Genetic variation among four closely similar isolates of Cyclocoelid trematodes based on random amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis

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Abstract: Four closely similar isolates of cyclocoelid trematodes were isolated from the air sacs of the waterfowl *Gallinula chloropus* from Sharkia province, Egypt. After whole mount preparation and redescribed the morphological characters, they were assigned to family: Cyclocoelidae, *Cyclocoelum microstomum*, as they were found to possess two testes arranged in a tandem forming a triangle with the ovary between them and the position of the genital pore relative to the pharynx. The genomic DNA was extracted from all the isolates, amplified by polymerase chain reaction based on random amplified polymorphic DNA (RAPD-PCR) technique using 11 arbitrary oligonucleotide 10-mer primers the products of amplification were run through agarose gel electrophoresis. All primers showed distinct banding patterns (RAPD profiles) for all the isolates. Different polymorphic bands and specific markers were produced by most primers. The similarity indices between isolates were calculated, hence a dendrogram was constructed. The molecular analysis was found to be consistent with the morphological findings in the fact that genetic and morphological variations are not so powerful to support the establishment of a genus or even a species. So these four isolates are members of the previously established species *Cyclocoelum microstomum* and are not new members of the recent genus *Selfcoelum*. From molecular analysis and morphological findings of the present study, isolates can be considered as a variety of the species *Cyclocoelum microstomum* and isolate A can be assigned as *Cyclocoelum microstomum* var. *gallinulae*, while isolates B, C and D can be considered as another variety and assigned as *Cyclocoelum microstomum* var. *chloropi.*

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Key words: Cyclocoelid trematodes; *Gallinula chloropus;* Sharkia province; DNA (RAPD-PCR).

1. Introduction

The family Cyclocoelidae represents an enigmatic group of digeneans living as adults primarily in the body cavities and air sacs of birds (Zamparoo *et al*., 2003).

Dronen (2007) recognized 2 genera of Cyclocoelinae based largely on the position of the genital pore relative to the pharynx: *Cyclocoelum* Brandes, 1892, in which the genital pore is prepharyngeal (ranging from anterior end of pharynx to midlevel of prepharynx) and *Selfcoelum* (Dronen *et al*., 2006), in which the genital pore is postpharyrgeal (ranging from midlevel of pharynx to near level of cecal bifurcation). As diagnosed by Dronen (2007) the vitelline fields of members of Cyclocoelinae are not confluent posteriorly as in the case of the both genera currently assigned to this subfamily.

The Genus *Cyclocoelum* Brandes, 1892 was established by Brandes (1892) with the description of the type species, *cyclocoelum mutabile* Zeder, 1800, described as *Monostoma mutabile* Zeder, 1800 from the abdominal cavities of the common moorhen, *Gallinula Chloropus* Linnaeus, 1758, and the Eurasian coot, *Fulica atra* Linnaeus, 1758, from Germany by Zeder (1800).

In Egypt, Gohar (1934) listed *Cyclocoelum problematicum* Stossich, 1902 from *Totanus calidris*. El – Naffar (1979) described two cyclocoeliidean parasites from the body cavity of moorhens in Assiut including *Cyclocoelum microstomum and Cyclocoelum problematicum* var. *Gallinulae* as n. variety. Sakla (1983) described *Typhlocoelum sp* and *Cyclocoelum sp* from moorhens in Assiut. Ahmed (1994) described three varieties (var. A, var. B and var. C) of the species *Cyclocoelum microstomum* from the common moorhen *Gallinula chloropus* from Sharkia and Giza provinces of Egypt and regarded them as new varieties because of differences in shape of testes, extension of vitellaria, position of genital pore and site of parasitism within the host (body cavity or air sacs). Wanas (2001) recorded two species of genus *Cyclocoelum* from air sacs of waterfowls collected from Egypt and presented full morphological and histological description for the adult stage of the species *Cyclocoelum microstomum* from moorhen *Gallinula chloropus* and the other species *Cyclocoelum obscurum* was collected from coot *fulica sp.* He also discussed some problems of the status of the genus *Cyclocoelum.*

Molecular approaches are the most effective and accurate means for the detection of many

organisms and for screening of genetic variation among populations (Wongsawad and Wongsawad, 2010).

Molecular techniques have become widely accepted through the world. They provide a more specific method than methods conventionally employed in epidemiological studies (Coote, 1990; Erlich *et al*., 1991, Barker, 1994, Rognlie *et al*., 1994; Karmer and Schnieder, 1998; Hekheroth and Tenter, 1999; Mostafa *et al*., 2003 and Aldemir, 2006). Molecular techniques such as PCR and its variants are used for the diagnosis of parasitic diseases and identification of parasites (Aldemir, 2006).

Molecular techniques based on genomics are very useful for epidemiological and diagnostic tools as well as for research on genetic variation of parasitic organisms (Mas-Coma *et al*., 2005 and Meshgi *et al*., 2008).

DNA Polymorphism assay based on random amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) has been proved useful for analyzing the inter- and intra- specific genetic variations and phylogenetic relationships (Gasser, 2005; Mas-Coma *et al*., 2005; Nuchprayoon *et al*., 2007 and Rokni *et al*., 2010).

The RAPD technique is based on amplification of a random DNA segment with a single primer of arbitrary nucleotide sequence and using polymerase chain reaction (Welsh and McClelan, 1990; Williams *et al*., 1990; Mohammedzadeh *et al*., 2007 and Nuchprayoon *et al*., 2007).

The technique is very rapid, simple and generates reproducible fingerprints of the PCR products. In addition, it neither depends on previous knowledge or availability of the target DNA sequences nor requires DNA hybridization (Mohammedzadeh *et al*., 2007; Nuchprayoon *et al*., 2007 and Sripalwit *et al*., 2007).

In the present study, 4 closely similar isolates of cyclocoelid parasites (A, B, C and D) were compared morphologically and random amplified polymorphic DNA (RAPD) analysis was used to examine the extent of genetic variation among these 4 isolates.

