

Original Article

Morphology and histology of the adult *Paramphistomum gracile* Fischeoeder, 1901

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In the present study, we evaluated the histological morphology of the adult *Paramphistomum (P.) gracile*. Adult flukes with bodies 5~15 mm in length and 2~7 mm in width were subjected to histological analysis. Longitudinal and transversal serial-sections were stained with hematoxylin and eosin, and examined. The body surface and longitudinal section of *P. gracile* were also assessed using scanning electron microscopy. In this species, the anterior sucker and posterior sucker (acetabulum) were present on an anterior and posterior part of the body, respectively. The major folds were located in the areas of the anterior sucker, genital canal, and posterior sucker. The fluke membrane was spineless at the tegument surface and in the tegument tissue. Histological data showed structural-systematic characteristics of the digestive tract, reproductive tract, excretory tract, copulatory organs, connective tissues, and muscle tissues. We attempted to elucidate the histological characteristics of *P. gracile* that might increase the knowledge and understanding of rumen fluke morphology.

Keywords: histology, light microscopy, morphology, *Paramphistomum gracile*, scanning electron microscopy

Introduction

Rumen flukes, also known as paramphistomes, are parasites that infect ruminants including cattle, goats, sheep, and water buffaloes. These organisms are a major cause of productivity and economic losses in many countries [5,18]. Rumen flukes belong to the Paramphistomoidea super family [24]. Paramphistomosis is a disease characterized by lower nutrition conversion, decreased milk production, and acute parasitic

gastroenteritis with high morbidity and mortality rates, particularly among young animals [12,13,23]. The prevalence of paramphistomosis is high throughout tropical and subtropical regions, particularly in Africa, Asia, Australia, Eastern Europe, and Russia [10,12,19]. It has been reported that specific parasites which cause the disease, including *Paramphistomum (P.) cervi*, *P. epiclitum*, and *Gastrothylax crumenifer* are most prevalent in tropical and subtropical areas [12,28]. Prasitirat *et al.* [21,22] reported that, cases of paramphistome infection in Thailand are mainly due to *Paramphistomum* sp.

A limited number of studies on the structure and histology of *Paramphistomum* sp., including vitelline cells [10], eggshell, and testis [8], have been performed. Identification of these trematodes is typically based on structural characteristics of parasites that have been fixed and compressed between two glass slides. However, these flattening techniques are unsatisfactory for species characterization, and little is known about structures and histology of their organ systems. In the present study, we evaluated the surface structure as well as the histology of various organs of *P. gracile* using scanning electron microscopy (SEM) and light microscopy (LM).

Materials and Methods

Collection of adult flukes

Adult *P. gracile* 5~15 mm in length and 2~7 mm in width across the mid-section were collected from the rumens of infected cattle sacrificed for consumption at a local abattoir (Pathum Thani Province, Thailand). The flukes were washed several times with 0.85% NaCl solution and processed immediately for SEM and LM according to the methods described by Panyarachun *et al.*

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[20] and Anuracpreeda *et al.* [1,3].

SEM

Adult parasites were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, USA), pH 7.2, at 4°C for 2 h. They were washed three times with the same buffer, and re-fixed in 1% osmium tetroxide (Sigma-Aldrich) with 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 1 h. After washing with distilled water, the flukes were dehydrated with increasing concentrations of ethanol (from 50% to 100%), and then dried in a HCP-2 critical point drying apparatus (Hitachi, Japan) using liquid carbon dioxide as a transitional medium for 15 min. The specimens were then mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus (SPI-Model sputter coater; Structure Probe, USA) for 4 min. Finally, the specimens were examined with a JSM-5400 electron microscope (JEOL, USA) operating at 15 kV.

LM

Carminic staining: Adult *P. gracile* were flattened between two glass slides (Sigma-Aldrich) and immersed overnight in alcohol-formal acetic acid fixative containing 10% formalin, 2% acetic acid, and 50% ethanol at room temperature. Next, the slides were washed with tap water and subsequently dehydrated in serial concentrations of ethanol (from 30% to 70%) for 1 h each before being immersed in Semichon's carmine at room temperature for 1 h. The slides were then washed in 70% ethanol and incubated in 1% acid alcohol (2% HCl in 70% ethanol) at room temperature until a pink stain was observed. Finally, the parasites were washed and dehydrated in a graded series of ethanol (80~100%) for 30 min each at room temperature, cleared with xylene (50~100%), and examined under a light microscope (BX51; Olympus, Japan).

