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Comparing the Ultrastructural Morphology of *Raillietina tetragona* and *Raillietina echinobothrida* in Local Hens at Sidoarjo Regency with a Scanning Electron Microscope (SEM)

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ABSTRACT

Background: *Raillietina* spp. is one of the cestodes class worms that attacks poultry. The *Raillietina* species that commonly attack poultry are *Raillietina echinobothrida* and *R. tetragona.* In general, *R. echinobothrida* and *R. tetragona* are usually identified using a light microscope, but in reality these two species are still difficult to differentiate using a light microscope because the size difference is too small and they have many differences. This study compared the ultrastructural morphology of *R. echinobothrida* and *R. tetragona*, which caused minor intestinal infections in native *Gallus domesticus* chickens that were purchased from different traditional markets in Sidoarjo Regency.

Methods: A total 57 small intestines from native chickens (*Gallus domesticus*) that were sold at Tulangan, Larangan, Wonoayu, Krian and Suko Markets served as the samples for this study. Using a scanning electron microscope (SEM), the samples were inspected both macroscopic and microscopically.

Result: With a prevalence of 57.89%, the results indicated that *R. echinobothrida* has a yellowish string-like body that is 16-23 cm long. It also has a round sucker and scolex that are both 0.07 × 0.08-0.08 × 0.09 mm in size, a rostellum that is armed with hooks that is 0.06 × 0.07-0.08 × 0.1 mm in size and an unsegmented, thick and short neck. The yellowish string-like body of *R. tetragona*, on the other hand, measures 20-28 cm in length. It has an oval scolex (0.16-0.2 mm in diameter), an oval sucker (0.04 × 0.09-0.05 × 0.01 mm in size), a rostellum armed with hooks that measures 0.03 × 0.05-0.04 × 0.06 mm and an unsegmented, thin, and long neck. Both *R. echinobothrida* and *R. tetragona* have a velvety, silky texture on their surface bodies.

Key words: Chickens, Intestine, *Raillietina echinobothrida*, *Raillietina tetragona*, SEM.

INTRODUCTION

Poultry worm infestations can result in large financial losses (Setyowati *et al*., 2022). Many factors, such as the pathogenicity of the parasite, the host-parasitic interaction, the infectious dose and the vulnerability of each host species, influence the severity of the infection (Sandhu *et al.,* 2009; Buckingham and Ashby, 2022). *Raillietina* spp. is one of the cestodes class worms that infects the poultry (Gamra *et al*., 2015) with two species of *Raillietina*, *i.e*. *R. echinobothrida* and *R. tetragona*, are known to frequently attack poultry (Panich *et al*., 2021). So far, morphological traits including the shape and size of the rostellum, suckers, scolex, and the location of the vaginal pores have made it possible to distinguish between the two species (Butboonchoo *et al*., 2016). *R. echinobothrida* and *R. tetragona* can infect poultry due to their eating of intermediary hosts, such as ants and flies, which harbor cysticercoid larval stage of the cestode (Eshetu *et al*., 2001). The adult form of these parasites subsequently resides in the small intestine of the animal that has been contaminated (Makwanise *et al*., 2020; Manjunatha *et al*., 2023).

The infectious stage of *Raillietina* is present in intermediate hosts, which harbor the disease, and the definitive host eats paratenics, which makes the transmission rate higher (Anindyta *et al*., 2022).

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Raillietiniasis frequently affects free-range chickens due of their unclean living conditions (Subedi *et al*., 2018). In addition, there is evidence that *Raillietina* is more likely to infect regions with high humidity, abundant biodiversity, and intermediate hosts like ants and beetles (Scott, 2023). One of the most dangerous cestode worms, *R. echinobothrida*, can cause nodules in the intestines and, in cases of severe infestation, the degeneration and necrosis of intestinal villi (Lalchhandama, 2010). In the meantime, *R. tetragona* can result in decreased egg production and weight loss (Belete *et al*., 2016).

Sidoarjo is a district located in the province of East Java, Indonesia. Due to its convenient location and near proximity to residential areas, Sidoarjo's traditional market is often bustling with vendors selling their items (Priyono, 2013). The chicken vendors transport the birds from their farms and then offer them at discounted prices in conventional markets with other vendors (Saraiva *et al*., 2021). However, because traditional marketplaces have unclean and filthy surroundings, there is a significant rate of infectious disease transmission among the chickens sold there (Kirana *et al*., 2023).

