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Integrative Description of Three Species of Paramphistomes Using Different Techniques

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Abstract: Disclosure of taxonomic valid characteristics is crucial for accurate identification of paramphistomes. Techniques often affect the internal and external structures and in turn affect description. In the present study, paramphistomes namely *Cotylophoron cotylophorum*, *Carmyerius gregarious* and *Fischoederius elongatus* were re-described from cattle, buffaloes and sheep respectively. Four different methods for fixation and specimen processing were adopted: whole mount flattened, hand cut section, histological paraffin section and scanning electron microscope techniques. Description and identification were based on characteristics revealed by the four techniques. The ability of each technique to reveal the taxonomic valid characteristics was critically evaluated. Both scanning electron microscopy and hand cut section technique were the most accurate methods for identification of the large thick reported trematode species.

Key words: Paramphistomes · *Cotylophoron cotylophorum* · *Carmyerius gregarious* · *Fischoederius elongates* · Flattened Whole Mount · Hand Cut · Histology · SEM

INTRODUCTION

Paramphistomiasis is a disease of prime economic importance to animal industry, causing serious loss in wool, meat and milk production. Immature flukes live in the small intestine and adults are found in the rumen or reticulum, they have complex life cycle which requires an intermediate host for completion [1].

The accurate identification of paramphistomes is very important to define pathogenicity, treatment and control strategies. Scientists disagree in defining taxonomic valid characteristics of these flukes. Controversies in identification arose from the fact that scientists used different techniques and consequently pointed to different characteristics as taxonomically valid [1]. Some overlooked the surface structures and highlighted the internal anatomy and other empowered the main organs and missed the concealed structures. Eduardo [1-4] made extensive studies on the taxonomy of paramphistomes and reviewed the various published schemes of classification of the family Paramphistomidae Fishoeder, 1901. Previous studies in Egypt [5-9] were based on whole mount flattened specimens alone, which was proved by Eduardo [10] to be insufficient for species identification.

The present study is conducted aiming to identify species of Paramphistomes in two governorates in Delta region using different techniques including whole mount flattened specimens, hand cut sections, paraffin sections and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Materials

Collection of Adult Flukes: Paramphistomes were collected from the rumen and reticulum of infected cattle, buffaloes and sheep from Tanta and Kafr El-Sheikh Governorates slaughterhouse .

Flukes were rinsed and kept in 0.9 % isotonic saline and transported to the laboratory. For each host, the collected specimens were accurately sorted depending on the shape, color, size and site within the rumen. Identical specimens were divided into four groups to be fixed for different adopted techniques.

Methods: The first was fixed in 70 % ethanol for hand cut technique [11], the second was fixed flattened in 10% formalin for flattened technique [12] the third was fixed in Bouin's fluid for paraffin section technique [13, 14] and

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the fourth was fixed in a mixture of formaldehyde and gluteraldehyde (4:1) buffered solution for scanning electron microscopy [15].

The identification of collected amphistomes was done according to Eduardo [2], Gibson *et al.* [16] and CAPI international [17]. All measurements were taken using an eyepiece micrometer. All measurements are in millimeter unless otherwise stated.

RESULTS

***Cotylophoron cotylophorum*:** Description is based on 8 hand cut, 8 flattened carmine stained specimens and 5 haematoxylin and eosin stained cryostat-cut sections.

Live adults are pinkish in color. Flattened mounted specimens are conical, bending ventrally and measure 11.97±1.68 X 4.25±0.67 (Figs. 1-3). Oral opening is terminal (Figs. 2, 3). Pharynx is calicophoron type, lacks diverticula, measures 0.68 ± 0.21 X 0.60 ± 0.21 (Figs. 1-3) and leads into an oesophagus which measures 0.28 ± 0.16 X 0.25 ± 0.15 and bifurcates into two caeca that run along both sides of the body (Figs. 2, 3). The right caecum measures 9.3 ± 1.9 and the left measures 9.0 ± 1.0 (Fig. 3). Testes are tandem and lobed (Figs. 1- 4). Anterior testis measures 1.56 ± 0.27 X 1.6 ± 0.46 and posterior testis measures 1.4 ± 0.36 X 1.58 ± 0.4. Vasa efferentia arise from anterior pole of each testis unite to form the vas deferens which leads to the seminal vesicle followed by the pars musculosa and pars prostatica (Figs. 1-3). Ovary is spheroid to ovoid, post testicular, situated dorsal to acetabulum and measures 0.50 ± 0.15 X 0.50 ± 0.13 (Figs. 1-3). Oviduct is a short convoluted tubule opening posteriorly in the ootype that is surrounded with Mehlis' gland (Figs. 2, 3). Vitelline glands are follicular, overlap caeca and restricted at the middle third of the body (Figs. 1, 4). Uterus is a wide coiled tube originating from the ootype and extends anteriorly to reach the genital opening (Figs. 1, 2). Genital atrium is of the cotylophoron type surrounded by muscularized genital sucker that measures 0.17 ± 0.01 X 0.14 ± 0.01 and opens at the end of first third of the body (Figs. 1, 3). The elongated excretory vesicle extends dorsally and opens by the excretory pore at the dorsal surface (Figs. 1, 3). Laurer's canal extends from the ootype and crosses the excretory vesicle (Figs.1, 3). Acetabulum is subterminal and measures 2.48 ±0.93 X 2.76 ±0.36 (Figs. 1- 4). The ratio of genital sucker to acetabulum is 1:9.

The body is bean shaped (Fig. 5). The body surface is slightly corrugated (Fig. 6). Dome-shaped papillae are present on the tegumental surface sometimes in groups of 3-15 or discrete in different parts of the body (Fig. 7). The oral opening is terminal and surrounded by wrinkled, extensively ridged tegument that forms concentric elevated rings encircling the oral opening (Figs. 6, 8). The tegument covering these rings are corrugated into small tubercle-like structures that are more pronounced around the mouth opening, but gradually diminish posteriorly (Fig. 9). Numerous dome-shaped papillae are seen on these tegumental tubercles (Fig. 9). The genital sucker is ventrally situated at the middle of the anterior third of the body (Figs. 5, 6), where it is surrounded by a tyre-shaped elevation of the tegument (Fig. 10). The tegument covering the genital opening is irregularly corrugated and lacks papillae (Figs. 10, 11). The tegument covering the outer border of the subterminal acetabulum is somewhat smooth, while that close to the center is corrugated in a bee-comb like structure (Figs. 12, 13).