Hematoxylin and eosin staining: Adult flukes were fixed in Bouin's fixative solution (Sigma-Aldrich) for 12 h and stored in 70% ethanol at room temperature. The specimens were dehydrated with a graded ethanol series (from 50% to 100%) and embedded in paraffin. Longitudinal, transverse, and coronal serial-sections (6- μ m-thick) were cut with a RM2125 microtome (Leica Biosystems, USA). The sections were stained with hematoxylin and eosin (Sigma-Aldrich) and observed under a light microscope (BX51; Olympus, Japan).

Results

SEM

When observed by SEM, the ventral and dorsal surfaces of adult *P. gracile* about 5~15 mm in length and 2~7 mm in width were highly corrugated with transverse folds (Figs. 1A and B). The anterior and posterior suckers were positioned at the anterior and posterior ends, respectively

(Fig. 1A). Both suckers were rounded, had rows of papillae arranged in clusters, and were covered with wide transverse folds (Fig. 1A). The papillary clusters in the anterior sucker

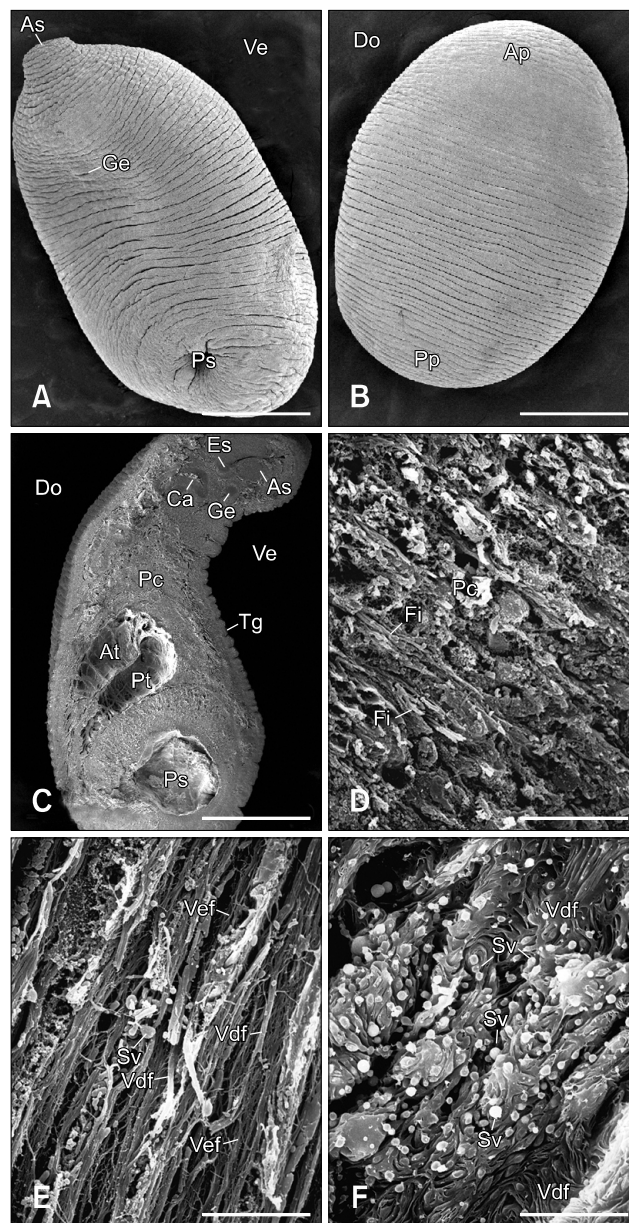


Fig. 1. Whole-body topography of the adult *Paramphistomum* (*P.* *gracile* Fischöeder, 1901 on the (A) ventral and (B) dorsal surfaces showing the anterior sucker, posterior sucker, and genital canal. (C) Longitudinal section of *P. gracile* showing the tegument, anterior sucker, esophagus, opening of the genital canal, caecum, parenchymal cells, anterior testis, and posterior testis. (D) Parenchymal cells and fibers. (E and F) Fine structures of the testes including the vas efferens, vas deferens, and seminal vesicle. As: anterior sucker, Ve: ventral area, Ge: genital canal, Ps: posterior sucker, Do: dorsal area, Ap: anterior part, Pp: posterior part, Es: esophagus, Ca: caecum, Pc: parenchymal cells, Tg: tegument, At: anterior testis, Pt: posterior testis, Fi: fibers, Vef: vas efferens, Vdf: vas deferens, Sv: seminal vesicle. Scale bars = 6 μ m (F), 8.6 μ m (D and E), 1 mm (A~C).

