Advances in Bioresearch
Adv. Biores., Vol 5 (1) March 2014: 92-99
©2014 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV 7.20 [Poland]

Advances in Bioresearch

ORIGINAL ARTICLE

Molecular Characterization of Paramphistomes from Cattle from Matebeleland Region (Zimbabwe) using Random Amplified Polymorphic DNA (RAPDs) and Amplified Ribosomal DNA Restriction Analysis (ARDRA)

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ABSTRACT
Paramphistome isolates collected from local abattoirs were genetically characterised using the Random Ampl41i47fied Polymorphic DNA (RAPD) technique and Amplified Ribosomal DNA Restriction Analysis (ARDRA). These isolates were morphologically characterized using median sectioning and five putative species were identified. Of the 18 isolates that were being investigated, 16 were positively identified: three belonged to Calicophoron calicophorum, two were Calicophoron microbothrium, one was Gigantocotyle symmeri, 6 were identified as Calicophoron raja and the other 6 were identified as Calicophoron clavula. A restriction digest of the amplified ITS-2 region of all isolates was done using two restriction enzymes Hae III and Sau 3A1 and the fragments obtained did not show any detectable polymorphisms on all isolates. A total number of 110 bands were generated by RAPD-PCR and 91.82% of these were polymorphic with an average genetic distance of 0.4810+/- 0.185 that showed substantial variability among the paramphistome isolates. The RAPD-PCR technique however, gave banding patterns that on analysis were able to cluster (on the dendrogram) the isolates into their respective species groups and even aid in identifying the two isolates that were not positively identified morphologically as Calicophoron raja. A fragment of approximately 1300bp was generated from primer OPB 07 on Calicophoron microbothrium isolates which can be used as a selectable marker for this species. The findings of the present study therefore showed that the RAPD- PCR technique can be used for molecular identification of paramphistomes.
Key words: Paramphistomes, Calicophoron, Gigantocotyle, RAPD-PCR, Zimbabwe.

Received 14/12/2014 Accepted 20/02/2014

How to cite this article:
M.S. Sibula, Z. Dhlamini, S. Dube. Molecular Characterization of Paramphistomes from Cattle from Matebeleland Region (Zimbabwe) using Random Amplified Polymorphic DNA (RAPDs) and Amplified Ribosomal DNA Restriction Analysis (ARDRA). Adv. Biores., Vol 5 [1] March 2014: 92-99**.** DOI: 10.15515/abr.0976-4585.5.92-99.

INTRODUCTION

Paramphistomes are digenetic trematodes that exhibit a heteroxenous lifestyle that includes an intermediate host (fresh water snails) and a definitive host (cattle, sheep and goats) and fall under a number of genera among which include *Calicophoron, Gigantocotyle* to mention but a few [1]. Species of paramphistomes that have been reported in Zimbabwe include *Calicophoron microbothrium, C. calicophorum, C. phillerouxi, C. raja, C. sukari, C. sukumum, Cotylophoron cotylophorum, Cotylophoron jacksoni, Carmyerius bubalis, and Camyerius spatiosus* [2, 3].

Although there are several species of paramphistomes in Zimbabwe, only *Calicophoron microbothrium* is of veterinary significance as it may cause acute paramphistomosis [3]. This disease is mainly caused by immature paramphistomes as they migrate through the host causing mild to severe damage that may even result in death [4, 5]. Paramphistomosis in domestic ruminants results in serious economic losses to the wool, meat and milk industries [3]. Ruminants that play host to paramphistomes are goats, sheep and cattle [3, 5, 6] including wild ruminants like the water buck, buffalo and the wildebeest.

Traditionally, paramphistomes were identified using morphological features of the adult parasite [7, 8]. This morphological characterization entailed sectioning through thick robust bodies in order to visualize the internal organs [9] making mere identification relatively difficult. With increase in genetic variability, it would be rather unjustifiable to just make generalizations or predictions [10] about paramphistomes solely based on morphological features, host and geographical ranges. This makes molecular methods that are rapidly becoming sophisticated, an attractive tool for parasitologists and evolutionary biologists [10]. Identification of paramphistomes has often been limited to the adult stage of the worm making it difficult for diagnosis in cases of outbreaks of diseases like paramphistomosis. Molecular techniques will therefore enable such limitations to be overcome so that identification of paramphistomes can be done even on the larval stages from different samples.