***Carmyerius gregarious*:** Description is based on 8 hand cut, 6 flattened carmine stained specimens and 5 haematoxylin and eosin stained cryostat-cut sections.

Live adults are reddish in color. Flattened mounted specimens are conical and measures 16.49 ± 3.74 X 6.62 ± 2.06 (Figs. 14-16). Oral opening is terminal (Figs. 14-16). Pharynx lacks diverticula, measures 0.56 ± 0.09 X 0.51 ± 0.15 and leads to oesophagus that measures 0.33 ± 0.12 X 0.25 ± 0.10 (Figs. 14-16). Oesophagus bifurcates into two caeca which extend until the level of the ovary (Figs. 14-17). The right caecum measures 9.22 ± 4.22 and the left measures 9.17 ± 2.10. Ventral pouch is ovoid, extending till the level of the testes and measures 7.93 ± 2.44 X 3.39 ± 1.00 with crescent-shape opening (Figs.15,17). Acetabulum is small, terminal and measures 1.50 ± 0.20 X 2.4 ± 0.31 (Figs. 14-16). Testes are lobed, slightly tandem and subequal (Figs. 14-17). The right testis measures 1.22 ± 0.57 X 1.08 ± 0.52 while the left measures 1.65 ± 0.38 X 1.11 ± 0.32. The ovary is small, spherical and intertesticular and measures 0.35 ± 0.23 X 0.30 ± 0.10 (Figs. 14-16). Vitelline glands are follicular, overlap caeca and restricted at the middle third of the body (Figs. 14,15,17) Genital opening opens ventrally in the ventral pouch in the anterior third of the body (Figs. 16, 17).

The body is pear-shaped (Fig. 18) and covered with transversely wrinkled tegument (Fig. 19). Tegumental papillae are irregularly distributed on the body surface densely aggregating around the terminal oral opening (Fig. 20) and sparsely around acetabulum (Figs. 22, 25, 26). High magnification of the corrugated tegument around the oral opening shows irregularly distributed tubercle-like structures that carry numerous ciliated papillae (Fig. 21).

Table 1: Comparison of the measurements of adult *C. cotylophorum* recorded in the present study with those recorded in previous studies (in mm)

Structures	Eduardo (1982b) (Hand cut technique)	Eduardo (1985) (Hand cut technique)	El-Seify *et al*. (1999) (Flattened whole mount technique)	Racioppi *et al*. (1994) (Flattened whole mount technique)	Romero *et al*., (2009) (Flattened whole mount technique)	Romero *et al*., (2009) (Flattened whole mount technique)	Present study*
Body length	4.1-6.8	4.82-8.93	11.9	7-13	5.88-6.42	6.20-8.36	11.97± 1.68
Body width	2-3.35	2.14-3.45	4.3	-	3-312	2.02-2.61	4.25 ±0.67
Acet. Diameter	1.28-181	1.51-2.34	2.5	1.6-2.1	1.38-1.96	1.50-1.70	0.60 ± 0.21
Pharynx length	0.57-0.84	0.61-1.01	-	0..7-0.85	0.7-0.86	0.64-0.84	0.37 ± 0.14
Pharynx width	0.51-0.58	0.60-0.85	-	-	0.44-0.55	0.57-0.61	0.41± 0.10
Oesophagus	0.51-0.78	0.37-1.01	-	-	0.47-0.57	0.57-0.81	0.25 ± 0.15
AT long	0.53-0.97	0.61-1.05	1.8	-	1.04-1.39	0.98-1.20	1.56 ± 0.27
AT wide	1.01-1.54	1.20-2.02	1.9	-	1.3-1.35	1.00-1.36	1.6 ± 0.46
PT long	0.53-0.93	0.73-1.98	1.9	-	0.6-0.7	0.80-0.91	1.4 ± 0.36
PT wide	0.83-1.96	2.10-2.63	1.9	-	1.66-2	1.50-1.75	1.58 ± 0.4
Pars Prostatica	0.9-0.32	-	-	-	0.20-0.26	0.28-0.40	-
Ovary diameter	0.28-0.55	0.49-0.55	0.5	0.5-0.62	0.58-0.69	0.45-0.61	0.50 ± 0.13
Ratio Acetabulum-body length	1: 3-3.7	1:2.5-4.3	-	-	1:3.8-4.3	1: 4.1-4.9	-
Ratio pharynx-body length	1:6-8.6	1:7.3-10	-	-	1: 7.5-8.4	1: 9.7-11.2	-
Ratio pharynx-acetabulum	1:1.9-2.5	1:2-2.3	-	-	1:1.9	1:2-2.5	-
D. E. C.1 of Acetabulum	10-14	10-16	-	-	10-15	12-14	-
D. E. C.2 of Acetabulum	34-39	-	-	-	27-33	26-30	-
D. I. C. of Acetabulum	38-44	36-48	-	-	38-41	36-44	-
V. E. C. of Acetabulum	16-20	9-14	-	-	13-15	11-15	-
V. I. C. of Acetabulum	47-50	45-59	-	-	48-53	42-55	-
M. E. D. of Acetabulum	14-26	10-14	-	-	12-16	16-25	-

AT- anterior testis; DEC- dorsal external circular (of acetabulum); DIC- dorsal internal circularof acetabulum; MED-median external circular of acetabulum; PT- posterior testis; (in mm); VEC- ventral external circular of acetabulumand VIC- ventral internal circular of acetabulum.

* Description in the present study is based on hand cut, flattened whole mount and histological technique.

Acetabulum is tyre-shaped (Fig. 22), surrounded by concentrically corrugated tegument (Fig. 23) and formed of three concentric rings (Fig. 22). The tegument covering the outer ring is slightly corrugated with concentric farrows and papillae; the middle is extensively corrugated with numerous papillae, while the inner is feebly corrugated (Fig. 25). The tegument covering the area boarding the central opening of acetabulum is flaky (Figs. 24, 25). Dome-shaped papillae with minute pores are observed on the tegument on the middle and outer ring of the acetabulum (Fig. 26). The opening of the ventral pouch is crescent-shaped and covered with corrugated tegument (Fig. 27).

