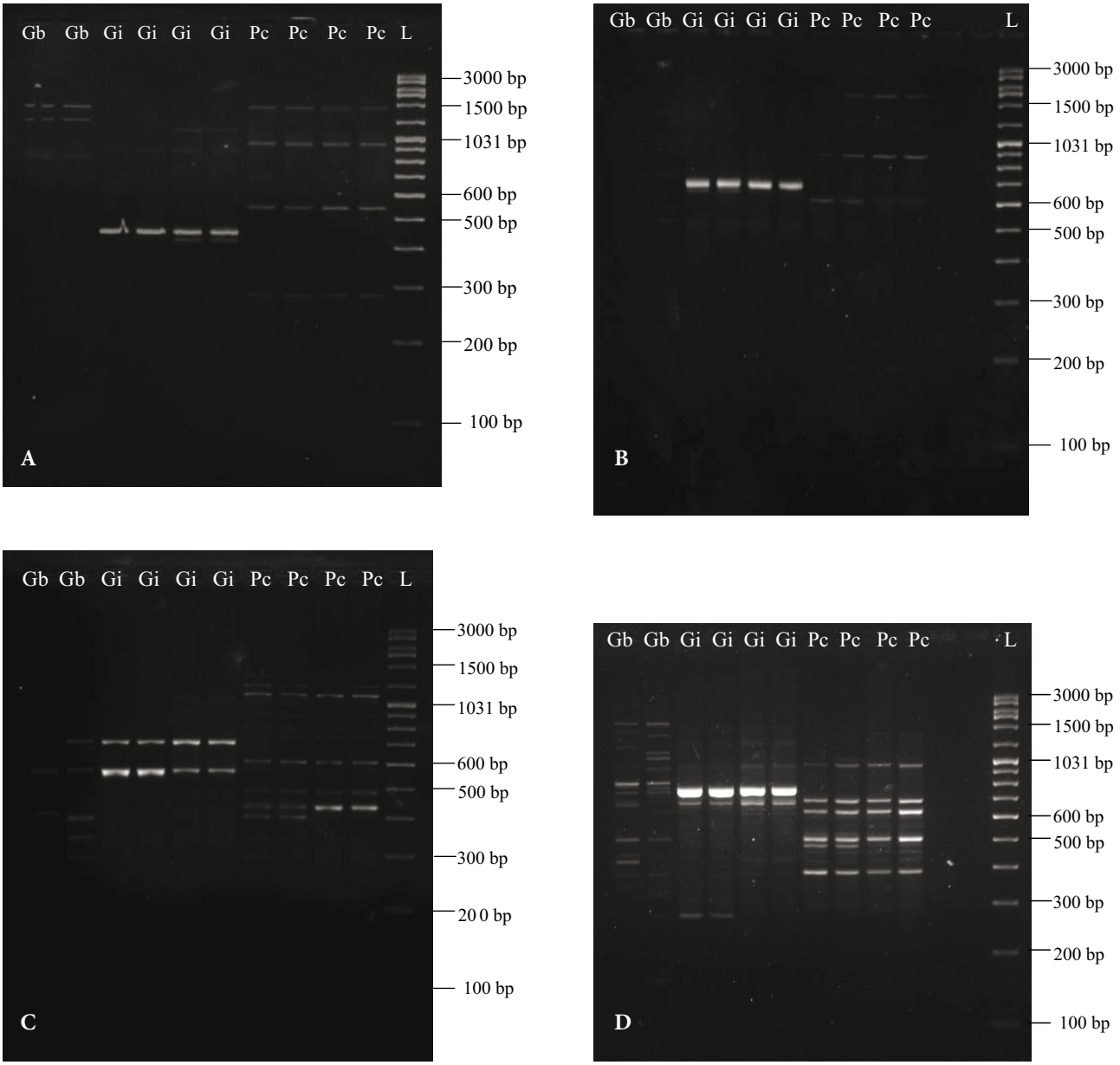

Figure 1. Agarose gel electrophoresis analysis of RAPD bands from different species of adult amphistomes amplified with random primers. **A)** RAPD bands from different species of adult amphistomes amplified with OPA2 primer. Gb: *Gigantocotyle bathycotyle*, Gi: *Gastrothylax indicus*, Pc: *Paramphistomum cervi*, L: GeneRuler 100 bp plus DNA ladder (Fermentas). **B)** RAPD bands from different species of adult amphistomes amplified with OPA4 primer. Gb: *Gigantocotyle bathycotyle*, Gi: *Gastrothylax indicus*, Pc: *Paramphistomum cervi*, L: GeneRuler 100 bp plus DNA ladder (Fermentas). **C)** RAPD bands from different species of adult amphistomes amplified with OPA8 primer. Gb: *Gigantocotyle bathycotyle*, Gi: *Gastrothylax indicus*, Pc: *Paramphistomum cervi*, L: GeneRuler 100 bp plus DNA ladder (Fermentas). **D)** RAPD bands from different species of adult amphistomes amplified with OPA9 primer. Gb: *Gigantocotyle bathycotyle*, Gi: *Gastrothylax indicus*, Pc: *Paramphistomum cervi*, L: GeneRuler 100 bp plus DNA ladder (Fermentas).