Random Amplified Polymorphic DNA (RAPDs) markers have been proven to be one of the molecular techniques that can be used for identifying polymorphic DNA markers within a population [11]. This technique has an advantage of being able to detect and distinguish between different strains of a particular parasite as well as different species [12]. RAPDs are relatively in-expensive and straight forward, requiring only the use of universal primers to detect polymorphisms within populations. [6] applied this technique on paramphistomes and described it as a technique that can support the taxonomist by distinguishing these parasites.

Nuclear ribosomal DNA is another region where useful genetic markers can also be obtained. It is particularly useful for molecular studies because it contains both variable and conserved regions [13]. The regions from the rDNA cluster that can be used for genetic variability studies within a population are variable ribosomal regions. These include Internal Transcribed Spacers (ITS 1 and 2) which have been successfully used for identification of Platyhelminths providing reliable genetic markers [14]. The ITS-2 has been used for molecular characterization of paramphistomes by [9, 14] and results showed that sequence comparisons of the ITS-2 regions, increased the ability to identify and distinguish between each individual paramphistome species and to recover their phylogenic relationships.

The aim of the study was to apply PCR based techniques for genetically characterizing paramphistomes.

MATERIALS AND METHODS

Collection and processing of Paramphistome isolates: Collection of paramphistomes was done from rumen of cattle slaughtered at abattoirs in Bulawayo. These isolates were washed thoroughly using normal saline and stored in 70% ethanol for morphological sectioning and DNA isolation. Paramphistomes stored in 70% ethanol were purposefully picked and had a small fragment cut from them. This was done in such a way as not to distort the internal organs in each individual isolate for morphological sectioning. The small fragment of each isolate was taken for genomic DNA extraction while the rest of the worm was taken for histological sectioning.

Histological sectioning: This entailed dehydrating the paramphistomes in 70%, 80%, 90%, 95% and absolute ethanol successively for one hour in each series. The paramphistomes were then cleared in xylene for ten minutes and were thereafter infiltrated in liquid paraffin overnight. These were then embedded in wax to make blocks that could be sectioned using a microtome. Histological sectioning was then done, cutting these to 7 µm and these were mounted on slides. Staining of the sections was done using Haematoxylin and Eosin and viewed under the microscope. The paramphistomes were assigned groups depending on the genital atrium, testes, acetabulum and pharynx according to the keys of [7, 15]. The dimensions of these features were then recorded enabling for correct identification.

Genomic DNA extraction: Sections of paramphistomes were homogenized in 200 µl of sterile lysis buffer (8mM EDTA pH 8, 10mM Tris- HCl and 0.4M NaCl) for 10-15s. Then 45 µl of 20 % SDS and 2 µl of 20 mg/ml proteinase K were added and mixed well. The samples were incubated at 55- 65^{0}C for at least 1hour after which 1/10 the sample volume of 5M NaCl was added to each sample. This was incubated for 1 hour on ice after which centrifuging was done at 12 000g for 10 min. The supernatant was transferred to fresh tubes and spun down again at 12 000g for 5 min. The supernatant was again transferred to fresh tubes and two and a half times the sample volume of 95 % ethanol was added. Samples were incubated at -20^{0}C overnight to precipitate DNA. Thereafter the samples were centrifuged for 10 min at 12 000g to pellet DNA. The pellet was washed with 70 % ethanol, dried and finally dissolved in 50 µl of nuclease free water. The DNA for PCR reactions was diluted to a concentration of 50ng/µl using nuclease free water.

Amplified Ribosomal DNA Restriction Analysis: The ITS-2 rDNA plus flanking 5.8S and 28S sequences (ITS-2+) was amplified by PCR using the primers ITS-2 F (5`-TGTGTCGATGAAGAGCGCAG-3`) and ITS-2 R (5`TGGTTAGTTTCTTTTCCTCCGC-3`). PCR was performed in a total reaction volume of 25µl each containing1µl of DNA template, and a PCR mix composed of 0.25 mM of each dNTP (Thermo Scientific, USA), 12.5 pmol of each primer (Inqaba Biotec, South Africa), 2 mM of $MgCl_2$ and 2.5 U of DreamTaq DNA

Polymerase used with Dream Taq PCR Buffer (Thermo Scientific, USA). The PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Singapore) under the following conditions: 95 ^{0}C for 3 min; 35 cycles at 95 ^{0}C for 1 min; 55 ^{0}C for 30 s; 72 ^{0}C for 1 min; and 72 ^{0}C for 10 min. PCR products were stored at 4 ^{0}C. The products were visualized in a 1.5 % agarose gel that was stained with 10 µl ethidium bromide and prepared with TBE.