R. tetragona and *R. echinobothrida* are typically identified using a light microscope. However, due to their small size differences and multitude of differences, these two species are actually still difficult to distinguish using a light microscope (Butboonchoo *et al*., 2016). Consequently, more methods for identification and characterisation are needed in order to achieve a better level of resolution and generate a more comprehensive picture of the different morphological structures. The international community of materials research has acknowledged and embraced scanning electron microscopy (SEM) as a viable substitute technique (Li and Yao, 2023; İlgün *et al*., 2018; Ali *et al*., 2023). SEM is a device that provides a general overview of a sample's structure and composition through the use of electron emissions.

Based on these problems, this research aims to determine the differences in the ultrastructural morphology of *R. tetragona* and *R. echinobothrida* isolated from chickens in the Sidoarjo traditional market using a Scanning Electron Microscope (SEM).

MATERIALS AND METHODS

Study area and sample collection

The research conductede from April 2024 to May 2024. Sampling was carried out at five traditional markets in Sidoarjo (Tulangan Market, Wonoayu, Larangan, Krian and Suko) with a total of 57 chicken small intestine samples. Surgery and sample examination will be carried out at the Parasitology Laboratory, Faculty of Veterinary Medicine, Airlangga University and SEM examination will be carried out at the Biosciences Laboratory, Brawijaya University.

Dissection of the small intestine for worm collection

Cutting off a portion of the small intestine, the contents are gradually removed, sifted through a filter and then checked for worms. The worms that were found were placed in a petri dish that had been given a physiological NaCl solution. The contents of the small intestine are filtered once more using flowing water to facilitate the collection of worms. The filtered water is collected in a tray to anticipate the worms being filtered out. Using surgical scissors and a scalpel, the small intestine is opened once its contents have been filtered. To release the worms that are adhered to the mucosa, the small intestine is gently massaged and cleaned under flowing water. A tray holds the washing water in anticipation of the worms being rinsed.

Following the removal of every worm, the number of worms is counted and divided by genus. When viewed macroscopically, *Raillietina* worms have a slender body formed of segments that resemble ribbons, growing larger from the front to the posterior. Following the counting process, a portion of the worms were preserved in a 5% glycerin alcohol solution for carmine staining, while the remaining worms were destroyed in hot water at a temperature of ±700°C.

Carmine staining

Following collection from the the small intestine, worms were momentarily immersed in a physiological NaCl solution. Semichen-Acetic Carmine (Khulman, 2006) technique employed for staining. The worms were first cleaned by immersing them in 5% glycerin alcohol for a full day. After that, they were placed between two glass items and rope-tied at both ends. Next, bathed it in 70% alcohol for five minutes. After that, immersed it for four hours in a carmine solution that had been diluted with 70% alcohol at a 1:2 ratio. After removing the worm from the glass slide, immersed for two minutes in an acidic alcohol solution (70% alcohol+HCl) and then for another twenty minutes in an alkaline alcohol solution (alcohol+$NaHCO_3$) to completed the destaining step for organ/tissue diffraction.

The following step was the dehydration stage, which used alcohol concentrations of 70%, 85% and 95% for five minutes each to remove the water content. The next step was the clearing stage, which involved soaking the worms in Hung's I solution for 20 minutes. After that, the worms were mounted on a glass surface, covered with a glass cover and a drop of Hung's II solution. The last stage was examination using a binocular microscope with a 100× magnification.

Scanning electron microscope (SEM)

The adult *R. tetragona* and *R. echinobothrida* worm samples that were to be investigated were first cleaned in physiological NaCl solution. Following this, the samples underwent the first fixation step, which involved soaking them in 1% glutaraldehyde solution for three hours. PBS (phosphate buffer saline) solution was then used three more times for washing (Rianto, 2022).

Osmium tetraoxide solution was used for one hour during the second fixation step. Next, graded alcohol levels (30%, 50%, 70%, 80%, 90% and 100%) were used to induce

dehydration. The sample is then coated with a conducting substance, such as carbon, to complete the drying stage, also known as Critical Point Drying (CPD). After that, the samples were inspected and captured on camera with a SEM Model JOEL type JSM-T100.

