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RESEARCH ARTICLE

Ultrastructural Study of the Three Trematodes *Plagiorchis muris, Pleurogenoides japonicus* and *Loxogenes liberum* Collected from Dragonflies

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ABSTRACT

This study explains morphological differences among three trematode species collected from dragonflies in the fields. The trematodes were tentatively identified under light microscopy, and then precisely examined by scanning electron microscopy (SEM) on the basis of unique features at the tegumental spines, cuticles and internal organs of the worms. Despite the previous investigations regarding the ultrastructural details including the tegumental spines of the trematode *Plagiorchis muris*, very few efforts have been made in both the trematodes, *Loxogenes liberum* and *Pleurogenoides japonicus*, partly because the latter two species cause less damage to host animals compared to the former trematode *P. muris.* Since the three species of trematodes are readily found in the same dragonflies, correct identification of these species is important to predict and prevent the disease caused by these trematodes. Herein, we characterize by SEM new ultrastructural details in the three trematodes which were not previously described in *P. japonicas* and *L. liberum*. Three morphologically different spines, suckers and internal organs (ovary, vitellaria and uterus) were observed and described. Based on these results, we assert that the newly recognized morphological features should be used for species differentiation in the future.

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INTRODUCTION

Trematodes, also called flukes, cause various human and animal diseases. They are flattened oval or worm-like animals, and their most distinctive external feature is the presence of two suckers, one close to the mouth, and the other on the bottom of the animal (Barnes, 1982). There is usually only a single ovary, which is connected, passing through a pair of ducts to a number of vitelline glands on either side of the body that produce yolk cells (Dawes, 1963). The ovary is often also associated with a storage appendage for sperm, and a copulatory duct termed Laurer's canal (Dawes, 1963).

The trematodes in the genus *Plagiorchi* are the most abundant species in Republic of Korea (ROK), which include *P. koreanus*, *P. orientalis*, *P. corpulentus*, *P. vespertilionis*, *P. rhinolophi* and *P. kyushuensis* (Kifune *et al.*, 1983; Hong *et al*., 2006; Shin *et al*., 2008; Chai *et al*., 2009). Among this genus, *Plagiorchis muris* is one of the best characterized species regarding to its morphology, and frequently collected from wild and house rats captured in a range of collecting sites in ROK *(*Lee *et al*., 1990; Seo *et al.*, 1964). Recently, our study also indicated this species along with other two trematodes: *P. japonicus* and *L. liberum* were found in dragonflies, suggesting dragonflies as an intermediate host of trematodes during the summer.

Key morphological features of *P. muris* include a laterally located ovary, two tandem testes, an extensive distribution of the vitellaria, and large eggs (Chai and Lee,

2002). In addition, tegumental spines, and type I and II sensory papillae concentrated around the oral sucker of *P. muris*, which resemble those of intestinal and heterophyid trematodes (Hong *et al.*, 1998). Although there was previous morphological study of the *P. japonicus* and *L. liberum* trematodes (Lee *et al.*, 1976), a detailed ultrastructural study was scarce. Therefore, in this study, we elucidate the ultrastructural details of three trematodes including *P. muris*, *L. liberum* and *P. japonicus* which were first collected from dragonflies.

MATERIALS AND METHODS

Collection of metacercaria in dragonflies and infection test in animal hosts

Dragonflies were caught near villages and streams in Daejeon, Chungnam, Chonbuk provinces in ROK during the summer (June-August in 2009). These dragonflies were identified according to Shin's method (Shin, 1993) and grouped in species of dragonfly. After the discard of wings, each group of dragonflies was artificially digested for 2 hrs in artificial gastric juice (pepsin 6 g, HCl 7 ml in 1L D.W.) at 37^0C. The digests were washed and sedimented several times with 0.45% saline at room temperature. Six types of metacercaria harvested from dragonflies were classified as described previously (Park *et al.*, 2003). The metacercariae were collected from the sediment of digest under the dissecting microscopy and grouped under the light microscopy. Each grouped metacercariae was fed to mouse, rat, rabbit, chick, duck, dog and frog with a gastric needle.

Light microscopy

The feces from the animals were examined by Formaline-Ether method for the detection of eggs 4 days later. To validate the infection success, the small intestines of the infected animals were examined 19 days after the metacercarial infection. The recovered flukes were stained with Semichon's acetocarmine and identified using light microscopy.