***Fischoederius elongatus*:** Description is based on 8 hand cut, 6 flattened carmine stained specimens and 5 haematoxylin and eosin stained cryostat-cut sections.

Live adults are pink in color. Flattened mounted specimens are elongate measuring 14.3 ± 1.24 X 5.1± 0.89 (Figs. 28-31).Pharynx lacks diverticula and measures 0.18 ± 0.06 X 0.19± 0.04 (Figs. 28-30). Oesophagus measures 0.18 ± 0.02 X 0.18 ± 0.01 (Figs. 28, 30). Ventral pouch is ovoid in shape, extending from oesophagus till the level of the testes and measures 4.86±0.9 X 2.79 ±0.4 (Fig. 31). Acetabulum is terminal and measures 2.5 ± 0.20 X 2.63 ± 0.21 (Figs. 28-30).Caeca are un-equal, the right extends to the end of the second third of the body and measures 8.4 ± 2.4 in length while the left extends to the level of the ovary and measures 6.2 ± 3.00 (Figs. 28-30). Testes are subequal, lobed and slightly oblique and posteriorly located (Figs. 28, 30, 31). The right anterodorsal testis measures 2.0 ± 0.5 X 2.16 ± 0.46, while the left measures 1.8 ± 0. X 2.2 ± 0.47. Ovary is antero-dorsal to left testis and measures 0.90 ± 0.19 X 0.94 ± 0.24 (Figs. 28-31). Vitelline follicles are small, extending in the lateral sides from the level of the anterior margin of the testes until the oesophagus (Figs. 28-31).

The body is elongate with a concave ventral and convex dorsal surface (Figs. 32, 33). Body tegument is transversely wrinkled (Figs. 32, 34). The oral opening is surrounded with concentric tegumental ridges (Figs. 39-41). High magnification of the tegument surrounding oral opening reveals the presence of dome shaped tegumental papillae that may be ciliated or carry minute pores (Figs. 40, 41). Acetabulum is terminal (Fig. 33) and carries aggregation of dome-shaped papillae (Fig. 37). Papillae are also distributed in different parts of the body (Fig. 38). Papillae are dome shaped; some may have cilia or minute pores (Fig. 38). An oval subterminal opening of ventral pouch is located posteroventral to the oral opening (Fig. 34). The tegument surface surrounding the opening of ventral pouch is extensively ridged and tuberculated and carry numerous dome shaped papillae that may have pores or cilia (Fig. 35).