2. Materials and Methods:

Parasite preparation. Adult flukes were collected alive from the air sacs of naturally infected and recently killed common moorhen, *Gallinula Chloropus* Linnaeus, 1758, from Sharkia province, Egypt. The collected parasites were rinsed several times with 0.65% saline solution. Some were mounted as a whole and prepared as permanent slides for further morphological investigation and differentiation. The flukes were fixed with 70% ethyl alcohol at room temperature under slight pressure between two glass slides; hydrated in descending alcoholic series stained with aceto – acid carmine dehydrated in ascending alcoholic series cleared with clove oil and mounted permanently by Canada balsam. The specimens were drawn by camera lucida. The remaining flukes were kept in 70% ethyl alcohol at – 20^{o}C for DNA extraction. Identification of the flukes to subfamily and genus was based on the key of Dronen (2007).

Genomic DNA extraction:

Genomic DNAs were isolated on a small scale from 1000 µl of adult worms using multisource genomic DNA, Mini-Prep Kit- Axgene Biotechnology-U.S.A Cat. No. 110420-25, according to manufacture manual.

RAPD analyses:

Primers for RAPD were tested in parasite isolates and eleven of them were selected due to successful amplification for all isolates. Names and sequences of primers are as shown in table (1).

Table (1): RAPD primers

Name	Sequence
A12	5'-TCGGCGATAG-3'
A15	5'-TTCCGAACCC-3'
A20	5'-GTTGCGATCC-3'
C12	5'-TGTCATCCCC-3'
C19	5'-GTTGCCAGCC-3'
O1	5'-GGCACGTAAG-3'
O5	5'-CCCAGTCACT-3'
O 10	5'-TCAGAGCGCC-3'
Z10	5'-CCGACAAACC-3'
Z17	5'-CCTTCCCACT-3'
Z20	5'-ACTTTGGCGG-3'

PCR analysis was performed in 25 µl volume containing 2.5 mM Mgcl2, 0.2 mM of dNTPS, 20 µM primer, 50 ng genomic DNA and 1.0 unit Taq DNA polymerase (Bioren, Germany). All reactions were performed in a Perkin Elmer 2400 Thermal cycler. RAPD program was performed as 1 cycle of 94^{o}C for 4 min (primary denaturation), 40 cycles of 94^{o}C for 1 min (denaturation), then 35^{o}Cfor 1 min. (annealing), 72^{o}C for 1 min (extension) and a final extension step of 72^{o}C for 10 min.

Detection of PCR products

The products of both RAPD based PCR analyses were detected using agarose gel electrophoresis (1.2% in 1X TBE buffer), stained with ethidium bromide (0.3 µg/ml), visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK).

Data analysis

Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered as a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing it with 1-kb ladder (Invitrogen, USA) using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA). The binary data matrices were entered into the NTSYSpc (Ver. 2.1) and analyzed using qualitative routine to generate similarity coefficient and used to construct a dendrogram using unweighed pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

3. Results

Morphological investigations

Using the most recent key for Cyclocoelids by Dronen (2007), these four isolates are assigned to Cyclocoelinae Stossich, 1902 because they have an intertesticular ovary (ranging from the level of the posterior end of the anterior testis to the anterior end of the posterior testis) that forms a triangle with testes. The testes in each strain are tandem to slightly diagonal and usually smooth. The vitelline fields are not united posteriorly and the uterus is intercaecal, further supporting placement of these flukes within subfamily Cyclocoelinae.

However, the position of the genital pore differs from one isolate to another. It in isolate A the genital pore opens ventrally on midline of the body, nearly at the midlevel of the pharynx. In isolate B opens ventrally, but slightly dextral to midline of the body at one side of the prepharynx, immediately at the level of the anterior end of pharynx. In isolate C opens ventrally, dextral to midline of the body immediately at the level of the posterior end of the pharynx near intestinal bifurcation, while in isolate D the pore opens ventrally and slightly dextral to midline of the body, immediately at the level of the anterior end of the pharynx.

Moreover, every isolate has some unique morphological characters and there are some morphological variations among them. Isolate A is somewhat longer than the other isolate, with narrower prepharynx, smaller pharynx, without oesophagus and it has a uterus with complicated uterine coils, larger testes lying sinistral to the midline of the body with larger distance separating them from each other and from the ovary which lies dextral to them. Posterior testis is kidney shaped and vitelline follicles extend anteriorly to near intestinal bifurcation level. Isolate B shows more curvation sinistrally with wider and longer oesophagus. The uterine coils are more complicated and more intervening. Testes are dextral with a small distance separating them from each other and from the ovary which is sinistral to them. The excretory bladder is disappearing. Isolate C has a very short prepharynx, no oesophagus. The uterus consists of uterine coils. Testes are dextral and the ovary sinistral. Isolate D has a wider prepharynx and pharynx, a uterus with simple uterine coils, sinistral testes, dextral ovary and vitelline follicles extending anteriorly near to the level of intestinal bifurcation. The other characters are nearly the same or differ a little in size (Fig. 1 and 2).

DNA was successfully extracted from all isolates. All primers provided distinct patterns of amplified genomic DNA. The RAPD profiles of the 4 isolates with every primer are shown in figure (3) and table (2). The analysis of RAPD profiles indicated the presence of genetic variation between the 4 isolates as there was a considerable variation in the RAPD profiles among them using the 11 primers on the basis of number and intensity of bands.

The 11 primers selected produced clearly distinguishable band patterns and yielded a total of 226 scoreable RAPD fragments. The 226 bands ranged between 130 and 1722 bp (base pair) in length by comparison with a 1- Kb ladder . Of the 226 bands, only 184 bands (81.41%) were shared by all the isolates and 42 bands (18.58%) were polymorphic among the 4 isolates. The number of bands generated by every primer, number of polymorphic bands and number of bands of each isolates are indicated in table (2). The highest number of bands scored by a certain primer was generated by primer A12, amplifying the 4 isolates, while isolate 1 produced the highest number of DNA fragments (62 DNA fragments) using the 11 primers.

Table (2): Number of amplified fragments markers of four isolate based on RAPD-PCR analysis.