RESULTS AND DISCUSSION

Results of a laboratory examination of chicken intestines from markets in the Sidoarjo Regency revealed that, out of the 57 samples tested, 33 had *R. echinobothrida* infection, with a total prevalence rate of 57.89%, and 12 had *R. tetragona* infection, with a total prevalence rate of 21.05%. Table 1 displays information on the prevalence of *R. tetragona* and *R. echinobothrida*. Macroscopically, the adult of *R. tetragona* and *R. echinobothrida* wereyellowish white in color and thin like ribbon. The results indicated that *R. echinobothrida* had a yellowish string-like body that is 16-23 cm long and the yellowish string-like body of *R. tetragona*, on the other hand, measured 20-28 cm in length.

The presence of cestode worms such as Raillietina spp. is influenced by the unhygienic conditions of chicken coop and traditional markets. Free-range chickens, which were at Sidoarjo Market, were suspected of being raised in a dirty environment. Chickens kept in traditional systems and in rearing areas have a greater chance of being infected with endoparasites (Malatji *et al*., 2016). Apart from that, the density of chickens in the Sidoarjo traditional market is very high so that the transmission of *Raillietina* spp. between individuals occurs more quickly.

The dirty environmental conditions in traditional markets cause the *Musca domestica* fly species to be found as one of the intermediate hosts for *Raillietina* (Alsaad, 2023). The small intestines of free-range chickens serve as definitive hosts for the adult stages of *R. tetragona* and *R. echinobothrida*, while ants and beetles also serve as definitive hosts (Siddiqui *et al*., 2023). Chickens afflicted with raillietiniasis typically lose weight, giving them a lean, frail, and listless appearance (Islam *et al*., 2020). *R. echinobothrida* is considered a more pathogenic species than *R. tetragona* due to its ability to develop nodules and hyperplastic enteritis (Panich *et al*., 2021).

The results of observations using a binocular microscope showed the presence of the anterior regions of *R. tetragona* and *R. echinobothrida*, as well as the scolex, four suckers, rostellum and neck, varies in size and shape depending on the species. The razor-sharp hooks encircling the suckers and rostellum are not very evident. *R. echinobothrida* has a broad, short neck, four circular suckers, and a round head. In contrast, *R. tetragona* has a longer and thinner neck than *R. echinobothrida*, as well as an oval head and four oval suckers. Table 2 and Fig 1 and 2 presents a comparison of the morphological features of *R. echinobothrida* and *R. tetragona*.

Fig 1 shows a comparison of the anterior portion morphology between *R. echinobothrida* and *R. tetragona* using a binocular microscope fitted with a camera lucida at a 100× magnification. Fig 2 shows a comparison of the posterior portion morphology between *R. echinobothrida* and *R. tetragona* using a binocular microscope fitted with a camera lucida at a 100×magnification.

Free-range chickens can be infected with *Raillietina* worms because they eat intermediate hosts (beetles and ants) which contain cystercercoids (Hariani and Simanjuntak, 2021). The ingested cystersercoids will later develop and settle in the small intestine of the definitive host and excrete mature body segments along with their feces (Blecharz-Klin *et al*., 2022). Several studies had shown the same thing as this study that free-range

Table 1: Comparison of prevalence between *R. echinobothrida* and *R. tetragona*.

Market	*R. echinobothrida*	*R. tetragona*
Tulangan	6 (60%)	2 (20%)
Wonoayu	4 (40%)	1 (10%)
Larangan	7 (58.33%)	3 (25%)
Krian	10 (66.67%)	4 (26.67%)
Suko	6 (60%)	2 (20%)
Total	33 (57.89%)	12 (21.05%)

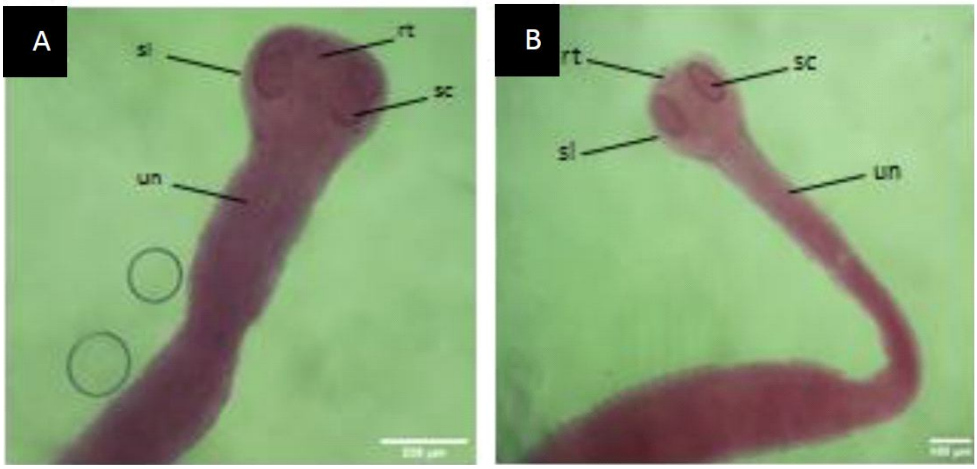
Table 2: Comparison of morphological characteristics of *R. echinobothrida* and *R. tetragona.*