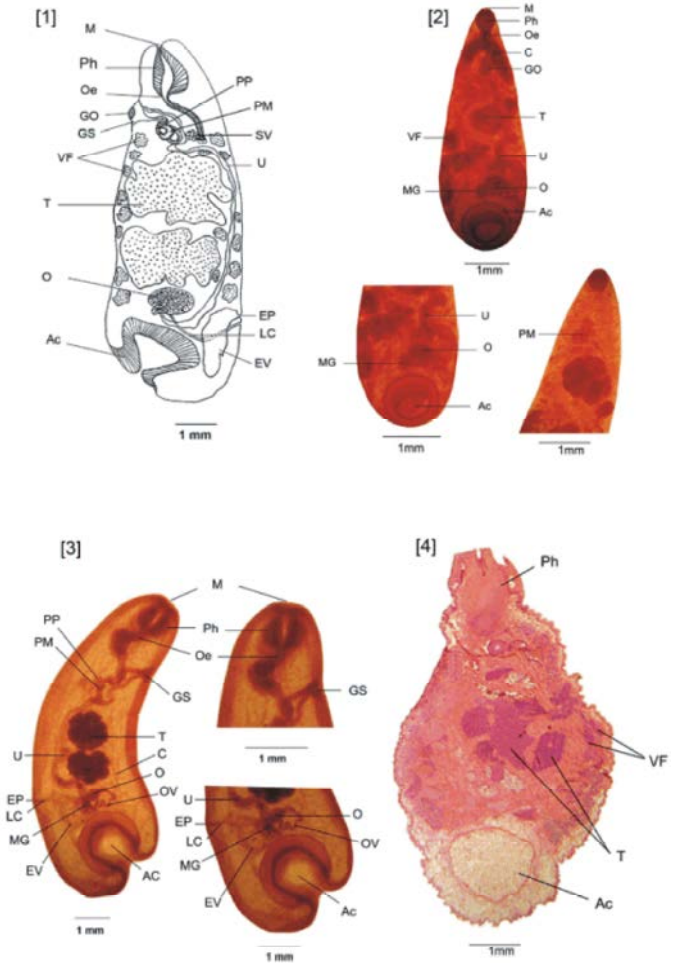


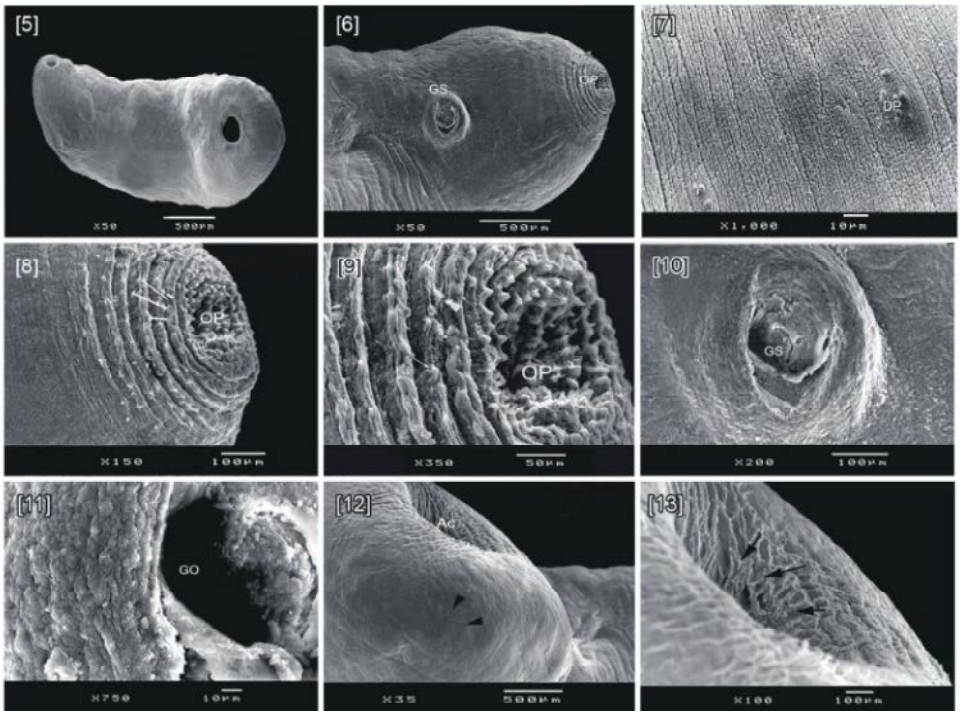
Fig. 1: *Diagrammatic drawing of hand cut section of Cotylophoron cotylophorum: Ac (acetabulum), EP (excretory pore), EV (excretory vesicle), GO (genital opening), GS (genital sucker), LC (Laurer's canal), M (mouth), MG (Mehils' gland), O (ovary), Oe (oesphagus), Ph (pharynx), PM (pars musculosa), PP (pars prostatica), SV (seminal vesicle), T (testis), U (uterus) VF (vitteline follicles). Fig. (2): Photomicrograph of flattened whole mount C. cotylophorum (carmine stain): Ac (acetabulum), C (caecum), GO (genital opening), M(mouth), MG(Mehils' gland), O (ovary), Oe (oesphagus), Ph (pharynx), PM (pars musculosa), T (testis), U (uterus) and VF (vitelline follicles). Fig. (3): Photomicrograph of hand cut section of C.Cotylmphorum (carmine stain): Ac (acetabulum), EP (excretory pore), EV (excretory vesicle), C (caecum), M (mouth), GO (genital opening), GS (genital sucker), LC (Laurer's canal), MG (Mehils' gland), O (ovary), Oe (oesphagus), OV (oviduct) Ph (pharynx), PM (pars musculosa), PP (pars prostatica), T (testis) and U (uterus). Fig. (4): Photomicrograph of histological section of C. cotylophorum (haematoxylin and eosin stain): Ac (acetabulum), Ph (pharynx), T (testes) and VF (vitelline follicles)*

DISCUSSION

Identification of the three encountered species was based on examination of numerous sagittal sections for each species to examine the shape, structure and relative position of organs with valid taxonomic characteristics (Pharynx, acetabulum, testes, genital atrium, etc…) as referred to in the taxonomic keys [1, 2, 16]

The morphometric comparison of adult *C. cotylophorum* described in the present study with the previous studies is listed in Table 1.

According to Eduardo [1], the occurrence, distribution and shape of the tegumental papillae have an important taxonomic value and can be employed to support other characteristics in the specific identification of some mammalian amphistomes. The author reported



Figs. 5-13: *Scanning electron micrographs of Cotylophoron cotylophorum*
Fig. (5): whole body of adult C. Cotylophorum. Fig. (6): General view of anterior part of the body showing terminal oral opening (OP) and genital sucker (GS) in the first third of the body. Fig. (7): Body surface is slightly corrugated with dome-shaped papillae (DP) arranged in groups. Fig. (8): Terminal oral opening (OP) surrounded by wrinkled tegument forming concentric elevated rings (arrows) encircling the mouth opening. Fig. (9): Corrugated tegument surrounding oral opening (OP) with tubercle-like structures (arrows) that are more pronounced around the oral opening. Fig. (10): Genital sucker (GS) opens in the center of an ovoid tyre-shaped elevation of the tegument. Fig. (11): Irregularly corrugated tegument surrounded genital opening (GO). Figs. (12, 13): Posterior end showing the subterminal acetabulum (Ac) note that the tegument covering the outer border of the acetabulum is smooth (arrow heads), while that close to the center is corrugated in a bee-comb like structures (arrows)

that among the genus *Cotylophoron*, the tegumental papillae are located anteriorly and are dome to conical shaped and either ciliated (*C. bareilliense*), non-ciliated (*C. cotylophorum*) or even absent (*C. macrosphinctris*).

In the present study, dome-shaped papillae were distinguished on the tegumental surface, distributed in groups or discrete in different parts of the body. These papillae were also detected on the tubercle-like structures of the corrugated rings around the oral opening. This distribution pattern agrees with the previous description based of SEM observations [18-25]. However, Eduardo [1] described the papillae as conical to dome-shaped and Hiekal and Hilali [26] reported that both the anterior and posterior thirds of the worm were smooth with button-like papillae. In accordance with Eduardo [2], the ventrally curved conical shaped body, absence of ventral pouch and cirrus sac, presence of genital sucker, post testicular position of ovary and Mehlis' gland and Laurer's canal crossing the excretory vesicle are the most taxonomic valid characteristics that confirm the identification of *C. cotylophorum*.

The tegument surrounding the terminal oral opening revealed in the present study as wrinkled and extensively ridged, forming concentric elevated rings which are corrugated into small tubercle-like structures, was previously described as balloon-like projections [26]. In agreement with Eduardo [2] and Tandon and Mairta [27], the posterior third of the body of the present material is transversely wrinkled ventrally and dorsally. On the other hand, Veerakumari and Paranthman [28] reported that the whole tegument of the worm is smooth without corrugations.

Although the structure of the pharynx is one of the most important taxonomic valid characteristics of Paramphistomes, scientists disagreed on its description even when referring to the same species. Based on the