PCR-RFLP was performed utilizing 5µl of product that was digested with 1unit of one of two restriction endonucleases [*Hae III* and *Sau 3A1* (Thermo Scientific, USA)], thus producing two different digested products. Digestion took place in a total volume of 15 µl for 30 min at 37^0 C. These two digested products and an undigested PCR product (5 µl) were subjected to electrophoresis in 1.5% agarose gel stained with 10µl ethidium bromide, and then photographed (UV) and analysed using the UVTEC silver pro gel documentation system (Cambridge, UK).

RAPD PCR Procedure: The RAPD analysis was performed in 10 µl total reaction volumes containing 2 µl of genomic DNA, 1µl PCR buffer l0x (Thermo Scientific, USA), 0.2 mM dNTPs (10 mM of each), 2 µl of 25mM $MgCl_2$,1µl Q solution (Qiagen), 2.5 U Dream Taq DNA polymerase (Thermo Scientific, USA) and 0.1µM of each one of the following primers (OPN- 12, OPN-13, OPAB- 08, OPAE- 14, OPAQ- 16, OPAR- 17, OPB- 07, OPK- 03, OPAH- 11, OPAR- 08, OPAR- 16).

The thermal cycling profile consisted of one cycle of 2 min at 95°C (Primary denaturation) and 40 cycles of 30 sec at 95°C (denaturation), 45 sec at 40°C (Annealing), 1.5 min at 72°C (extension) and one cycle of 15 min at 72°C (Final extension) carried out in the GeneAmp PCR System 2700 (Applied Biosystems, Singapore).

A negative control tube without DNA template was included in each PCR reaction. For agarose gel electrophoresis, individual RAPD products (10 µl) were loaded on 1.5 % agarose gel (w/v TBE 1x) and subjected to electrophoresis at 100 V for 1.5 h using TBE buffer (1x), stained with 10 µl ethidium bromide and then visualized with UV illumination followed by recorded photography using the UVTEC silver pro gel documentation system (Cambridge, UK). For estimation of band profiles, a 1kb base pair DNA ladder marker (Thermo Scientific, USA) was used. The major criteria for taking a fragment into account were reproducibility and distinctness of the fragments; both faint and bright bands were scored as the same.

RAPD Data Analysis: The different band profiles obtained were analysed using the Popgene 32 Software version 1.32 (University of Alberta, California). The bands were visualised and scored for using 1 for their presence or 0 for their absence in each genotype. A dendogram of the paramphistome genotypes based on Nei (1978) similarity coefficients using Unweighted Pair-Group Method of Arithmetic (UPGMA) method was generated and a phylogenic tree was obtained.

RESULTS AND DISCUSSION

Morphological characterization: On sectioning the 18 isolates (P1- P18), five putative species were identified and these are shown on Fig.1. These species include *Calicophoron calicophoron, Calicophoron clavula , Calicophoron raja, Calicophoron microbothrium and Gigantocotyle symmeri.* The species obtained in this study are similar to those observed and described by [2] on doing a prevalence study in the Mashonaland West of Zimbabwe.

Molecular Characterisation

DNA Extraction: Genomic DNA was successfully extracted from all isolates used in this study. Although it is not easy to detect RNA, proteins and other contaminants using gel electrophoresis, a lot of DNA isolates showed little or no heavy smear on the gel signifying satisfactory DNA quality. Nevertheless, the amount of DNA extracted was adequate since RAPD and ITS PCR amplifications do not require large amounts of DNA.

ARDRA: The PCR analyses of the ITS-2 from all paramphistome isolates produced identical fragments whose length was approximately 500bp as shown on Fig.2 A. A restriction digest using *Hae III* produced two fragments of approximately 400bp and 100bp and that of *Sau 3A1* also produced two fragments of approximately 330bp and 170bp for all the isolates as shown on Fig. 2 B1 and B2.