were larger than those in the posterior sucker. The genital canal was positioned at one-third the distance between the anterior and posterior tips, and surrounded by circular folds (Fig. 1A). Longitudinal sections of *P. gracile* contained various organs including the tegument, anterior sucker, posterior sucker, esophagus, genital canal, caecum, parenchymal cells, fibers, anterior testis, posterior testis, vas efferens, vas deferens, and seminal vesicles (Figs. 1C~F). No spines or ventral pouch were found. The sectional morphology included a Calicophoron-type pharynx, Paramphistomum-type posterior sucker, and Gracile-type genital canal opening (Fig. 1C). In addition, both the anterior and posterior testes were deeply lobed and tandem at the posterior half of the parasite's body. Moreover, the internal surface located near the testes contained numerous vas efferens, vas deferens, and seminal vesicles (Figs. 1E and F).

LM

Carmine staining: The whole mount of adult *P. gracile* subjected to carmine staining contained an anterior sucker, esophagus, caecal bifurcation, caecum, uterus, eggs within the uterus, testes, and posterior sucker (Fig. 2).

Hematoxylin and eosin staining: For the histological examination, longitudinal, transverse, and coronal sections of the parasites were cut as shown in Fig. 3. In the longitudinal section, the tegument appeared as folds with alternating grooves and no spines (Fig. 4A). The tegument formed a thick, continuous, acidophilic, and homogeneous layer that covered all parts of the adult fluke (Figs. 4A~C). The tegumental cells were located deep among the

parenchymal tissues (Figs. 4C~E) partitioned from the tegument and two underlying muscle layers by a clear basement membrane. Below the tegument were two layers of muscle: an external circular muscle and an internal longitudinal muscle. The interior of the parasite was filled with the parenchyma composed of loose connective tissues and parenchymal cells (Figs. 4D and E). The male reproductive organs (*i.e.*, the prostate gland and seminal vesicle) were adjacent to a genital canal around the posterior sucker (Fig. 4A). The female reproductive organs (*i.e.*, the uterus) were located between the posterior testis and posterior sucker (Figs. 4A and B). In the anterior region, the esophagus extended from the pharynx to an area close to the genital canal (Fig. 4C).

The transverse sections contained various organs starting from the round anterior sucker. The anterior sucker was lined by a fine thick tegument lacking spines and resting on a basement membrane. Below this membrane, four layers of muscles were observed (Figs. 4F and G). The first formed a fine circular layer. The second was a radial layer orientated in a position perpendicular to the first layer. The third and fourth were longitudinal and circular layers, respectively, surrounded by loose connective tissue. The anterior sucker connected the mouth with the pharynx (Figs. 5A and B). The pharynx had a circular or ovoid structure with four layers of muscles similar to the anterior sucker (Figs. 5A and C). The esophagus extended posteriorly from the pharynx to the posterior sucker. Layers of internal circular and external longitudinal muscles surrounded the esophagus (Figs. 5D~F). In the anterior part of the digestive tract, the cross section of the esophageal lumen had various forms. At the posterior part of the digestive tract, before the bifurcation of the intestinal

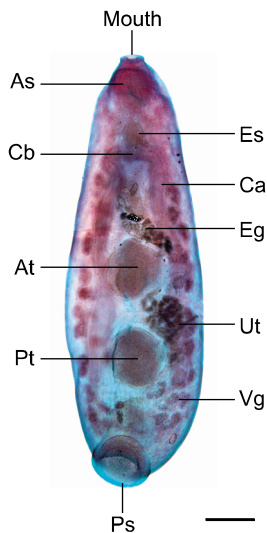


Fig. 2. Whole mount of an adult *P. gracile* Fischeoeder, 1901 showing the anterior sucker, esophagus, caecal bifurcation, caecum, uterus, eggs within the uterus, anterior testis, posterior testis, and posterior sucker. Cb: caecal bifurcation, Ut: uterus, Eg: eggs within the uterus. Scale bar = 50 µm.