Scanning electron microscopy

Samples were prepared by a slight modification of a method previously described (Hong, 2009). The flukes were washed several times briefly with 0.2M cacodylate buffer (pH 7.3) and fixed in 2.5% glutaldehyde solution at 4^0C After washing in three changes of distilled water, they were dehydrated through increasing concentrations of ethanol, and dried in a critical point drying machine using liquid carbon dioxide as a transitional medium. Thereafter, specimens were mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus, SPI-Model sputter coater for 4 min. Specimens were examined by SEM (S-4800, Hitachi) operating at 15 kV.

RESULTS

Morphological characterization of *Plagiorchis muris* Tanabe, 1922

The adult worms were harvested in the small intestine of mouse, and it measures 1.85-2.05 mm long by 0.59-0.63 mm broad and the ratio of length to width is 3.14-3.25:1 (Fig 1 and 8). The cuticle is armed with spines over the whole body (Fig.11 and Fig. 13). The oral sucker is circular and measures 0.20-0.24 mm and the pharynx 0.08-0.09 mm long (Fig. 2 and Fig. 9). The ventral sucker is situated at the level of one third from anterior end and measures 0.19-0.22 mm. The two branches of the intestine are noticeably long. The intestinal caeca reach near to the posterior extremity. The testes lie in the posterior part of the body (Fig. 3); they are situated diagonally and are circular. Anterior and posterior testes measure 0.48-0.52 mm and 0.50-0.55 mm, respectively. The cirrus pouch is C-shaped and dextral to the ventral sucker (Fig. 1). The cirrus is protruded at the genital pore in the some of adult. The genital pore opens in front of the ventral sucker (Fig. 10).

An ovary is situated to the right of the median line posteriorly at the ventral sucker and measures 0.25-0.29 mm (Fig. 3). The uterus has wide folds, which runs from the posterior end of the body to the ventral sucker (Fig. 3 and Fig. 5). The Laurer's canal opens dorsally in front of the ventral sucker (Fig. 12). The vitellaria are placed mainly laterally and between the portion of posterior pharynx and end of body (Fig. 1, 3 and 5). Eggs measure 35.13-37.80 × 21.75-23.25 μm (Fig. 7) and they are yellow brown, having a distinct operculum and a blunt point at the opposite end. Moreover, eggs are unembryonated when laid, and their prepatent period is 5 days.

Morphological characterization of *Pleurogenoides japonicus* Yamaguti, 1936

Adult worms were harvested in the small intestine and the harvest ratio was 95 %. Adult worm measures 1.80-2.20 mm long by 0.70-0.91 mm broad and the ratio of length to width is 2.57-2.42:1 (Fig. 15 and Fig. 18). The cuticle is armed with spines over the whole body (Fig. 19, 20, 22, 23 and 24). The tegumental spines are digitate and the digitations of spines are 6-11 pointed (Fig. 20). The oral sucker is circular and measures 0.18-0.24 mm, and the pharynx is 0.05-0.07 mm long (Fig. 16). The two branches of the intestine are very short. The intestinal caeca reach near the front of testis (Fig. 16). The length of esophagus is long, and the ventral sucker is situated at 1/3 level from anterior end and measures 0.17-0.23 mm (Fig. 17 and Fig. 22). Five big spines arm the inner side of ventral sucker (Fig. 22 and Fig. 23).

A Y-shaped excretory is situated at the posterior portion of testis (Fig. 15). Two testes lying laterally at the ventral sucker measure 0.25-0.34 mm (Fig. 17). The cirrus pouch, 0.433-0.69 mm, is club-shaped, situated on the left side in front of the ventral sucker (Fig. 16). The genital pore opens at the left side of body (Fig. 21). An ovary is situated to the left side in front of left testis and measures 0.24-0.29 mm (Fig. 16).