		RAPD primers											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**total**
A	AF	4	6	8	4	6	6	7	6	7	5	3	62
	SM	1	2	3	0	3	0	1	0	2	1	2	15
B	AF	3	8	5	4	3	6	6	6	7	4	3	55
	SM	0	0	0	0	0	0	0	0	0	0	0	0
C	AF	3	8	5	3	3	6	6	6	7	4	4	55
	SM	0	0	0	0	0	0	0	1	0	0	0	1
D	AF	3	8	5	3	3	6	6	7	6	4	3	54
	SM	0	0	0	0	0	0	0	2	1	0	1	4
TSM		1	2	3	0	3	0	1	3	3	1	3	20
TAF		13	30	23	14	15	24	25	25	27	17	13	226
PB		1	6	3	2	3	0	1	9	7	1	9	42

TAF: total amplified fragments; **PB**: polymorphic bands; **AF**: amplified fragment; **SM**: marker including either the presence or absence of a band in a certain isolate; **TSM**: total number of specific markers across a certain isolate.

Table (3): Markers resulting from RAPD-PCR analysis.

	Positive marker	**Negative marker**	**Total**
A	11	4	15
B	0	0	0
C	0	1	1
D	1	3	4
Total	12	8	20

Table (4): Similarity indices resulting from RAPD-PCR analysis.