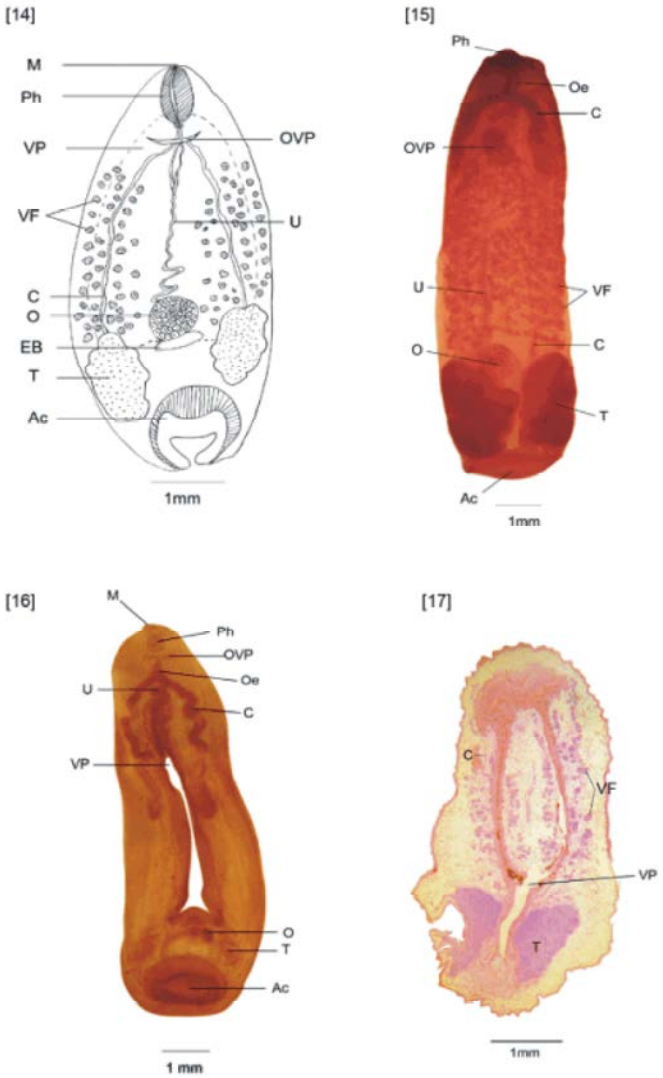
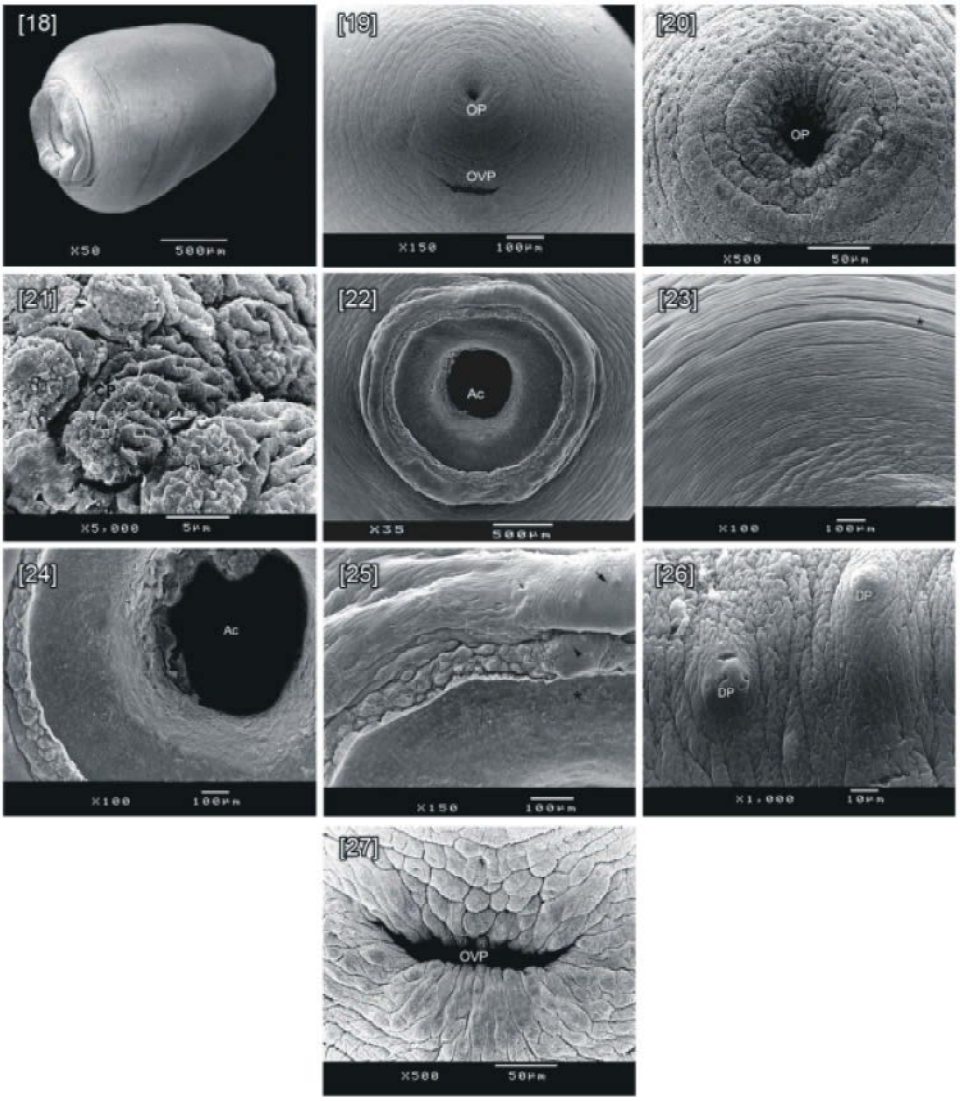


Fig. 14: *Diagrammatic drawing of hand cut section of Carnyerius gregarious showing: Ac (acetabulum), C (caecum), EB (excretory bladder), OVP(opening of ventral pouch), (genital opening), M (mouth), O (ovary), Oe (oesophagus), Ph (pharynx), T (testis), U (uterus), VF (vitteline follicles) and VP (ventral pouch). Fig. (15): Photomicrograph of hand cut section of C. gregarious (carmine stain):Ac (acetabulum),C (caecum),OVP(opening of ventral pouch), M (mouth), O (ovary), Oe (oesophagus), Ph (pharynx), T (testis), U (uterus) and VP (ventral pouch). Fig. (16): Photomicrograph of flattened whole mount of C. gregarious (carmine stain): Ac (acetabulum), C (caecum), O (ovary), Oe (oesophagus), OVP (opening of ventral pouch), Ph (pharynx), T (testis), U (uterus) and VF (vitteline) Fig. (17): Photomicrograph of histological section of C. gregarious (haematoxylin and eosin stain): C (caecum), T (testis), VF (vitteline follicles) and VP (ventral pouch)*

arrangement and development of musculature, Eduardo [1] and Näsmark [29], agreed on typing of the pharynx into nine types. Gupta and Nakhasi [30] by applying flattened technique, Eduardo [1] by applying SEM technique, Miranda and Costa [31] and Abou Laban [32] by applying cryostat section and SEM technique, described the phaynx of *C. cotylophorum* as a highly muscularized organ following the oral opening, with no mention of an oral sucker. However, when Hiekal and Hilali [26] described flattened specimens and El-Seify [9] and Veerakumari and Paranthman [28] described cryostat sections and SEM, they referred to the musculature of the pharynx as an oral sucker surrounding the mouth opening.