Species	Morphological characteristics						
	Scolex shape	Scolex diameter (mm)	Suckershape	Sucker size (mm)	Rostellum size (mm)	Neck shape	Length (cm)
R. echinobothrida	Round	0.27-0.3	Round	0.07x0.08 -0.08x0.09	0.06x0.07 -0.08x0.1	Unsegmented, thick, and short	16-23
R. tetragona	Oval	0.16-0.2	Oval	0.04x0.09 -0.05-0.1	0.03x0.05-0.04x0.06	Unsegmented, thin, and long	20-28

chickens were infected with cestode worms. The study by Braz (2021) found that the incidence of gastrointestinal parasites in free-range chickens increased extensively in Brazil with the discovery of cestoda worms of 64.7% and *Raillietina echinobothrida* (100%) was the only cestoda identified. The study by Makwanise *et al*. (2020) showed that from 34 intestinal samples collected from 9 different areas in the Matabeleland-Zimbabwe region, tapeworms were detected in all samples examined and morphological characterization confirmed that those used in the study belonged to the *Raillietina sp*.

Using a SEM, the second stage of observation was used to look for and identify adult *R. echinobothrida* and adult *R. tetragona* in the small intestine of free-range chickens. Ultrastructural structures, such as the scolex, sucker, rostellum, and neck, were discovered in the anterior region of *R. tetragona* and *R. echinobothrida*. These forms may be viewed in detail in 3-dimensional photographs. In the meantime, a segmented body development with a porous surface is seen on the posterior side. The surface ultrastructural morphology of *R. tetragona* and *R. echinobothrida* was observed more closely and three-dimensional images were produced using a SEM (Fig 3 and 4) as a SEM can be used to directly view the surface of solid objects (Smith and Oatley, 2004; Deniz and Kuloðlu, 2024).

The results of the identification of adult *R. echinobothrida* and adult *R. tetragona* based on SEM indicated that these worms were 16-23 cm and 20-28 cm long, respectively. *Rechinobothrida* had a round scolex with a diameter of 0.27-0.33 mm, while *R. tetragona* had a more oval scolex with a diameter of 0.16-0.2 mm. On the scolex four suckers were found and the rostellum is surrounded by sharp hooks. The suckers on *R. echinobothrida* were round with dimensions of 0.07 × 0.08-0.08×0.09 mm and the rostellum wasalso round 0.06 × 0.07-0.08×0.1 mm,



A) Anterior part of *R. echinobothrida* using binocular microscope at 100x magnification B) Anterior part of *R. tetragona* using binocular microscope at 100x magnification.

Fig 1: Morphological comparison of the scolexpart between *R. echinobothrida* and *R. tetragona*.

A) Posterior part of *R. echinobothrida* using binocular microscope at 100x magnification
B) Posterior part of *R. tetragona* using binocular microscope at 100x magnification.

Fig 2: Morphological comparison of the neckpart between *R. echinobothrida* and *R. tetragona*.