for *F. elongates*. Primer OPH11 produced species-specific markers of 560, 640, 805, and 930 bp for *O. streptocoelium* and of 260, 435, and 590 bp for *F. elongatus*, whereas primer OPA5 did not produce distinguishable bands for analysis. It is not possible to compare our results as the primer and species of amphistomes studied by the above workers were different. Banding patterns might differ depending upon the primer, reaction conditions, and species of parasite.

Out of 4 primers used to carry out RAPD for adult specimens, OPA8 placed hepatic amphistomes (*G.*

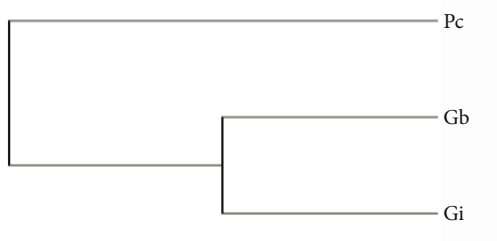

Figure 2. Dendrogram of adult amphistomes from pooled data from different primers. Pc: *Paramphistomum cervi*, Gb: *Gigantocotyle bathycotyle*, Gi: *Gastrothylax indicus*.

Table 2. Sequence length of different species of adult amphistomes from different primers.

Species	Primers	No. of sequences	Sequence length	Accession number	BLAST search
Gigantocotyle bathycotyle	OPA2	1	696 bp	HN148447	No significant homology with the sequences available in the NCBI database
	OPA4	-	-		
	OPA8	2	349 bp	HN148450	
			367 bp	HN148451	
	OPA9	3	204 bp	HN148446	
			413 bp	HN148448	
			388 bp	HN148449	
Gastrothylax crumenifer	OPA2	1	458 bp	HN148437	
	OPA4	2	765 bp	HN148438	
			458 bp	HN148439	
	OPA8				
	OPA9				
Paramphistomum cervi	OPA2				
	OPA4				
	OPA8	4	190 bp	HN148440	
			162 bp	HN148441	
			193 bp	HN148442	
			163 bp	HN148443	
	OPA9	2	416 bp	HN148444 HN148445	
			383 bp	HN148445	

bathycotyle) into one cluster and ruminal amphistomes (*P. cervi* and *G. indicus*) into another cluster, whereas OPA9 classified them into pouched amphistomes (*G. indicus*) and unpouched amphistomes (*G. bathycotyle* and *P. cervi*). Hence, OPA8 can be used to differentiate between hepatic and ruminal amphistomes, whereas OPA9 can be used to differentiate pouched and unpouched amphistomes. While constructing a phylogenetic tree from HAT-RAPD data, Sripalwit et al. (7) grouped *P. epiclitum* and *O. streptocoelium* (unpouched amphistomes) together and placed F. *elongatus* (pouched) into another group. The similarity index between *P. epiclitum* and *O. streptocoelium* was 0.2286, between *P. epiclitum* and *F. elongatus* was 0.0645, and between *O. streptocoelium* and *F. elongatus*