	A	**B**	**C**	**D**
A	1			
B	0.87	1		
C	0.84	0.96	1	
D	0.81	0.94	0.95	1

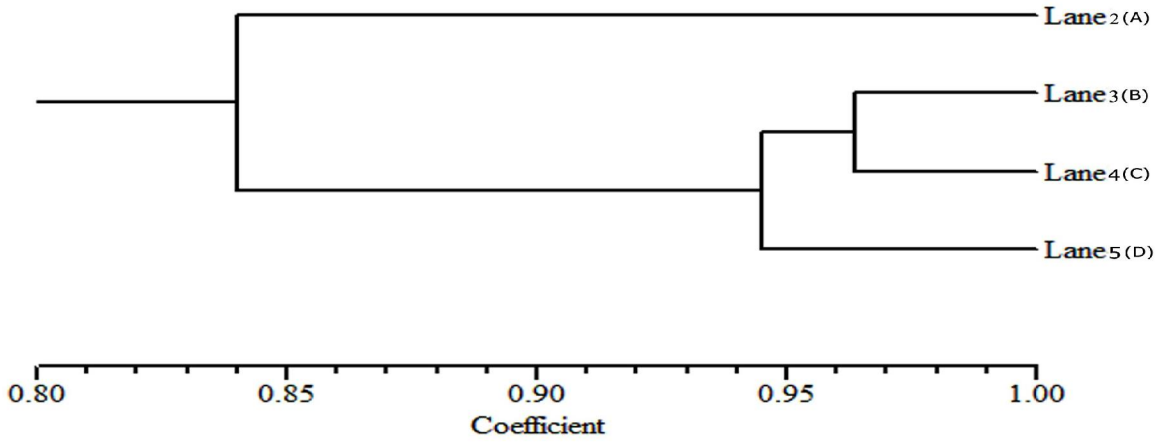


Figure (3): Dendrogram resulting from RAPD-PCR analysis

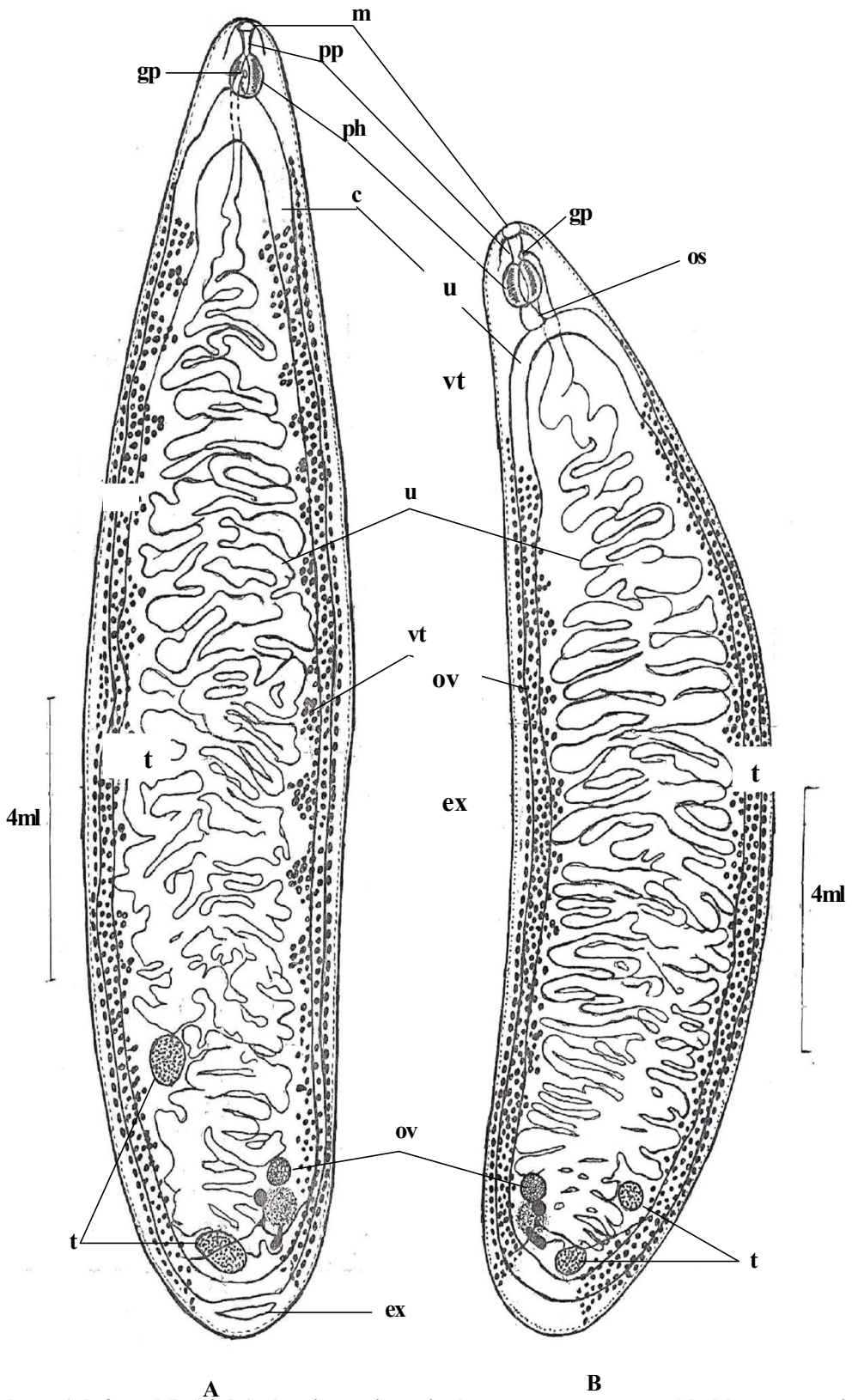


Fig. (1): Isolates A left and B (right) showing c, intestinal caeca; ex, excretory bladder; gp, genital pore; m. mouth; ov, ovary; pp, prepharynx; ph, pharynx; os, oesophagus; vt, vitelline follicles; t, testis and u, uterus.

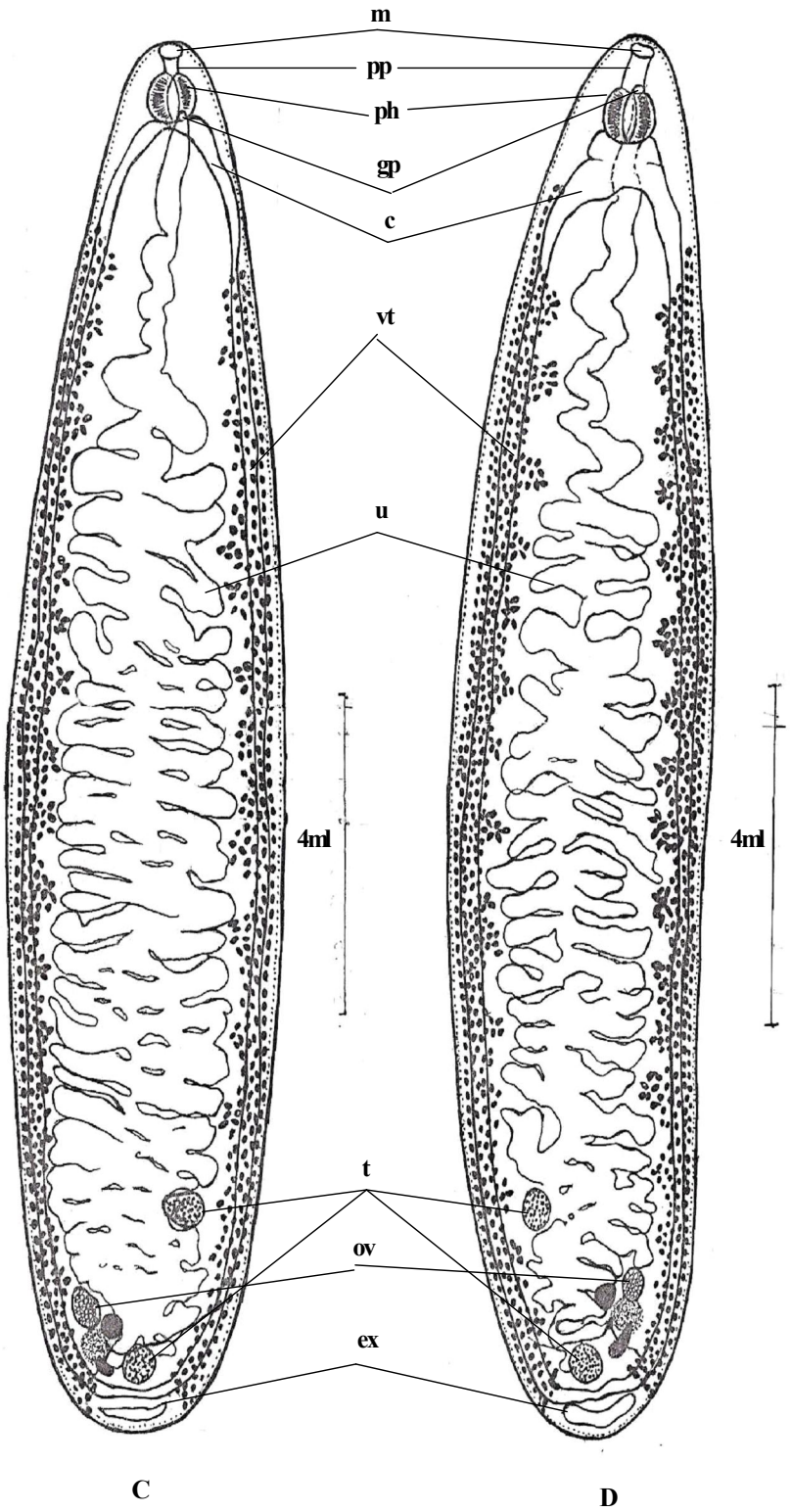
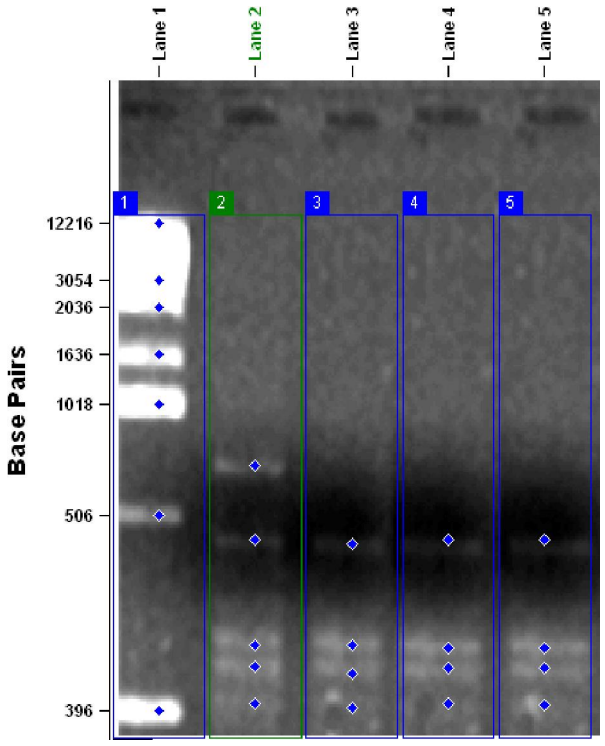
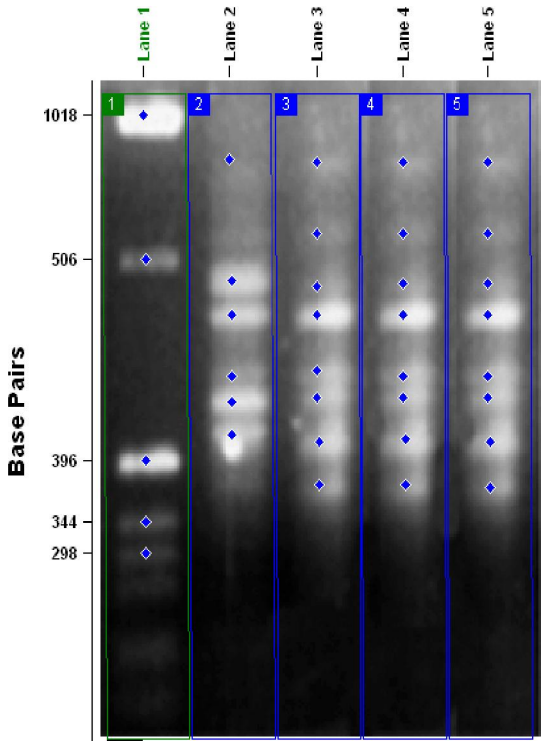