Morphological characterization of *Loxogenenes liberum* Seno, 1908

Adult worms were harvested in the small intestine of frog and the harvest ratio was 97.16 %. It measures 1.11-1.18 mm long by 0.70-0.84 mm broad and the ratio of length to width is 1.4-1.6:1 (Fig. 26, 29, 30 and 31). The cuticle is armed with sharp spines over the whole body (Fig. 29, 30 and 31). The oral sucker is circular and measures 0.17-0.21 mm while the ventral sucker is 0.17-0.20 mm long and muscular pharynx 0.06-0.07 mm long

(Fig. 26). The two branches of the intestine are considerably short. The intestinal caeca reach near the front of testes. The ventral sucker is situated at the median portion (Fig. 26 and Fig. 29). The V-shaped excretory, situated at the posterior portion of testes, opens at posterior end of body (Fig. 34). Two testes situated laterally in front of ventral sucker, measured 0.14-0.18 × 0.11-0.16 mm. The cirrus pouch situated obliquely between the right testis and ventral sucker measured 0.40-0.45 mm (Fig. 28).

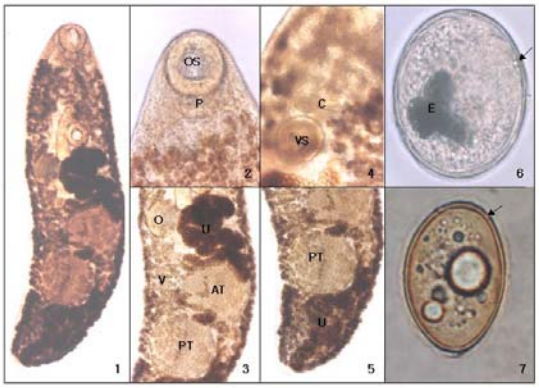

Fig. 1-7: Light microscopic view of metacercaria, adult and egg of *Plagiorchis muris*. **Fig. 1**: A whole worm. Ovary and cirrus pouch are dextral to ventral sucker. The vitelline follicles distributed in the preacetabular region. **Fig. 2:** Anterior end of adult worm showing oral sucker (OS) and well developed pharynx (P). **Fig. 3:** Mid-body of adult worm showing a circular ovary and two testis, anterior (AT) and posterior (PT). Two testes situated obliquely. **Fig. 4:** The coiled cirrus situated anterior portion of ventral sucker. **Fig. 5:** Posterior end of adult worm. Uterus prolonged at the anterior end. **Fig. 6:** A metacercaria collected from dragonfly, *Sympetrum drawinianum*. A stylet (arrow) is visible in oral sucker. **Fig. 7:** An egg with operculum (arrow) from feces of *P. muris* infected mice.

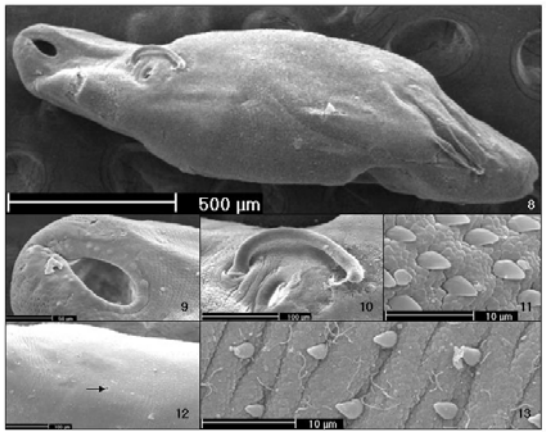

Fig. 8-13: SEM view of 19-days old adult of *Plagiorchis muris*. **Fig. 8:** A whole worm, ventral view, showing prominent oral sucker, ventral sucker and the cirrus protruding from the genital pore. **Fig. 9:** The oral sucker showing some papillae. **Fig. 10:** The genital pore situated at the anterior of the ventral sucker. **Fig. 11:** The tegumental spine at the ventral portion of adult. **Fig. 12:** The dorsal view of the worm. Laurer's canal (arrow) opening at the dorsal tegument. **Fig. 13:** The tegumental spine at the dorsal portion of adult.

The genital pore opens at the left side of ventral sucker (Fig. 21). An ovary is situated near the front of ventral sucker and measures 0.17-0.23 mm (Fig. 28). The uterus is situated widely at the anterior and posterior portion of the body (Fig. 26). The vitellaria are placed close to posterior of oral sucker. Brown-colored eggs measures 22-24 × 11-13 µm, having a distinct operculum and a blunt point at the opposite end (Fig. 27).