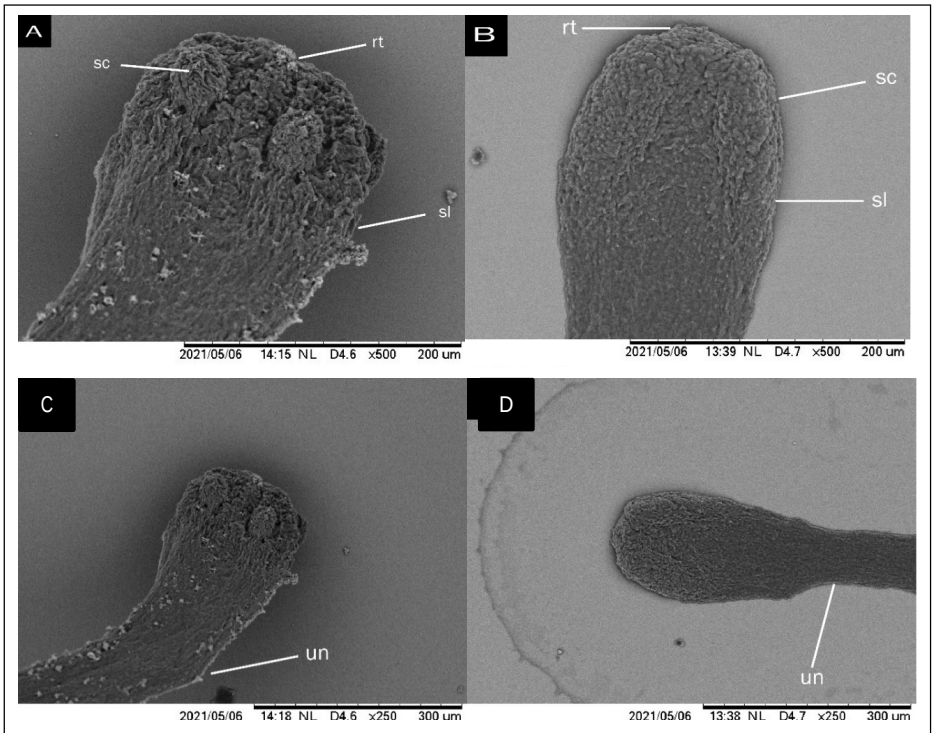
while the suckers on *R. tetragona* were round. oval-shaped with dimensions of 0.04 × 0.09-0.05×0.1 mm and a small, oval-shaped rostellum measuring 0.03×0.05-0.04×0.06 mm. Every species seemed s to have suckers that stick out from the scolex's surface.

R. echinobothrida and *R. tetragona* worms come from the same genus but have slight differences. One of the differences is the size and shape of the rostellum and sucker. *R. echinobothrida* worms have a round rostellum and sucker, while *R. tetragona* worms have a small round rostellum and ovoid sucker (Lalchhandama, 2009). The second difference lies in the unilateral genital pore. The *R. echinobothrida* worm has a unilateral genital pore located in the mediolateral to posteriolateral while *R. tetragona* are located in the anterolateral to mediolateral in each segment (Panich *et al*., 2021). Raillietina's scolex was slightly protruding like a knob, bulging in a spherical shape, and had a rectangular symmetrical structure (Butboonchoo *et al*., 2016). The body/strobilla's smooth, soft surface had microscopic holes that were visible through and cause bulges and indentations.

Since they lack a digestive tract, *R. echinobothrida* and *R. tetragona* are members of the cestode class. As a result, food can enter through the pores on the surface of the tegumen, which are also covered in microvilli that resemble the lumen of a mammalian intestine and are useful for transportation (Panich *et al*., 2021). Cestoda are hermaphroditic organisms that have male and female productive systems in each proglottid (Belete *et al*., 2016). Mature proglottids are found at the most posterior region of the body, whereas immature proglottids are closer to the neck and have not fully formed genitalia (Flisser, 2013).