Figs. 18-27: *Scanning electron micrographs of Carmyerius gregariou*

Fig. 18: Pear-shaped body covered with transversely wrinkled. Fig. (19): Terminal oral opening (OP) surrounded by wrinkled tegument and subterminal opening of ventral pouch (OVP). Fig. (20): Tegumental papillae irregularly distributed on the body surface, densely aggregated around the oral opening (OP). Fig. (21): Tegument around oral opening shows irregularly distributed wrinkled trubecule-like structures carrying ciliated papillae (CP). Fig. (22): Acetabulum (Ac) tyre-shaped and formed of three concentric rings. Fig. (23): Tegument surrounding the acetabulum is concentrically corrugated (star). Fig. (24): The middle ring surrounding acetabulum (Ac) is extensively corrugated, while the inner is feebly corrugated with flaky inner surface. Fig. (25): Part of acetabulum showing part of the outer ring (arrow) with few concentric farrows and papillae, the middle (arrow head) which is extensively corrugated with numerous papillae and the inner (star) which is feebly corrugated. Fig. (26): Dome-shaped papillae (DP) with minute pores on the tegument covering the middle ring of the acetabulum. Fig. (27): Subterminal crescent-shaped opening of ventral pouch (OVP)

In agreement with Eduardo [1] and Miranda and Costa [31], median sagittal sections in the present material showed the presence of calicophoron type pharynx which is a characteristic feature of the genus *Cotylophoron*. On the other hand, Gupta and Nakhasi [30] recorded the presence of Paramphistomum type in *C. cotylophorum*.

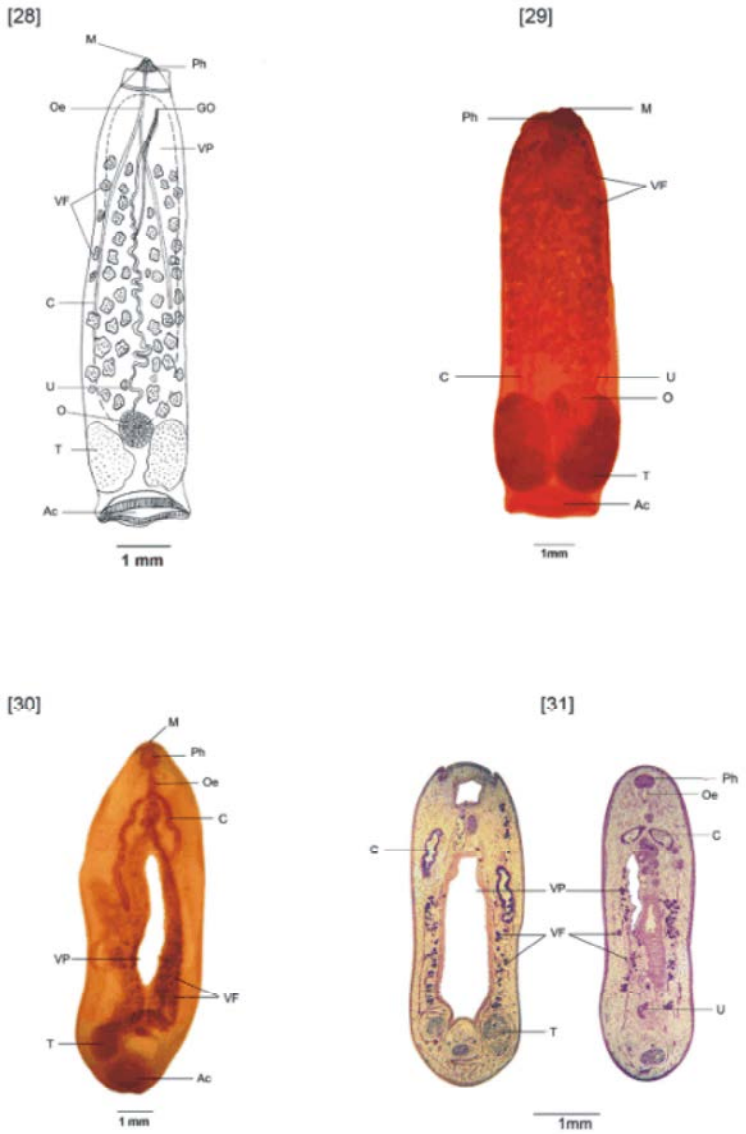
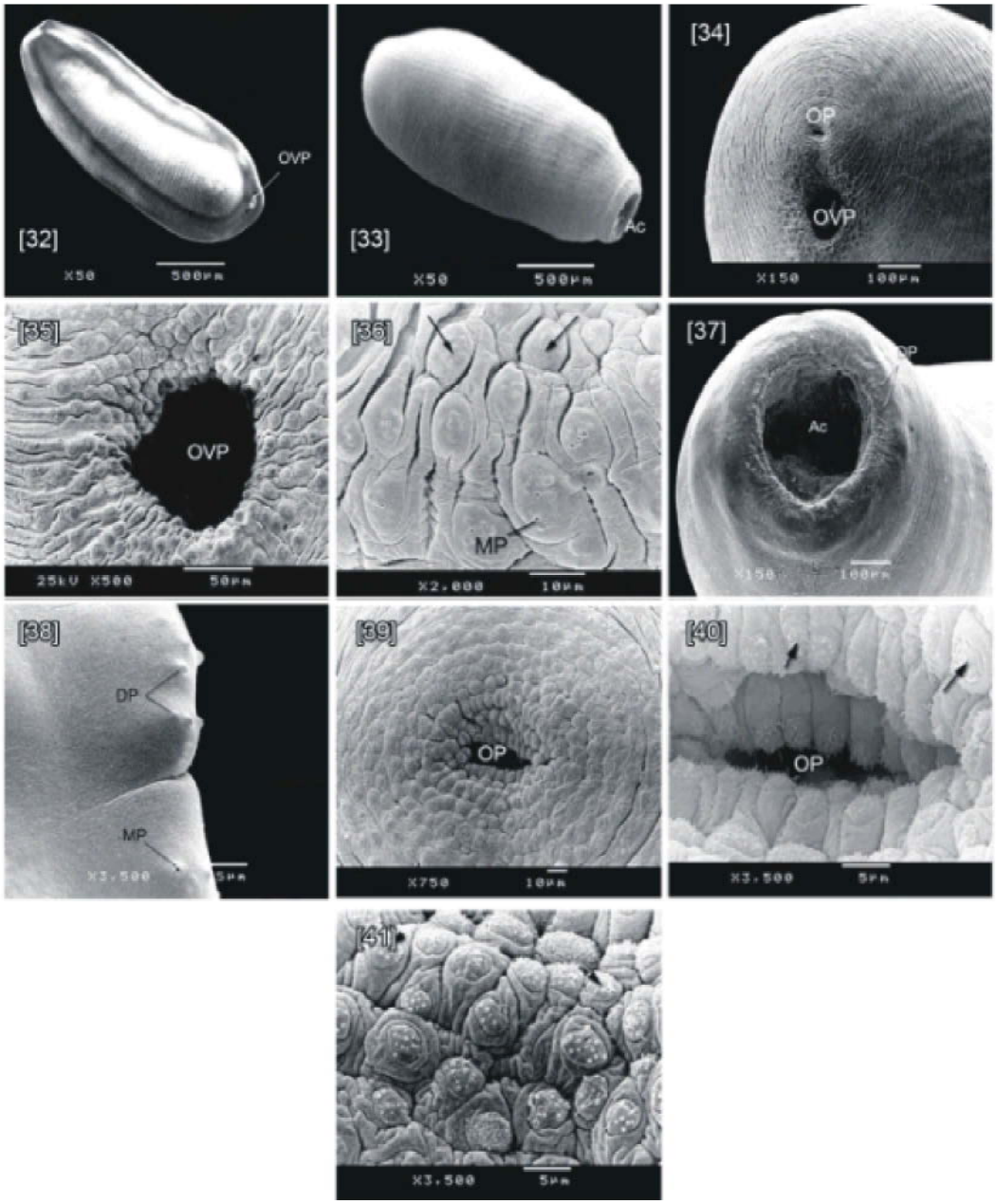