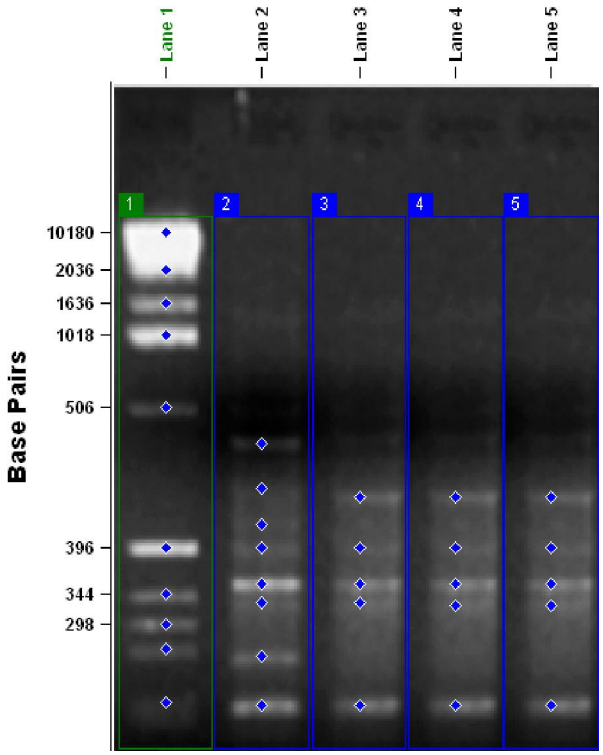
Fig. (2): Isolates C left and D (right) showing c, intestinal caeca; ex, excretory bladder; gp, genital pore; m. mouth; ov, ovary; pp, prepharynx; ph, pharynx; os, oesophagus; vt, vitelline follicles; t, testis and u, uterus.