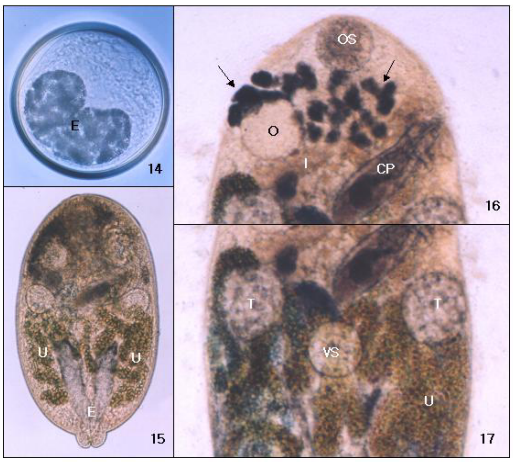

Fig. 14-17: Light microscopic view of metacercaria and adult worm of *Pleurogenoides japonicus*. **Fig. 14:** A metacercaria collected from dragonfly, *Sympetrum drawinianum*. The metacercarial wall is thick. **Fig. 15:** The whole adult worm. Uterus (U) situated laterally and the V-shaped excretory bladder (E) is large. **Fig. 16:** Anterior end of adult worm. The vitelline follicle situated between the oral sucker and intestine. Intestines (I) are very short. A ovary is circular dextral portion to the esophagus. A cirrus pouch is situated obliquely at the left side. **Fig. 17:** Mid-body of the worm. Two circular testes are lateral. Ventral sucker is at the median. A lot of eggs filled in uterus.

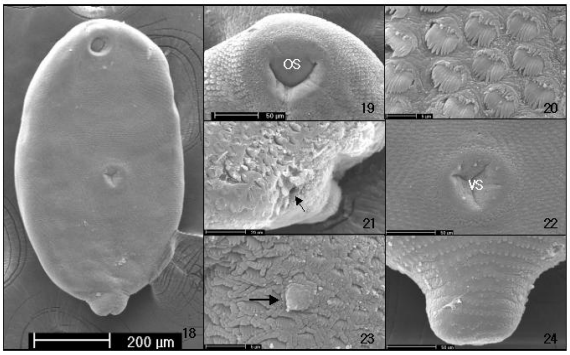

Fig. 18-24: SEM view of 19-days old adult of *Pleurogenoides japonicus*. **Fig. 18:** A whole worm, ventral view, showing prominent oral sucker, ventral sucker. **Fig. 19:** The oral sucker is smooth. **Fig. 20:** Tegumental spines on the ventral surface between oral and ventral sucker. Digitations of spines are 6-11 pointed. **Fig. 21:** The genital pore opening at the left margin between oral and ventral sucker. **Fig. 22:** Ventral sucker with 5 big hooks. **Fig. 23:** The big hook (arrow). **Fig. 24:** The tegumental spine around the excretory pore.

DISCUSSION

The morphological observations by using SEM and light microscopy identified ultrastructural details among three metacercaria, *P. muris*, *P. japonicus*, and *L. liberum*.

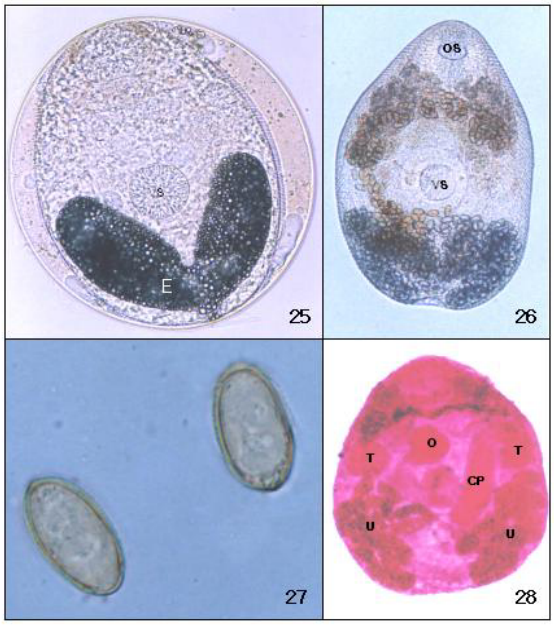

Fig. 25-28: Light microscopic view of metacercaria, eggs and adult worm of *Loxogenes liberum*. **Fig. 25:** A metacercaria collected from dragonfly, *Sympetrum drawinianum*. The excretory bladder (E) is V-shaped. **Fig. 26:** The whole adult worm. Uterus (U) is C-shaped and dextral to the ventral sucker (VS). A lot of eggs filled in uterus. Vitelline follicles are between oral sucker and uterus. **Fig. 27:** Eggs from uterus. **Fig. 28:** The stained whole adult worm. An ovary (O) is at the posterior of ventral sucker. A cirrus pouch (CP) is situated obliquely at the left side between ventral sucker and left testis.