Fig. 28: *Diagrammatic drawing of hand cut section of Fischoderius elongatus: Ac (acetabulum), C (caecum), GO (genital opening), M (mouth), O (ovary), Oe (oesophagus), Ph (pharynx), T (testis), U (uterus), VF (vitteline follicles) and VP (ventral pouch). Fig. (29): Photomicrograph of hand cut section of F. elongatus (carmine stained): Ac (acetabulum), C (caecum), M (mouth), Oe (oesphagus), Ph (pharynx),T (testis), VF (vitelline follicles) and VP (ventral pouch). Fig. (30): Photomicrograph of flattend whole mount of F. elongatus (carmine stain): Ac (acetabulum), C (caecum), M (mouth), O (ovary), Ph (pharynx), T(testis), U(uterus), VF(vitteline follicles). Fig. (31): Photomicrograph of histological sections of F. elongatus (haematoxylin and eosin stain): C (caecum), O (ovary), Oe (oesphagus), Ph (pharynx), T (testis), U (uterus), VF (vitelline follicles) and VP (ventral pouch)*

The main characteristic features of the present material agree with previous reports, such as the extension of the intestinal ceaca to the acetabulum level [2,9,32]; the subterminal acetabulum with cobblestone-like tegument and dome-shaped papillae at its edge [2, 26, 32], (While El-Seify [9] described the acetabulum as terminal); the tandem testes [2, 9, 30, 32]; the spheroid ovary [9, 28] and the extent of the vitelline glands from the level of caecal bifurcation to the middle third of the body [30].

Näsmark [29] used the term terminal atrium to define the whole terminal structure of the genital system which opens to the exterior through the genital pore.



Figs. 32-41: *Scanning electron micrographs of Fashioderius elongates*

Fig. 32: *Elongated body with concave ventral surface, transversely wrinkled tegument and subterminal opening of ventral pouch (OVP). Fig. (33): Elongated body with convex dorsal surface, transversely wrinkled tegument and terminal acetabulum (Ac). Fig. (34): Subterminal opening of ventral pouch (OVP) and terminal oral opening (OP). Fig. (35): Subterminal opening of ventral pouch (OVP) surrounding with numerous papillae. Fig. (36): High magnification of the tegument surrounding the opening of ventral pouch showing the presence of teguemental papillae (arrows) that carry minute pores (MP). Fig. (37): Acetabulum (Ac) carries aggregations of dome-shaped papillae (DP). Fig. (38): Aggregation of dome-shaped papillae (DP) with minute pores (MP) distributed in different parts of the body. Fig. (39): Oval oral opening (OP) surrounding by tubercaluted structures that carries papillae. Fig. (40): Tubercaluted structures carrying ciliated papillae (arrows) surrounding oral opening (OP). Fig. (41): Magnification of Fig. 40.*

Table 2: Comparison of measurements of adult *C. gregarious* in the present and those recorded in previous studies (in mm)

Structure	El-Seify *et al*. (1999)	The present study
Body dimension	12.8X4.6	16.49 ± 3.74 X 6.62 ± 2.06.
Right testis	1.4X0.9	1.22 ± 0.57 X 1.08 ± 0.52
Left testis	1.7X1.0	1.65 ± 0.38 X 1.11 ± 0.32
Ovary	0.4X0.4	0.35 ± 0.23 X 0.30 ± 0.10
Caeca	-	right caecum:9.22 ± 4.22
		left caecum: 9.17 ± 2.10.
Ventral pouch	-	7.93 ± 2.44 X 3.39 ± 1.00
Acetabulum	1.5 X 2.3	1.50 ± 0.20 X 2.4 ± 0.31
Body dimension	12.8X4.6	16.49 ± 3.74 X 6.62 ± 2.06.

This includes the genital papillae, the fold of the wall surrounding it and different cavities which the wall encloses. Eduardo [1] used the same term to refer only to the cavity surrounding the genital papillae and enclosed by the genital fold, to differentiate it from another cavity, the genital atrium. All these authors agreed that the genital atrium which was redescribed later as terminal genitalium, is the most valid characteristic used in differentiating species belonging to Paramphistomidae. Twenty two types of genital atrium have been reported by Eduardo [1], the present material possess a *Cotylophoron* type genital atrium which is characterized by the absence of genital sphincter, well development of radial fibers and ventral fold. The genital atrium is provided with well developed genital sucker that opens at the end of the first third of the body and is surrounded with well developed papillae, that was clearly observed in SEM. This finding agrees with the description of Eduardo [2], Hiekal and Hilali [26] and Romero *et al.* [33].