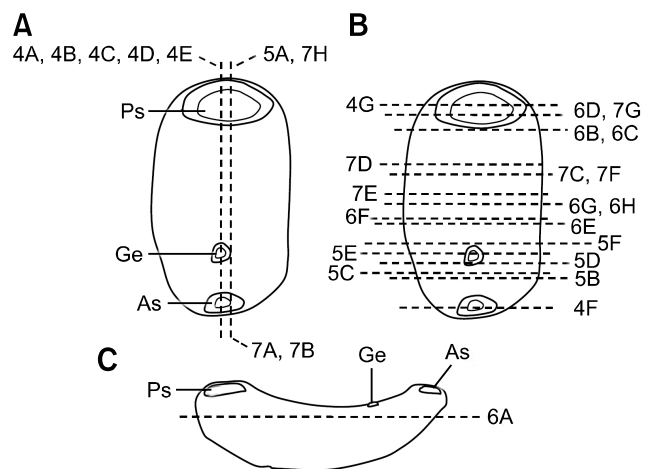


Fig. 3. Illustrations of the sectioning planes across the body of an adult *P. gracile* Fischeoeder, 1901. (A and B) Ventral views of the longitudinal and transverse sections, respectively. (C) Lateral views of the coronal sections.

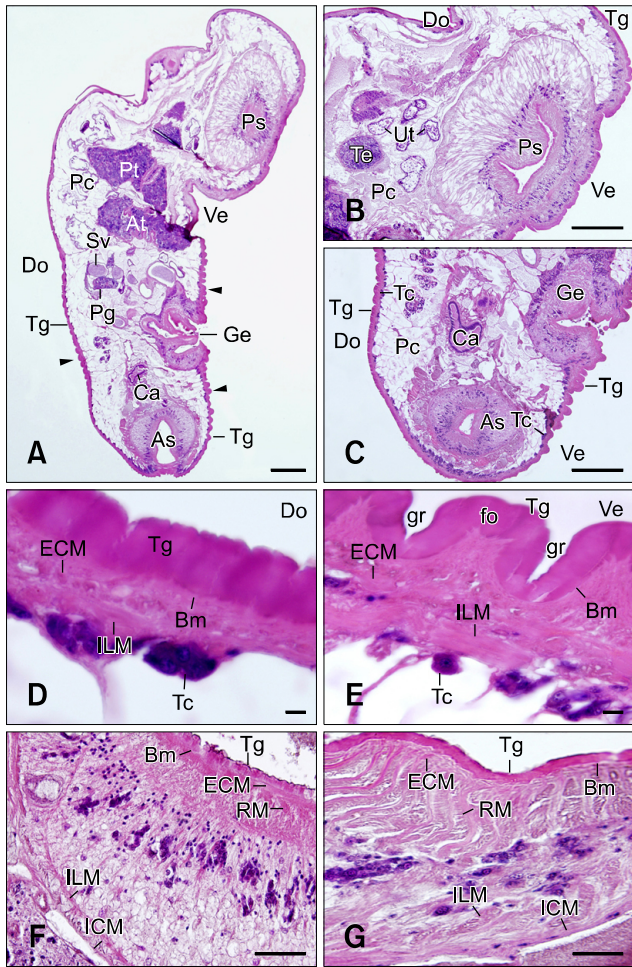


Fig. 4. Longitudinal sections of *P. gracile* Fischeoeder, 1901. (A) Whole body, (B) posterior portion, and (C) anterior portion containing the tegument, tegumental cells, anterior sucker, genital canal, caecum, parenchymal cells, anterior testis, posterior testis, seminal vesicle, prostate gland, and uterus. Arrowheads indicate major folds. (D and E) Longitudinal sections of the tegument and underlining muscles, tegumental cells, major fold, invaginated major grooves, basement membrane, external circular musculature, and internal longitudinal musculature. (F and G) Transverse sections of the anterior sucker and posterior sucker showing the tegument, basement membrane, external circular musculature, radial musculature, internal circular musculature, and internal longitudinal musculature. Tc: tegumental cells, Pg: prostate gland, ECM: external circular musculature, ILM: internal longitudinal musculature, Bm: basement membrane, gr: grooves, fo: fold, RM: radial musculature, ICM: internal circular musculature. Scale bars = 50 µm (D and E), 100 µm (B, C, F, and G), 150 µm (A).