Amplifying the ITS 2 regions of rDNA revealed that fragments of approximately 500bp were obtained for all the paramphistome isolates. This shows that there were no size differences in the amplified intergenic spacer that could be detected by gel electrophoresis. Size differences of a few base pairs are difficult to distinguish by gel electrophoresis. Parasites like *Meloidogyne* species have demonstrated differences in ITS regions enabling species differentiation to be made [16]. Sequencing of ITS 2 PCR products carried out by [14] on *Calicophoron calicophoron*, *C. daubneyi* and *C. microbothrioides* showed only a few base pair differences in these species. According to the ITS 2 sequencing results [14] obtained, both *C. calicophoron* and *C. microbothrioides* had a total of 429 base pairs whilst *C. daubneyi* had 428 base pairs. This difference in the number of base pairs would be difficult to detect by gel electrophoresis.

A restriction digest of the ITS 2 using *Hae III* and *Sau 3A1* did not reveal any polymorphisms on all isolates. Instead, the banding patterns produced were all similar. RFLPs in the ITS 2 region were therefore not sufficient to distinguish between the different paramphistome isolates. This is in line with the findings by [17] who upon restricting *Calicophoron daubneyi* and other paramphistome species using *Hae III* did not get any polymorphisms but instead got results that were similar to ours, a 400bp fragment and a 100bp fragment. Although this technique is good for differentiation of *Fasciola gigantica* and *Fasciola hepatica* at species level [17], it was not sufficient for differentiation of paramphistomes in our study. *Sau3AI* digestion has been observed to produce clear RFLPs and particularly small fragments that are less than 100bp that are difficult to detect on agarose gels [18]. In our study use of *Sau 3A1*gave two clear fragments of about 230bp and 170bp and smaller fragments could not be detected by agarose gel electrophoresis for all isolates.

RAPD analysis: Of the eleven primers that were used, only 8 primers (OPAB-08, OPN-12, OPN-13, OPAH-11, OPAR-08, OPAE-14, OPB-07 and OPQ-16) produced bands that were used for scoring. The total number of DNA bands generated from the 8 primers used was 110 of which 101 were polymorphic. The percentage of polymorphic loci was therefore 91.8%. The sizes of the bands ranged from as high 1500bp to as low as 75bp. Of all these, the primer that produced a selectable marker specific for *Calicophoron microbothrium* (lane 10 and 18) was OPB-07 (Fig. 3A), producing a clear distinct band of approximately 1300bp that can be used as a SCAR marker. The number of scorable bands generated by a single primer ranged from as few as 8 bands (OPAR-08) to as many as 19 bands (OPB-07). Fig. 3B shows a typical example of patterns generated by a RAPD PCR reaction in this case using OPAE-14.