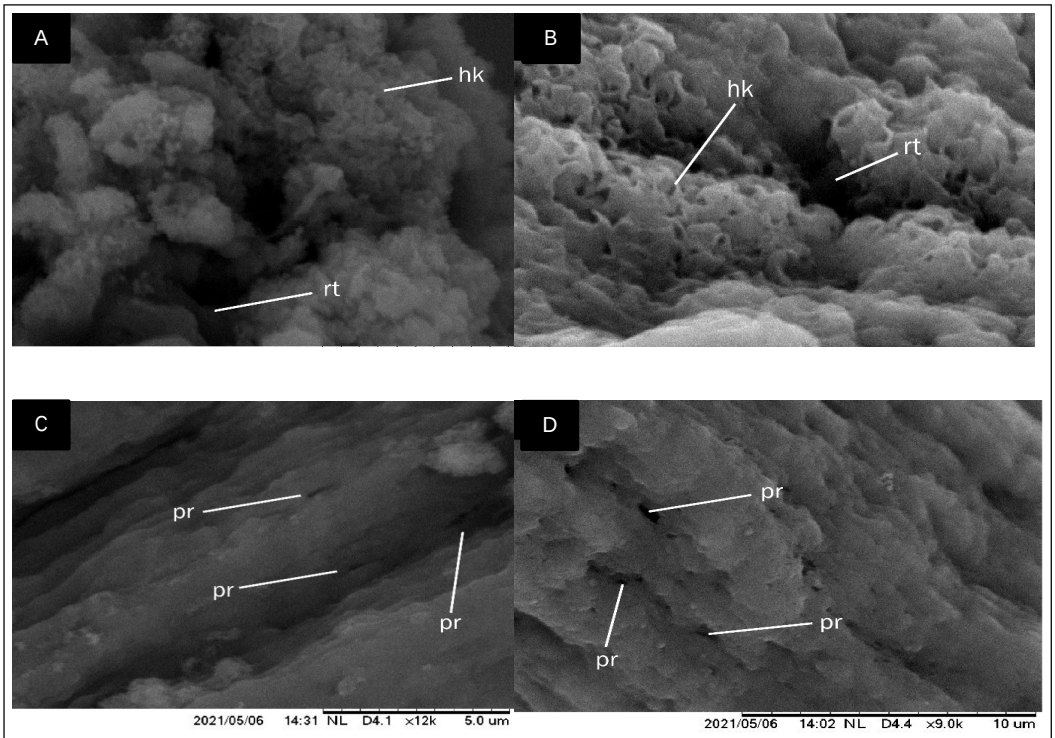
The types of Endoparasites that most often attack native chickens are *R. tetragona* and *R. echinobothrida*. The results of the study showed that the species of worms from the cestoda class were more commonly found. Cestoda worms are the ones that most often infest native chickens sold in traditional markets in Sidoarjo. This is because the environment where the chickens live contains many insects that are intermediate hosts for *Railletina* sp (Siddiqui *et al*., 2023). The large number of *Railletina* sp is due to the easy accessibility of intermediate hosts in the form of flies, beetles and ants in the yard where the native chickens live (Sapp and Bradbury, 2020). Cestoda infections in poultry cause stunted growth, enteritis diarrhea, bleeding, and hypovitaminosis B (Kerroucha *et al*., 2022).

The *R. echinobothrida* worm is one of the most pathogenic tapeworms compared to *R. tetragona* because it can cause nodules in the small intestine, hyperplasia enteritis associated with the formation of granulomas



A) Scolex of *R. echinobothrida* using a SEM at 500x magnification; B) Scolex of *R. tetragona* using a SEM at 500x magnification; C) Neck of *R. echinobothrida* using a SEM at 500x magnification D Neck of *R. tetragona* using a SEM at 500x magnification. Note: sl = Scolex, un = Unsegmented neck, rt = Rostellum, sc = Sucker, hk = Hook. Scale bar = 200 µm and 300 µm.

Fig 3: Morphological comparison of scolex and neck parts between *R. echinobothrida* and *R. tetragona* using a SEM.



A) Rostellum of *R. echinobothrida* using a SEM at 900x magnification; B) Rostellum of *R. tetragona* using a SEM at 900x magnification; C) Surface body of *R. echinobothrida* using a SEM at 1200x magnification; D) Surface body of *R. tetragona* using a SEM at 1200x magnification. Note: rt = Rostellum, hk = Hook, pr = Pores on the surface of the body/strobilla. Scale bar = 900 µm and 1200 µm

Fig 4: Morphological comparison of rostellum and surface body parts between *R. echinobothrida* and *R. tetragona* using aSEM.

which often causes intestinal necrosis (Lalchhandama, 2010). In addition, this is believed to be brought about by variations in the two species' morphological characteristics. Whereas *R. tetragona* only has one layer of sharp hooks enclosing its rostellum, *R. echinobothrida* has two layers (Lalchhandama, 2010). As the name suggests, Echino means thorny and Bothrida means a muscle structure that functions to clamp the host's tissue (Lalchhandama, 2009). The sucking mechanism of *R. echinobothrida* is equipped with several barb-like hooks. Owing to this anatomical feature, *R. echinobothrida* has the ability to inflict damage to host tissue by embedding its scolex deep within the muscularis mucosa, which in turn can result in the creation of intestinal nodules (Butboonchoo *et al*., 2016).

CONCLUSION

Scanning electron microscopy (SEM) and binocular microscopy observations of the morphological structure revealed that *R. echinobothrida* and *R. tetragona* differ in size and shape in the neck, rostellum, scolex and sucker segments.

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Conflict of interest

The authors declare no conflict of interest.

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