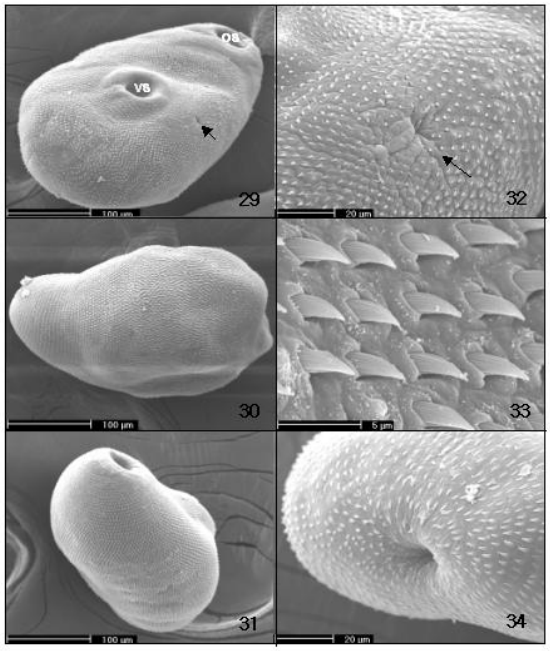

Fig. 29-34: SEM view of 19-days old adult of *Loxogenes liberum*. **Fig. 29:** A whole worm, ventro-lateral view, showing oral sucker, ventral sucker and genital pore (arrow). **Fig. 30:** The dorsal view of whole worm with a lot of tegumental spines. **Fig. 31:** The antero-lateral view of the worm. **Fig. 32:** The genital pore (arrow) openning left to ventral sucker. **Fig. 33:** Tegumental spines are sharp-pointed. **Fig. 34:** The tegumental spine around the excretory pore.

The surface ultrastructure of *P. muris* indicates the similar pattern of surface spines that was observed from the previous SEM study (Hong, 2009). The tegumental spines of *P. muris* show the pointed shape which is distributed evenly through the whole body of *P. muris.* In the other two trematodes, the cuticles are also armed with spines over the whole body. Interestingly, the unique patterns of tegumental spines were observed in these two species. In. *P. japonicus*, the spines are digitated with 6-11 pointed shape that is similar to clam-like figure. Since each unit of tegumental spines is quite different compared to those of the other two trematode species, the cuticle structure of *P. japonicas* is much more like wearing armor. In addition, although the spine shape of *L. liberum* is similar to that of *P. muris*, the tegumental spines of *L. liberum* are more populated through the whole body and have sharper edges compared to those of *P. muris.*

The morphology and the size of the oral suckers are similar among the three trematode species. However, unique structural differences near the ventral suckers were observed among the three species. In *P. muris*, the ventral sucker was positioned close to the posterior end, and on the right side of it, C-shaped cirrus pouch was found. This cirrus hanged over close to the genital pore that was located in front of the ventral sucker. On the other hand, the ventral suckers of *P. japonicus* possessed five spines inside of them. In *L. liberum*, the location of the ventral sucker was closer to the anterior compared to that of the other two species, in which the location was in the middle of the body.

Finally, the morphological and positional differences of internal organs were also found among the three trematodes. The ovary was located near the front of ventral sucker, and the vitellaria were placed in the posterior side of the body in the two trematodes, *P. muris* and *L. liberum*. In addition, the ovary of *L. liberum* was different from the other two trematodes, which was situated to the left side in front of left testis. Different sizes and shapes of the uterus were also observed among these species. The uterus had wide folds in *P. muris*. However, the uterus was elongated between the anterior and posterior portion of the body in *L. liberum.*

In conclusion, we summarize the morphological differences among three species by using light microscopy and SEM. Our ultrastructural study of these worms may clarify the morphological differences among three metacracria which have a significant infection potential in ROK.

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