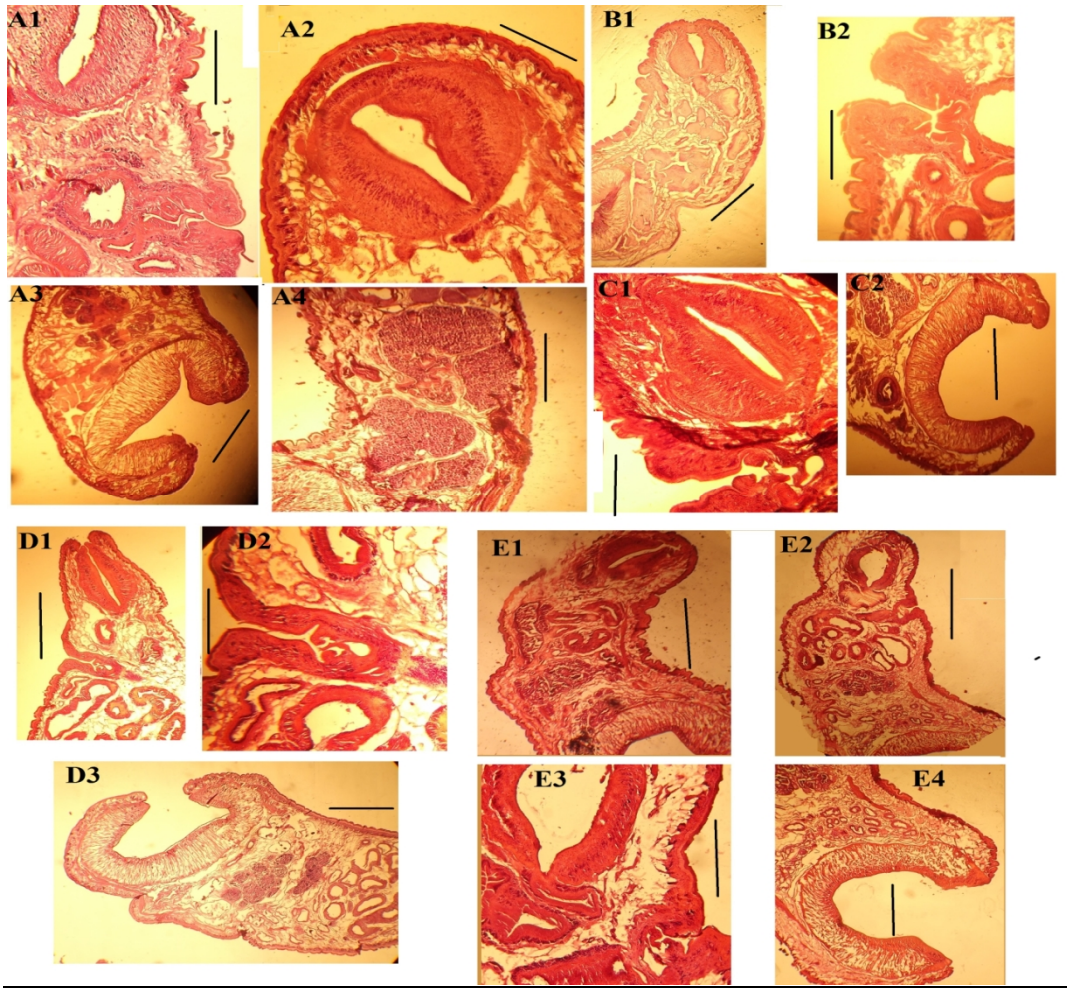

Figure 1: Histological sections of the paramphistome isolates showing diagnostic features. **A1- A4**: *Calicophoron microbothrium*: anterior region- A1- the pharynx and the genital atrium (scale bar = 700µm), A2- the pharynx (scale bar =300µm): posterior region- A3- the acetabulum (scale bar = 1000µm) A4- the testes (scale bar =700µm). **B1-B2:** *Calicophoron raja* B1-the testes and the pharynx (scale bar = 1000µm), B2- the genital atrium (scale bar = 200µm). **C1-C2**: *Calicophoron clavula* : anterior region- C1-the pharynx and the genital atrium (scale bar = 200µm): posterior region – C2- the acetabulum

(scale bar = 700µm). **D1-D3:** *Calicophoron calicophoron*: anterior region: D1- the pharynx and genital atrium (scale bar = 700µm), D2 – the genital atrium (scale bar= 200µm): posterior region-D3- the acetabulum and the testes (scale bar= 1000µm)**. E1-E4:** *Gigantocotyle symerri* E1- the pharynx and the testes (scale bar = 1000µm), E2- the pharynx, the genital atrium and the testes (scale bar = 1000µm), E3- the genital atrium and the pharynx (scale bar = 200µm), E4- the acetabulum (scale bar = 700µm).