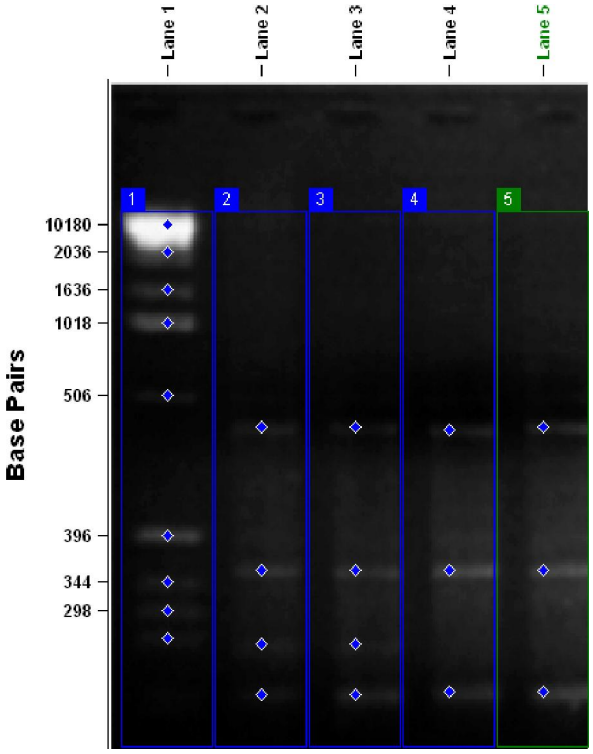
A12



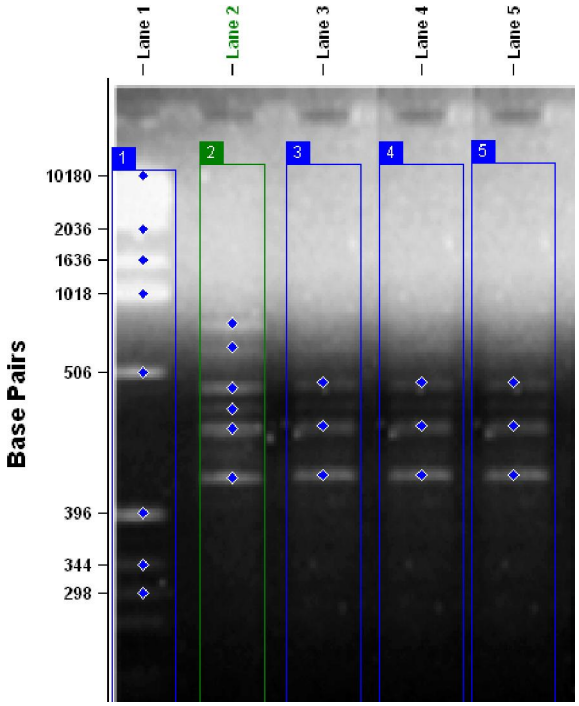
A15



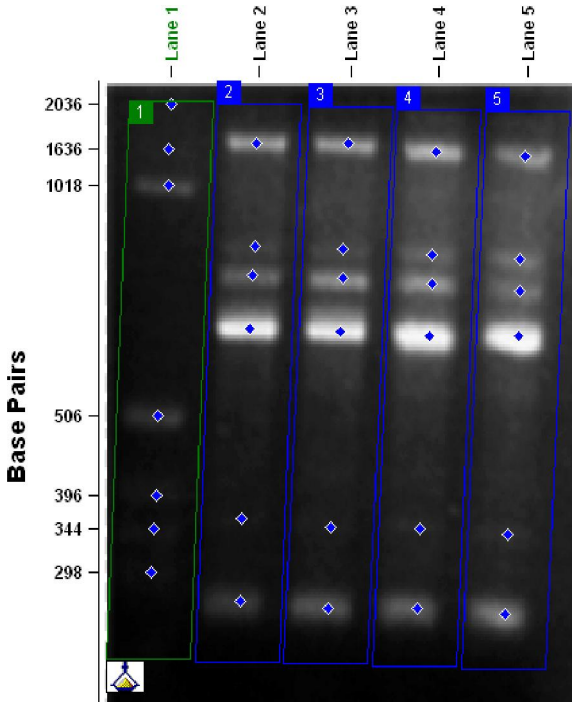
A 20



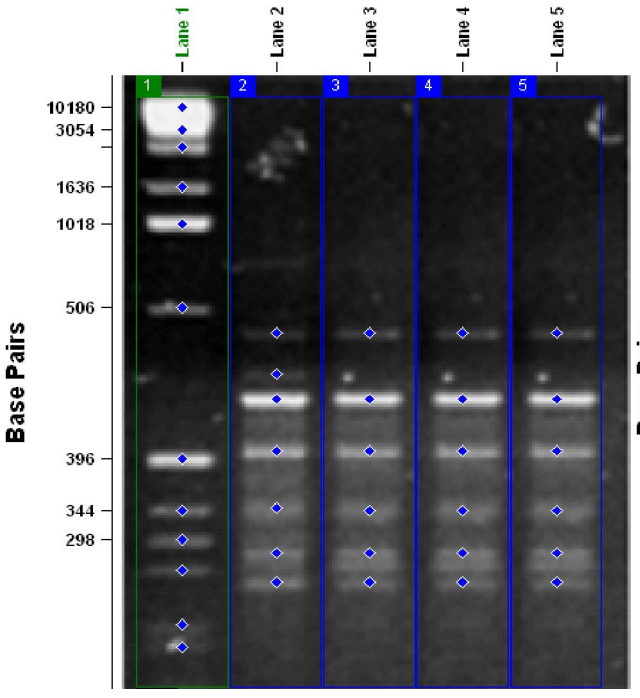
C 12


Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Base Pairs
10180
2036
1636
1018
506
396
344
298

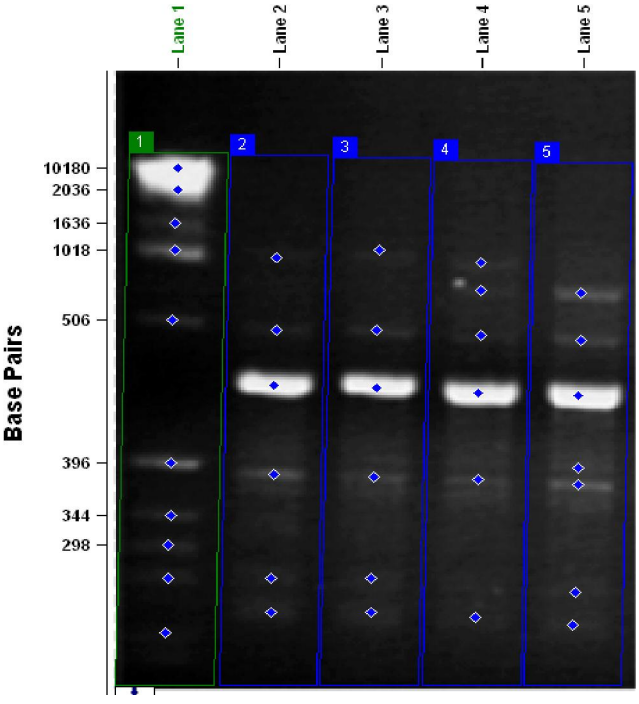
C19


Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Base Pairs
2036
1636
1018
506
396
344
298

O1


Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Base Pairs
10180
3054
1636
1018
506
396
344
298

O5


Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Base Pairs
10180
2036
1636
1018
506
396
344
298