caeca, the lumen was star-shaped (Figs. 5D and 6A). Numerous groups of glandular cells were lying along the pharynx and esophagus. The numbers of these glands decreased towards the end of the esophagus, and no glands were found in the caeca. Digestive glands appeared to be a major component of these glandular structures (Figs. 5A

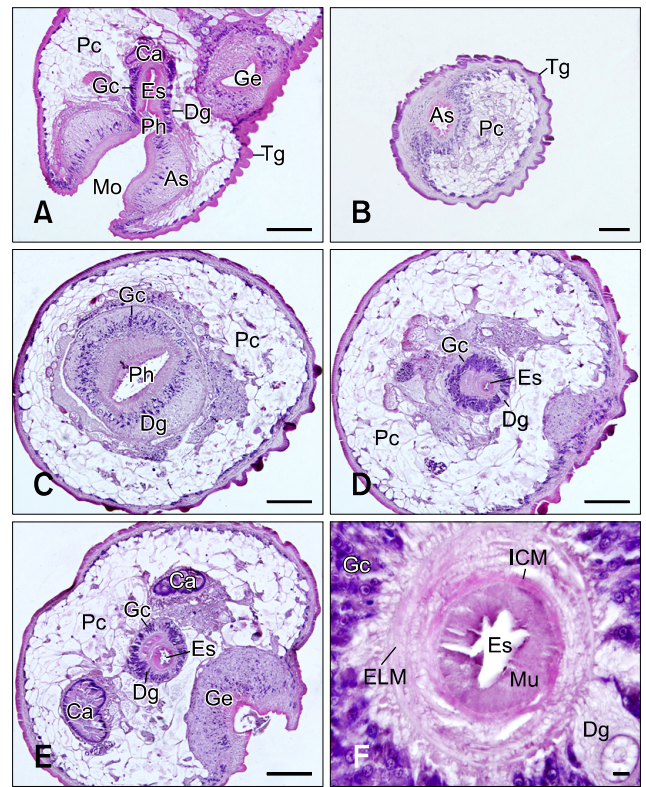


Fig. 5. (A) Longitudinal section of the digestive tract showing the mouth, anterior sucker, pharynx, esophagus, glandular cells, digestive gland, caecum, parenchymal cells, and genital canal. (B ~ E) Transverse sections of the digestive tract and beginning of the genital canal containing the anterior sucker, pharynx, esophagus, glandular cells, digestive gland, caecum, parenchymal cells, and genital canal. (F) High magnification of the transverse section of the esophagus showing the mucosa, external longitudinal musculature, internal circular musculature, and glandular cells. Gc: glandular cells, Dg: digestive gland, Ph: pharynx, Mo: mouth, Mu: mucosa. Scale bars = 50 µm (F), 100 µm (A, C, D, and E), 150 µm (B).

and C ~ F). The junction between the esophagus and caeca (Fig. 6A) was Y-shaped (the caecal bifurcation). The caeca were tubular structures with lumens of various sizes, and extended to the posterior part of the digestive tract. The caecum was surrounded by two layers of internal circular and external longitudinal muscles similar to the esophagus.

The genital canal was positioned ventrally and slightly anterior to the mid-point of the body. The ovoid ovary was located between the posterior testis and posterior sucker. It was surrounded by a thin layer of connective tissues and contained germ cells in various stages of differentiation (Fig. 6B). The oogonia were small basophilic cells with relatively little cytoplasm while the oocytes were larger cells with polygonal, ovoid or spherical shapes, larger nuclei, prominent nucleoli, and clear cytoplasm (Fig. 6C). The Mehlis' gland was positioned lateral and ventral to the ovary, towards the mid-point of the parasite. It was