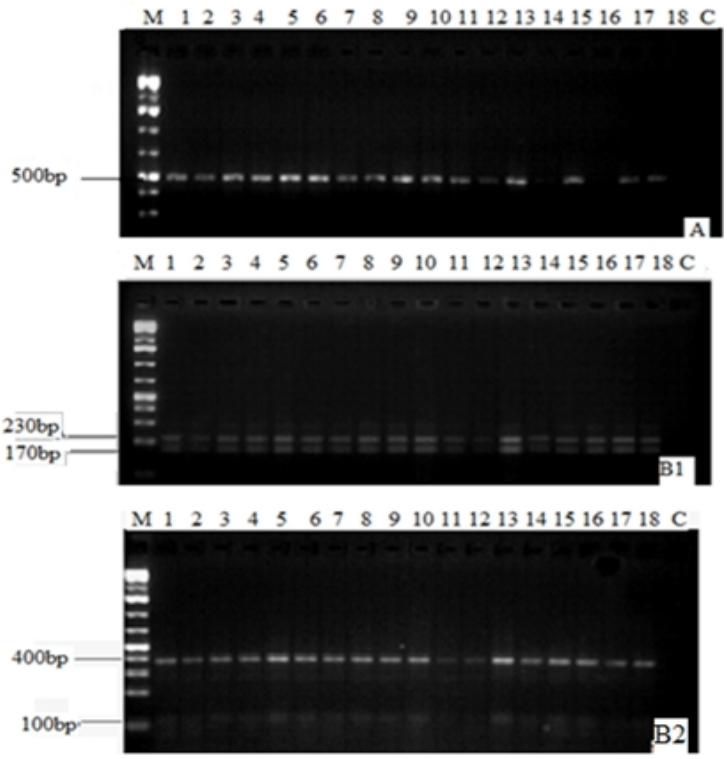

Figure 2: A- ITS-2 amplification. **B1-** ARDRA pattern of the ITS-2 after digestion with *Sau 3A1*. **B2-** ARDRA pattern of the ITS-2 after digestion with *Hae III.* On all gels *Calicophoron microbothrium* isolates are on lanes 10 and 18, *Calicophoron raja* lanes 11, 12, 14, 15, 16 and 17, *Calicophoron clavula* on lanes 2, 4, 7, 8, 9, 13, *Calicophoron calicophoron* lanes 1, 3, 5 and *Gigantocotyle symmeri* on lane 6.

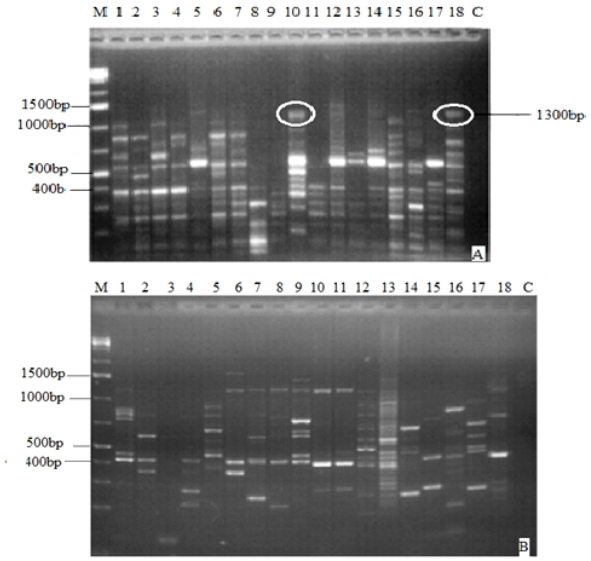

Figure 3: RAPD patterns obtained from: **A** OPB-07. **B-** OPAE- 14. On all gels *Calicophoron microbothrium* isolates are on lanes 10 and 18, *Calicophoron raja* lanes 11, 12, 14, 15, 16 and 17, *Calicophoron clavula* on lanes 2, 4, 7, 8, 9, 13, *Calicophoron calicophoron* lanes 1, 3, 5 and *Gigantocotyle symmeri* on lane 6. M is a 1Kb DNA ladder and C is the control.

A UPGMA dendrogram was generated from figures obtained according Nei's estimates of genetic distance and genetic variability and the results are shown in Fig. 4. The lowest genetic distance was 0,1572 between isolate 14 and 17 and the highest was 0.6061 between isolate 18 and 5. The average genetic distance for all the isolates was 0.4810 +/- 0.185. The average genetic distance within the species were as follows: *Calicophoron calicophorum* - 0.3536, *Calicophoron clavula* - 0.3040, *Calicophoron raja*- 0.3469 and *Calicophoron microbothrium* - 0.1787.