The comparison between measurements of adult *C. gregarious* recorded in the present study and those reported by other authors is recorded in Table (2).

In agreement with Gibson *et al.* [16]; the shape of the body; the presence of terminal acetabulum; presence of ventral pouch; the presence of pre-acetabular, symmetrical and lobed testes; intesticular ovary; the opening of genital pore inside the ventral pouch are very prominent and are definitely the most valid taxonomic characteristics of *C. gregarious*.

In the present material, the distributions of the ciliated papillae on the body surface as densely aggregating around the terminal oral opening and sparsely around acetabulum, agrees with the SEM description of Sey [34] and Eduardo [3]. However, the former author described the papillae as long and non-ciliated while the second referred to them as dome-shaped and ciliated. However, Eduardo described the papillae differently in different reports. In 1982a, he described the tegumental papillae of *C. gregarious* as short and stumpy without hair, while in 2004 the author described them as dome-shaped and ciliated, more recently, in 2005, he described them as cone-shaped. Although Sey [34] described dome to conical non-ciliated papillae distributed regularly around the genital opening, yet the present study did not reveal any on the surface of the opening of the ventral pouch which enclosed the genital opening.

The present hand cut sections revealed the presence of an ovoid ventral pouch, extending from the genital pore till the posterior level of the testes. According to the description of Eduardo [4] using SEM technique, the ventral pouch was roughly triangular and the apices bifid and dorsally directed. Using flattened whole mount technique, El-Seify [9] mentioned that the pouch extends until the acetabulum level. Such differences may result from variations in the used techniques.

The present hand cut and cryostat sections and flattened whole mount techniques revealed the testes as lobed and subequal, while El-Seify [9], using flattened whole mount technique, recorded oval and symmetrical testes. Such differences may result from the pressure used in flattened technique adopted by El-Seify *et al.* [9]; such technique may affect the size and orientation of the organs.

According to Eduardo [4] and Gibson *et al.* [16], the shape of the body, position of acetabulum, extent of the caeca posteriorly just anterior to acetabulum, lobed obliquely positioned testes in posterior half of the body, subspherical and post-testicular ovary and the narrow and limited ventral pouch are the most taxonomic valid characteristics of the genus *Fischoederius*, as revealed by examining sagittal sections taken at different plans of the present material.

Although the present SEM findings revealed tegumental ciliated papillae that carry minute pores, yet Eduardo [1], using the same technique, described the tegumental papillae as short and stumpy without hair and their surface carrying bleb-like structures or enclose minute pores. The cilia of the papillae are probably tactile that may fall off during specimen processing.

The tegumental surface of *F. elongatus* is provided with different structures that varied according to their location. The tegumental wrinkles around the oral opening carried round papillae with minute pores or cilia. Using SEM technique, Sey [34] described these papillae as dome to conical non-ciliated papillae. The present SEM findings revealed that the tegumental surface surrounding the opening of the ventral pouch is extensively ridged forming tuberculated structures that carry papillae similar in structure to those described by Sey [34].

To evaluate the techniques used to examine amphistomes,. hand cut, whole mount flattened and histological techniques in addition to scanning electron microscopy were employed to demonstrate the internal organs and external surface of *C. cotylophorum*, *C. gregarious* and *F. elongatus*, the use of these techniques had the following advantages: 1) obtain a full description and orientation of the suckers, digestive tract, genetalia, excretory organs and pouches, if present; 2) record accurate measurements and relative position of organs in relation to each other and to the body; and 3) complement the previous descriptions of the surface topography of the three species understudy, mainly the shape and distribution of tegumental papillae.

Lux *et al.* [12] reported that hand cut sectioning often provides an adequate method for rapid and inexpensive microscopic observation of the internal structures of large helminthes, that their organs are masked by dense overlying tissues. He referred to this technique as very simple and often results in high quality images. In *Cotylophoron*, it helped in detecting the shape and position of both oral opening and acetabulum, organs of the intestinal tract, both male and female genetalia and the excretory vesicle and its opening. In addition, it confirmed the presence of two valid characters of this genus; first, the presence of genital sucker surrounding the genital opening that opens at the end of the first third of the body and second, extent of Laurer's canal from ootype across the excretory vesicle.

In *Carmyerius,* in addition to the general description and position of oral opening, acetabulum, digestive tract and genetalia, examination of hand cut sections helped in detecting the subequality of the caeca, the ovoid shape of the ventral pouch and its extent posteriorly till the testes level and the crescent shape of genital opening. Such characteristics have not been clearly revealed in previous descriptions.

In *Fischoederius*, this technique helped to record unequal ceaca, slightly juxtaposed testes and the extent of the ventral pouch to the second third of the body and the vitelline follicles till the oesophagus level.

Whole mount flattened technique is usually used for examination of thick trematodes. Although such technique may destroy the specimen affecting the measurements and orientation and relative position of some organs, but it helps in detecting all the internal organs even those lying at different levels. In *Cotylophoron cotylophorum*, the whole mount flattened preparations revealed the lateral distribution of the vetilline follicles all over the body and confirmed the position of the oral opening and the ventral sucker. In *Carmyerius*, the flattened preparations confirmed the subequality of the ceaca and the juxtaposed position of the testes, while in *Fischoederius*, this technique confirmed the subterminal position of the terminal position of ventral sucker and the presence of un-equal caeca, where the right one extends to the end of the second third of the body while the left extends to the level of the ovary, this description has been well illustrated in hand cut preparations.

In histological sections of the three species, few characteristics could be distinguished depending on the level of which the section has been taken. However, sophisticated serial sections may overcome this drawback. The most important characteristics that were provided with such technique were the shape and extent of the ventral pouch in both *Carmyerius* and *Fischoederius* and the high muscularization of the acetabulum in all histological sections.

The most important characteristic revealed by scanning electron microscopy is the presence, distribution and structure of papillae that were recorded differentially in the three understudied species.

CONCLUSION

It could be concluded that the use of both scanning electron microscopy and hand cut section techniques simultaneously is essential as they provide powerful and accurate methods for identification and classifications of large and thick trematode species.

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