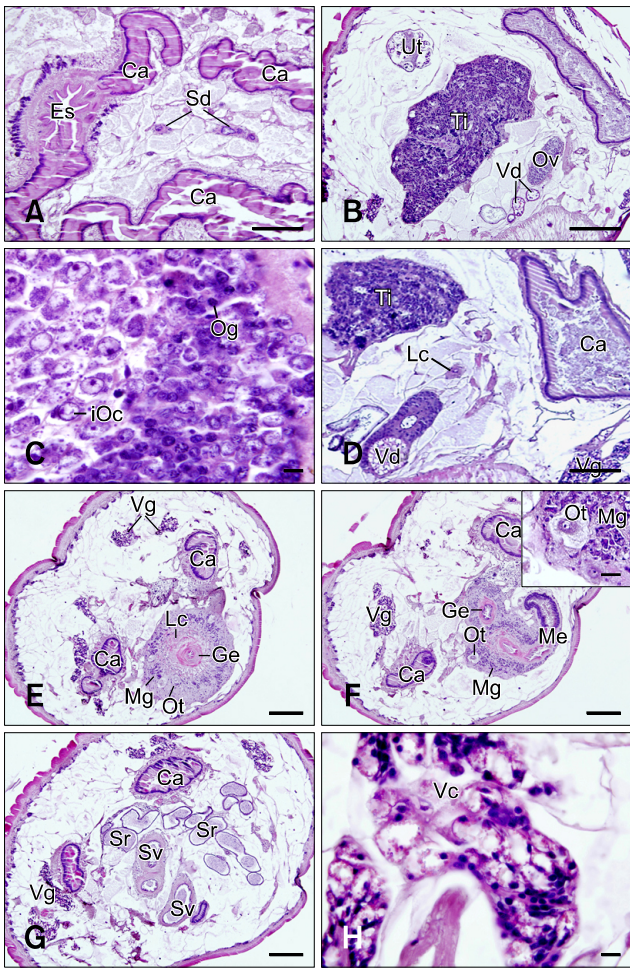


Fig. 6. (A) Coronal section of the digestive tract showing the Y-shaped caecal bifurcation at the distal end of the esophagus. (B ~ H) Transversal sections of the middle region of *P. gracile* showing the male and female reproductive organs comprised of the testis, seminal vesicle, ovary, vitelline duct, oogonia, immature oocytes, Laurer's canal, Mehlis' gland, ootype, vitelline gland, vitelline cells, genital canal, seminal receptacle, metraterm, and caecum. Sd: spermatid, Ti: testis, Ov: ovary, Vd: vitelline duct, Og: oogonia, iOc: immature oocytes, Lc: Laurer's canal, Mg: Mehlis' gland, Ot: ootype, Me: metraterm, Sr: seminal receptacle, Vc: vitelline cells. Scale bars = 50 μ m (C, F inset, and H), 100 μ m (A, B, and D), 150 μ m (E ~ G).

composed of a group of cells with slightly basophilic cytoplasm, large nuclei, and prominent nucleoli. The ootype was present in the center of this gland (Figs. 6E, F, and F inset). It appeared as a large duct lined by flattened cells and join with the vitelline ducts, seminal receptacle, and the beginning of Laurer's canal (Figs. 6B, D, E, and G). The seminal receptacle, lined by flattened cells, had an ovoid shape and was positioned lateral to the ovary. A large quantity of spermatozoa was present in the seminal receptacle (Fig. 6G). Vitelline glands filled with vitelline cells were located at both lateral sides of the ovary (Figs.