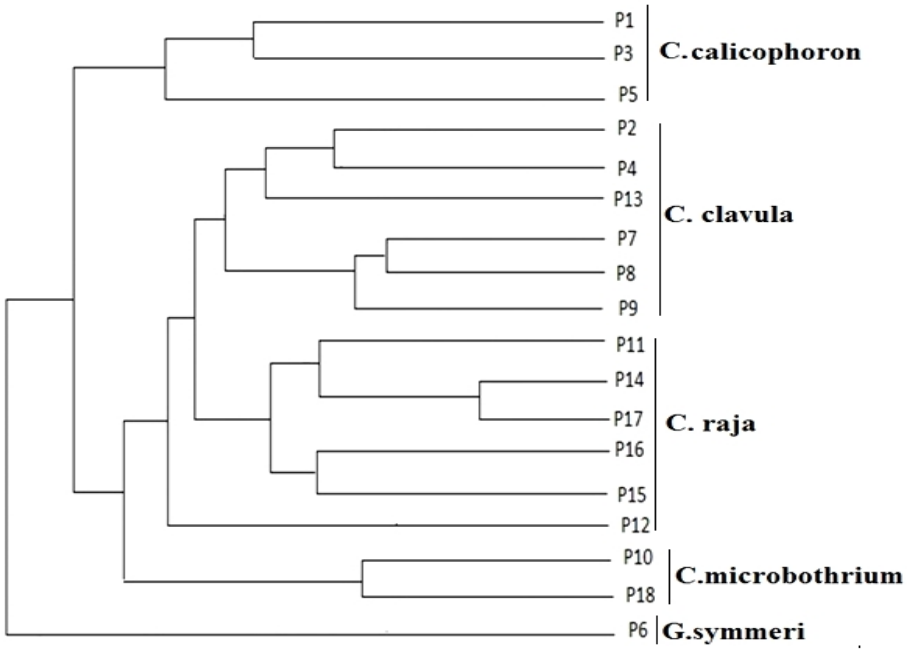

Figure 4: An Unweighted Pair-Group Method of Arithmetic (UPGMA) dendrogram of all paramphistome isolates from which DNA was extracted and sections were made.

The dendrogram generated in (Fig.4) had two distinct clusters. One cluster had 1 isolate and was *Gigantocotyle symmeri* and the other cluster had 17 isolates and was further divided into four clusters:- their respective species

Compared to PCR-RFLP of rDNA (ARDRA), RAPD analysis gives a much broader picture of genetic variation as RAPD data are based on the whole genome [19]. The average genetic distance of approximately 0.5 showed substantial variation amongst the paramphistomes. Genetic distance can be used to quantify similarities of individuals in order to try and classify them [20]. Lower genetic distances suggest that the individuals in the population have closer genetic relation among them, while higher genetic distance values suggest that the individuals in the population have farther genetic relation. According to the results obtained, *Calicophoron calicophoron* isolates had the highest average genetic distance within their population signifying some degree of intraspecies genetic variation. This could have been due to differences in the localities where they came from. Genotypes from the same geographic regions would most likely show closer genetic similarity (lower genetic distance) than those from geographically distant regions [21].

The *Calicophoron microbothrium* isolates had the lowest average genetic distance between them meaning they were distinctly similar. This species is of particular interest in this study as it causes outbreaks of paramphistomosis. Understanding its genetic variability and population structure would be of great importance to veterinary parasitologists- for treatment regimes and control of outbreaks. The low average genetic distance values within all the species under study mean that there is low intraspecies variation. Between the species, significantly high genetic distance values were observed particularly between *Gigantocotyle symmeri* and isolates in all the other groups. This means that *Gigantocotyle* falls into a cluster of its own and is different from the other isolates. These results correlate with results obtained from morphological characterization.

The dendrogram obtained confirmed the results from morphological characterization and was able to place the unidentifiable isolates into their respective groups or species. This further proves how molecular tools can be used to identify the morphologically indistinguishable features like larvae and eggs and classify them from the DNA profile. This confirms [6]'s suggestion that RAPD data analysis can

support the taxonomist by distinguishing parasites. The phylogenetic tree (dendrogram) and the genetic distance were in line with taxonomy.

The *Gigantocotyle* cluster only had one isolate and was therefore not further divided. The *Calicophoron* cluster however, was further divided into two with *Calicophoron calicophoron* having its own branch while the other 3 species took the other branch. This may be due to a fairly large genetic distance that was observed between some *Calicophoron calicophoron* isolates and isolates from the other three species. Primer OPB 07 from inqaba biotec gave a distinct band of about 1300bp on isolates 10 and 18 identified as *Calicophoron microbothrium*.

CONCLUSION

In conclusion, as has been demonstrated for other Helminths, molecular tools are useful for identification of paramphistomes. The RAPD technique proved particularly to be a molecular tool that can be further developed for epidemiological techniques for the study of *Calicophoron microbothrium* transmission patterns. The effect of paramphistomosis has often been underestimated [22] but with the emergence of molecular tools this can be determined. Restriction of ITS 2, however did not reveal much information about variability in the different paramphistome isolates and thus it is recommended that more restriction enzymes be used and that ITS 2 PCR products be sequenced to reveal these differences as was done by [14].

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