O10

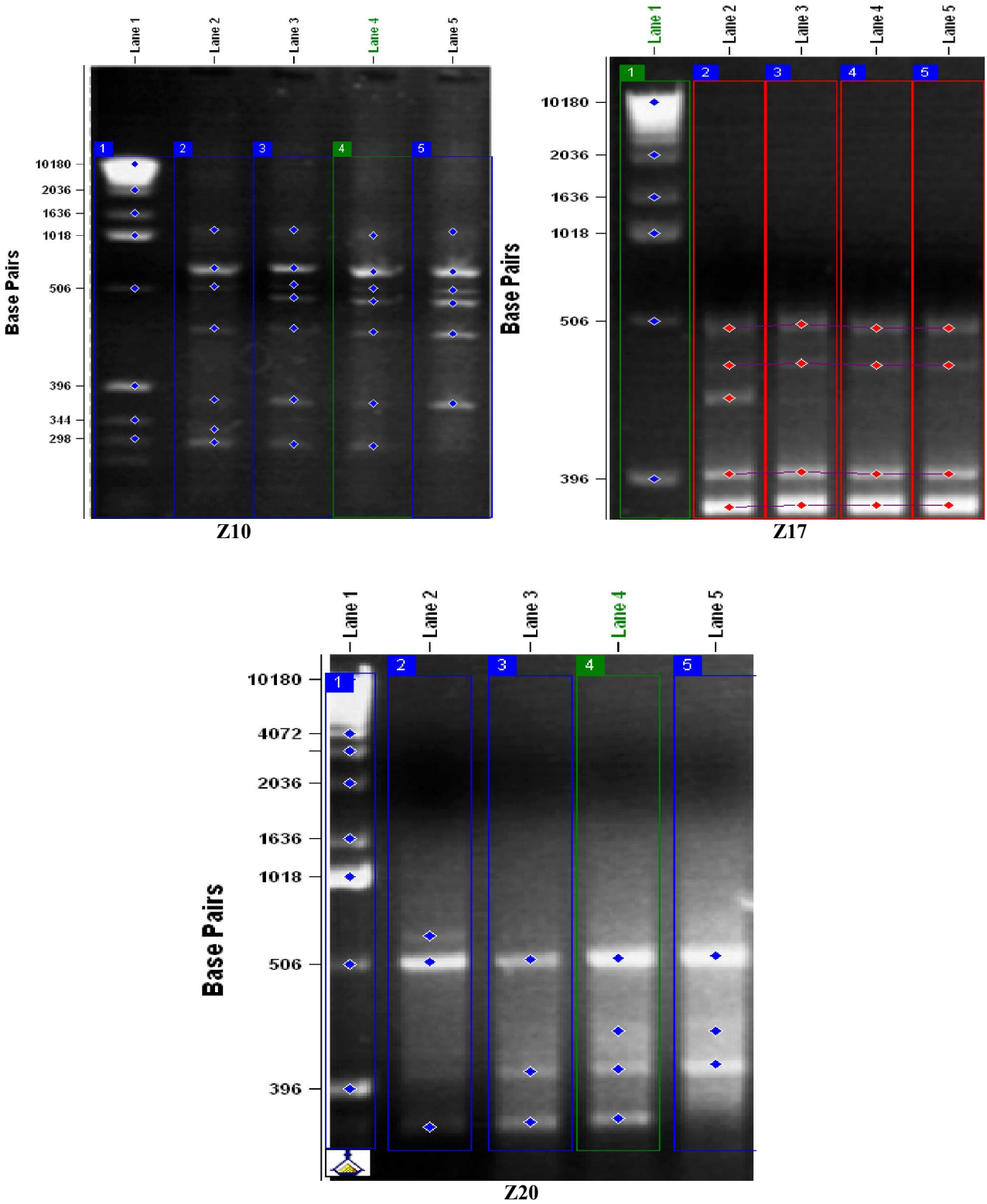


Fig. (3): agarose gel electrophoresis showing the RAPD profiles of the four isolates generated by 11 primers where; lane 1, 1 Kb, DNA ladder; lane 2, isolate (A); lane 3, isolate (B), lane 4, isolate (C) and lane 5, isolate (D).

All primers except primer C12 and O1 generated specific DNA fragments markers for a certain isolates. Only isolate B did not produce any specific marker with any of the 11 primers or by another meaning, none of the 11 primers could reveal any specific primer with isolate B. The total number of markers was 20 (15 for A, 0 for B, 1 for C and 4 for D), table (2). The specific marker may be either the presence of a unique band for a given isolate (positive marker) (11 with A and 1 with D) or the absence of a common band (negative marker) (4 for A, 1 for C and 3 for D), table (3). Primers (A20, C19, O10, Z10, and Z20) produced 3 different markers per each. Primers (A12, O5, and Z17) produced only one marker per each. Primer A15 produced 2 markers, table (2). The similarity indices resulting from RAPD-PCR analysis, table(4).

4. Discussion

Dronen (2007) recognized 2 genera based largely on the position of genital pore relative to the pharynx: *Cyclocoelum* Brandes, 1982 containing those species with a prepharynegeal genital pore and *Selfcoelum* in which the genital pore is postpharyngeal. As diagnosed by Dronen (2007), the vitelline fields of members of Cyclocoelinae are described as being not confluent posteriorly as is the case of both of the genera currently assigned to this subfamily. The four isolates are similar to *Cyclocoelum* and *Selfcoelum* in having testes arranged in tandem to be nearly diagonal and the ovary is between them forming a triangle with them. The vitelline fields are not united posteriorly so they are assigned to Cyclocoelinae, but isolate A and isolate C are most similar to *Selfcoelum* by having a genital pore which opens posteriorly relative to the pharynx supporting placement of these taxa in *Selfcoelum*, while isolate B and isolate D are most similar to *Cyclocoelum* by having an anterior genital pore relative to the pharynx supporting their placement in *Cyclocoelum.*

While isolate A and isolate C are most similar to the four species currently assigned to *Selfcoelum* by having a postpharynegeal genital pore, these isolates differ from every one of the four *Selfcoelum* species in a certain character: The two isolates have a uterus that is intercecal unlike *Selfcoelum limnodromi* and *Selfcoelum brasilianum* which have uterine coils extensively overreaching the caeca laterally and often reaching the general margin of the body. The two isolates also differ in having vitelline fields that don't reach the level of pharynx unlike the four species where vitelline fields may reach the anterior end of the pharynx. The two isolates also differ from these species in lacking an oesophagus which is longer than the prepharynx in these species and finally isolate A and C lack the oral sucker which is characteristic for *Selfcoelum capellum.* It was also noticed that isolate A and isolate C differ from each other in characters mentioned in the results.