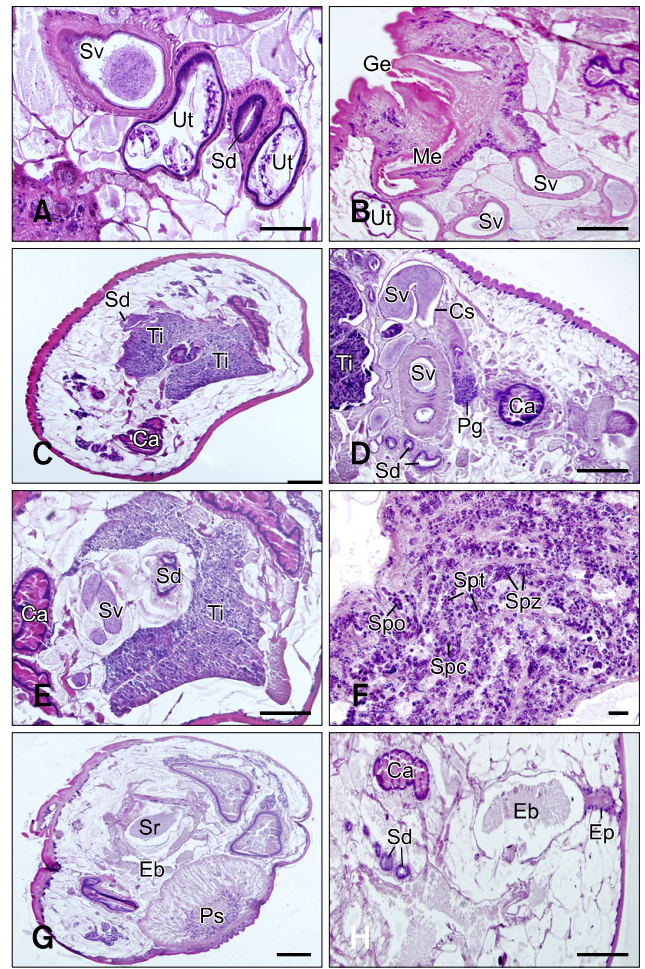


Fig. 7. (A and B) Longitudinal sections of the middle region of *P. gracile* Fischöeder, 1901 showing the male and female reproductive organs including the seminal vesicle, spermatid, uterine duct, metraterm, and genital canal. (C ~ F) Transverse sections of the middle to posterior region of the *P. gracile* male reproductive organs including the testis, spermatid, seminal vesicle, cirrus sac, prostate gland, spermatogonia, spermatids, and spermatozoa. (G ~ H) Transverse sections of the posterior region of *P. gracile* containing the excretory bladder, seminal receptacle, spermatid, excretory pore, and posterior sucker. Cs: cirrus sac, Spt: spermatids, Spt: spermatogonia, Spz: spermatozoa, Spc: spermatocytes, Eb: excretory bladder, Ep: excretory pore. Scale bars = 50 μ m (F), 100 μ m (A, B, D, E, and H), 150 μ m (C and G).

6D ~ H). They were ovoid, lobulated, and occupied areas from the caecal bifurcation to the posterior testis. The lobes were formed by a group of polygonal cells with central nuclei and cytoplasm full of basophilic granules (Fig. 6H). The uterus originated in the ootype area and extended posteriorly until the end of the body. The uterus was lined by flat cells and filled with a large number of eggs consisting of an egg cell surrounded by a thin shell (Fig. 7A). The metraterm, the thickened final part of the uterus, was surrounded by several glands and opened up at a

genital atrium close to the pore where the cirrus pouch opened up (Fig. 7B).

The testes had an irregular shape and were located in the distal one-third portion of the body. Both the anterior and posterior testes were surrounded by fine connective tissues (Figs. 7C~F). The testes contained efferent ducts leading to the seminal vesicle where they formed a single duct inside the cirrus pouch (Figs. 7D and E). The cirrus pouch extended transversely to the median line and contained the seminal vesicle, prostate glands, ejaculatory duct, and cirrus. The seminal vesicle was a long-coiled tubular structure containing numerous spermatozoa (Figs. 7D and E). Immature cells or spermatogonia were observed at the margin of the germinative epithelium while spermatocytes, spermatids, and spermatozoa were present in the center of testes (Fig. 7F). The spermatic duct opened at the end of the genital canal and extended into the testis (Figs. 7A~E and H). The diffuse prostate glands were formed by basophilic cells surrounding the cirrus (Fig. 7D). The cirrus pouch opened up at the genital atrium and closed at the metraterm pore (Figs. 7A and B). The excretory bladder was located dorsally near the posterior sucker and extended towards the excretory pore (Figs. 7G and H).

Discussion

The surface of the *P. gracile* tegument had some unique features. No spines were present in the tegument, suggesting that the spine was not necessary for movement of this fluke. Additionally, the tegument formed corrugations of alternating grooves and folds that vastly increased the surface area for absorption. This characteristic is also found in other trematode parasites such as *P. cervi* [5,20] and *Fischoederius cobboldi* [3]. The posterior sucker (acetabulum) was particularly large and had thick muscular layers. This was not surprising since this structure might tightly anchor the parasites to the wall of the host's rumen. In addition, the testes were involved in spermatogenesis of the parasite. The seminal vesicle consisted of spherical bodies filled with mature spermatozoa.

The histological components of the body wall consisting of the tegument, basement membrane, tegumental cells, and muscle layers were similar to those of other closely related trematode parasites such as *Fasciola gigantica* [2,4]. The anterior sucker was found at an anterior location while the posterior sucker (acetabulum) was present on the posterior tip similar to many species of rumen flukes including *Paramphistomum ichikawai*, *P. bombayiense*, *Calicophoron (C.) crassum*, *C. calicophorum*, *C. cauliorchis* [9], and *Fischoederius cobboldi* [3]. In other trematode parasites such as the intestinal fluke *Fasciolopsis buski*, the posterior sucker is located at the middle part of the body on the ventral side [15]. This extreme posterior position of the posterior sucker is a

specific characteristic of rumen flukes since these parasites use the suckers to attach themselves to the host rumen epithelium.