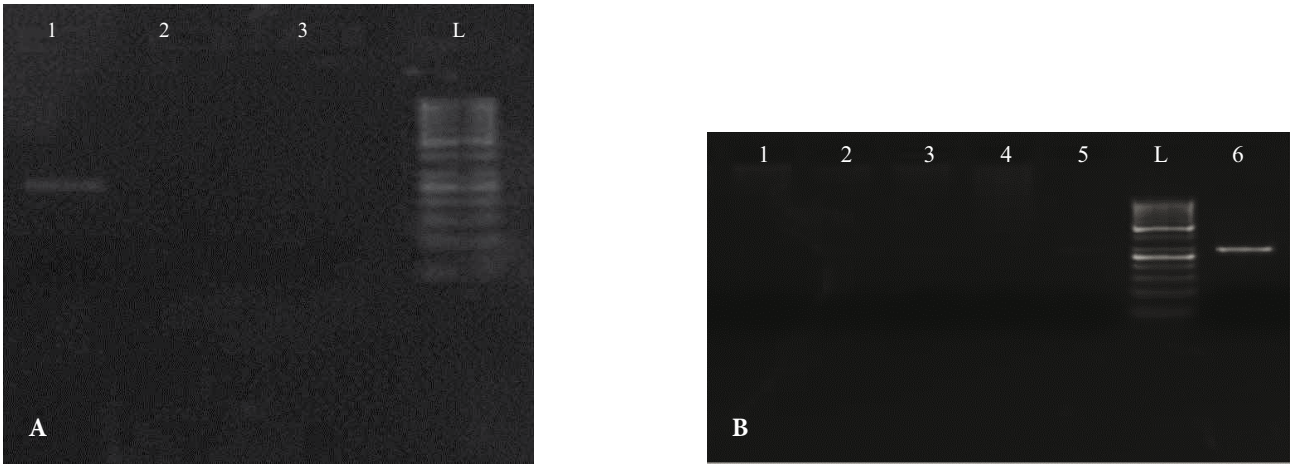

Figure 3. Analysis of specificity of amplification by SCAR primers. **A)** Amplification of different amphistome species with SCAR primers. Lane 1: *Gastrothylax indicus*, Lane 2: *Gigantocotyle bathycotyle*, Lane 3: *Paramphistomum cervi*, L: GeneRuler 50 bp DNA ladder (Fermentas). **B)** Amplification with DNA from ruminant species and ruminal flora. Lanes 1, 2, 3, 4, and 5: Amplification with DNA from cattle, buffalo, sheep, goat, and rumen microflora, respectively; Lane 6: amplification with DNA from *Gastrothylax indicus*; L: GeneRuler 50 bp DNA ladder (Fermentas).

was 0.0500. The phylogenetic tree was built using the parsimony method of the PAUP program with *S. falcatus* as an outgroup, and the 3 species fell into 2 groups. The upper part of the tree consisted of 2 species, *P. epiclitum* and *O. streptocoelium*, supported by 96% bootstrap replicas. The lower branch consisted of a single species, *F. elongatus*, whereas *S. falcatus* was placed in an external position.

In the present study, out of 31 fragments cloned, a total of 15 sequences were obtained, which included 6 sequences (190, 162, 193, 163, 416, and 383 bp) from *P. cervi*, 3 sequences (45, 765, and 458 bp) from *G. indicus*, and 6 sequences (696, 349, 367, 204, 413, and 388 bp) from *G. bathycotyle*. Similar sequencing studies were carried out by Itagaki et al. (17) from 3 bovine amphistomes *Calicophoron calicophorum*, *O. streptocoelium*, and *Homalogaster poloniae*. They obtained ITS-2 sequences of 284 bp from *C. calicophorum* and *O. streptocoelium* and of 285 bp from *H. poloniae*. The sequences included 9 variable sites including 1 gap and the degree of variation was 4.2%–5.3% among the 3 species. However, Rinaldi et al. (18) obtained an ITS-2+ sequence of 428 bp from *C. daubneyi* collected from cattle, buffalo, and sheep. A sequence of 285 bp plus 2 partial flanking conserved sequences of 5.8S (99 bp) and 28S (47 bp) rRNA were obtained from each host. There were no sequences available in GenBank for buffalo amphistomes until the preparing of this manuscript for comparison of results.

Cloning of the unique selected bands from RAPD products gave a total of 15 sequences, which included 6 sequences of 190 bp (GenBank Accession No. HN148440), 162 bp (HN148441), 193 bp (HN148442), 163 bp (HN148443), 416 bp (HN148444), and 383 bp (HN148445) from *P. cervi*; 3 sequences of 458 bp (HN148437), 765 bp (HN148438), and 458 bp (HN1 48439) from *G. indicus*; and 6 sequences of 696 bp (HN148446), 349 bp (HN148447), 367 bp (HN148448), 204 bp (HN148449), 413 bp (HN148450), and 388 bp (HN148451) from *G. bathycotyle*. The BLAST analysis of these sequences showed that the sequences obtained were unrelated and had not been previously characterized.

In the present study, we generated one SCAR marker from RAPD-derived sequence amphi1 from the sequence of *G. indicus*, which worked as a species-specific marker for *G. indicus*. Various authors have demonstrated the usefulness of RAPD in yielding sequence-specific SCAR markers (19). RAPD markers can be useful for the identification of species and strains of a range of parasite groups, including protozoa and helminths (20).

In conclusion, our study resulted in the development of a species-specific SCAR marker that can be used to identify the paramphistomes species *G. indicus*. The RAPD fingerprint also suggested close relatedness between *G. bathycotyle* and *G. indicus* as compared to *P. cervi*. Further unique sequences identified for *G. bathycotyle*, *G. indicus*, and *P. cervi* could be used to develop new SCAR markers for identification of these species.

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