Previously the only genus in *Cyclocoelinae* where the ovary forms a triangle with the testes as is seen in *Selfcoelum* was *Cyclocoelum*. However, species of *Cyclocoelum* are unlike species of *Selfcoelum* by having a prepharyngeal genital pore rather than a postpharyngeal genital pore and uterine loops that are intercaecal.

Applying the most recent key for Cyclocoelinae published by Dronon (2007), it was found that isolate B and isolate D are assigned as previously to Cyclocoelum and are closely similar and morphologically identical to *Cyclocoelum microstomum* described by El – Naffar (1979) and Ahmed (1994), but differ from that described by Sakla (1983) in the finding that they lack a ventral sucker (acetabulum) which was also observed in histological sections prepared for *Cyclocoelum microstomum* by Wanas (2001) who reported that the acetabulum wasn't easily to be seen in whole mount preparations. Wanas also reported in the same study the presence of a very weakly developed and rudimentary oral sucker which was not observed neither in this study nor in specimens of *Cyclocoelum* described by El – Naffar (1979), Sakla (1983) and Ahmed (1994). It was also noticed that the two isolates B and D have some morphological differences between each other.

The identification of closely similar species based on morphological characters could be difficult. This is particularly the case for soft bodied animals such as digenean trematods. However, recent advances in molecular biology, in particular the amplification of specific DNA regions via the polymerase chain reaction (PCR) and the improvement of direct deoxy sequencing techniques, may allow to distinguish closely related species by comparing their DNA (Coote, 1990, Erlich *et al*., 1991; Barker, 1994; Mc Manus and Bowles, 1996 and Mostafa *et al*., 2003).

RAPD – PCR has been used for several phylogenetic studies. The validity of this test has been approved for genomic differences in different studies (Motazedian *et al*., 1996; Intapan *et al*., 2004; Zemanova *et al*., 2004).

The RAPD-PCR technique allows to amplification of short regions of an organism's genome without prior sequence information. This technique has a great potential in identifying genetic markers, tagging genes and chromosomes and performing population studies. Morover, this method evidences species – specific DNA fragments which may be used as diagnostic probes among organisms and consequently would allow determination of their genetic relatedness (Espinosa and Borowsky, 1998 and Williams *et al.,* 1990).

Currently the yardstick for delineating species strains on the basis of genetic differences is unresolved in literature and remains contentious (Morgan and Blair 1995; Haag *et al*., Blouin *et al*., 1998; Sorenson *et al*., 1998). Although some studies have proposed that if within species the variation at particular genetic loci is low this supports the existence of a species (Hang *et al*., 1999), others suggest that caution is required in interpreting the genetic data in the absence of any supportive biological data (Thompson and lymbery, 1995; Blouin *et al*., 1998; Sorensen *et al*., 1998; Thrompson *et al*., 1998; Tibaryrenc , 1998; Macnish *et al*., 2002).

The present study is the first study to use genetic data to define further the taxonomic status of closely similar cyclocoelid parasites. As classifying and identifying these four isolates according to their morphological variations was a difficult matter, in the present study, 11 different primers gave different DNA fragments in size demonstrating that these fragments were specific for every phenotype.

The analysis of RAPD profiles showed considerable variation among these isolates using the eleven primers. Nine of the eleven primers produced RAPD fragments of genomic DNA that could be used as specific markers for three only of the four isolates. Many primers can be considered specific for a certain isolate. Primers A12, A15, A20, C19, O5 and Z17 produced DNA markers that are specific only for isolate A; primer A12 produced the specific marker 1055 bp, primer A15 produced the markers, 357 and 667 bp and primer A20 produced the markers 182, 451 and 787 bp. Primer C19 produced the markers 755, 1135, and 1323 bp, primer O5 produced the marker 644 bp while primer Z17 produced the marker 442 bp for isolate A only. Primer O10 produced the markers, 185 bp for isolate C and 432 and 1677 bp for isolate D. Primer Z10 produced 251 and 880 bp for isolate A and 218 bp for isolate D. Finally primer Z20 produced 305 and 840 bp for A and 201 bp for D. From RAPD profiles and similarity indices data, isolate A was found to be genetically distant from the other three isolates. Isolate B and isolate C are the most similar, and isolate A and isolate D are the most genetically distant.

The molecular analysis was found to be consistent with the morphological findings in the fact that genetic and morphological variations are not so powerful to support the establishment of a genus or even a species. So these four isolates are members of the previously established species *Cyclocoelum microstomum* and not new members of the recent genus *Selfcoelum*. Also, the morphological and taxonomical findings of this study matched with those of Ahmed (1994) in the principle that the position of the genital pore and other characters are just relative characters that can only establish varieties of the species *Cyclocoelum microstomum*. So Ahmed (1994) classified the members of *Cyclocoelum microstomum* he described under three varieties named *Cyclocoelum microstomum* var. A, *Cyclocoelum microstomum* var. B and *Cyclocoelum microstomum* var. C. From molecular analysis and morphological findings of the present study, isolate A can be considered as a variety of the species *Cyclocoelum microstomum* and can be assigned as *Cyclocoelum microstomum* var. *gallinulae*, while isolates B, C and D can be considered as another variety and assigned as *Cyclocoelum microstomum* var. *chloropi*. Molecular together with morphological results contradicted the key provided by Dronen (2007) which appeared to be unreliable and can not be applied in the present study concerning the principle of the genital pore that can not establish two genera (*Cyclocoelum* and *Selfcoelum*). RAPD markers established in the present study provide the foundation to conduct further detailed studies on the genetic variation among members of the family Cyclocoelidae using a variety of molecular and biochemical techniques (e.g. restriction fragment length polymorphism, single-strand conformational polymorphism, DNA sequencing and multilocus enzyme electrophoresis) and to detect at different genes to detect whether morphological variations are just individual variations or may establish different species.

5. Conclusion

The members of some genera or some species of the family Cyclocoelidae may exist as a number of phenotypes and genotypes that are closely similar and can not be recognized morphologically but can be identified using molecular assays.

The RAPD-PCR technique has proved to be a reliable technique in detecting intra-specific genetic variation between closely similar and closely related parasitic members of the same species.

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