The *P. gracile* digestive system was a tubular tract with the mouth as the pore opening to the exterior. The radial muscle of the pharynx was highly developed as in the anterior sucker. This was similar to the structural organization of the anterior sucker and pharynx of most digenean parasites including *Haematoloechus medioplexus*, *Haplometra cylindracea*, *Opisthioglyphe ranae*, *F. hepatica* [7], and *F. gigantica* [16]. On the other hand, *Diplodiscus subclavatus*, *Gorgoderina vitellioloba*, and *Gorgoderina cygnoides* do not have a true pharynx; instead, they possess a highly muscular esophagus [7]. Taken together, *Schistosoma mansoni* pharynx was poorly developed and difficult to discern [7]. Most small digenean trematodes, such as *H. medioplexus*, *H. cylindracea*, *O. ranae*, *D. subclavatus*, *G. vitellioloba*, *G. cygnoides*, and *S. mansoni*, have two main caeca bifurcated from the esophagus [7] similar to *P. gracile*. On the other hand, *F. hepatica* and *F. gigantica* have two main caeca from which numerous medial and lateral branches extend to all parts of the body [7,16].

The *P. gracile* ovary was located near the posterior sucker similar to other rumen flukes [9]. In comparison to other trematodes whose posterior sucker is present in ventral area of the middle body, the ovaries were located near the posterior sucker [25,26]. In *P. gracile*, oogenesis in the tubules of the ovary includes at least four stages of development [27]. The oogonia were located at the periphery of the tubules and moved into the central area during their development into oocytes. The oocytes developed into immature ova and mature ova surrounded by vitelline cells similar to *F. gigantica* [27] and *Schistosoma margrebowiei* [6].

The present study showed that Laurer's canal in *P. gracile* runs from the dorsal surface of the body downwards and enters the Mehlis' gland. Finally, this canal connected with the oviduct in the central portion of the Mehlis' gland. Unicellular glands surrounded the Laurer's canal at the periphery of the Mehlis' gland. These gland cells had cytoplasmic processes extending through the muscle of the canal and connected to the epithelium. The function of these gland cells is not known. The cells may stimulate the capacitation of spermatozoa passing through the canal during copulation or may produce nutrients for the spermatozoa. In *P. gracile*, the eggs were surrounded by vitelline cells similar to those in *F. gigantica* [17]. In the proximal uterus, vitelline cells are stimulated by the secretions of the Mehlis' gland cells and release shell granules to form the main part of the egg shell [17]. At the end stage of egg formation, the nuclei and cell membranes of surrounding vitelline cells degenerate [17]. Nutrients stored in the yolk granules and cytoplasm are released to supply the ovum to promote miracidium development [17].

Two testes were located in the distal one-third region of the body in *P. gracile*. This was in contrast to the testes of both *F. hepatica* [7] and *F. gigantica* [2] that are located side by side in the central part of the body. In general, spermatogenesis in *P. gracile* is similar to that of other digenean parasites in that spermatogonia undergo three mitotic and two meiotic divisions to give rise to 32 spermatids that are transformed into 32 spermatozoa [11,29]. Our study showed that *P. gracile* efferent ducts extended from the testes to the middle part of the body and enlarged to become the seminal vesicle located dorsal to the posterior sucker. Anterior to the seminal vesicle was the male genital canal surrounded by the prostate gland. Both the seminal vesicle and prostate gland were surrounded by thick circular and longitudinal muscles. All of these structures were similar to the cirrus sac found in other digenean parasites described by Hyman [14]. The prostate gland of *P. gracile* was composed of unicellular gland cells located at the peripheral side of the gland with cytoplasmic processes extending into the prostatic duct in the central part of the gland. Some materials were transported into the lumen of the duct and stored as secretory granules. These granules may contain nutrients for the spermatozoa while they are passing through the duct. Both spermatozoa and the prostate gland secretions are ejaculated out of the body by contraction of the strong cirrus sac muscles [9]. Morphology of the *P. gracile* genital canal was similar to those of other rumen flukes including *P. bombayiense*, *C. calicophorum*, and *Gigantocotyl siamense* as reported by Gupta and Nakhasi [9].

In this study, we attempted to elucidate the morphological and histological characteristics of *P. gracile* that could increase the knowledge and understanding of rumen fluke morphology.

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