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RESEARCH ARTICLE

Investigation of complete life cycle and species identification of a digenean gill parasite *Centrocestus* sp. infesting Koi carp (*Cyprinus carpio* Linnaeus, 1758) using morphology and morphometric characters

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Abstract: This study was done to describe the morphology and morphometric characters of the life cycle stages of the digenean gill parasite, *Centrocestus* sp. that infests Koi carp (*Cyprinus carpio*) at Rambadagalle and Ginigathhena ornamental fish breeding centres of Sri Lanka and to identify the species of the parasite. The snail, *Melanoides tuberculata* (Müller, 1774) that inhabited the earthen ponds with Koi carps were induced to shed cercariae and 45 days old Koi carps were experimentally infested by them. The infected fish with mature metacercariae were fed to chicks (*Gallus gallus*) and eggs of the parasite released with faeces and adult stages of the parasite developed in the intestine were recovered. After keeping the eggs in water for several days ciliated miracidia were observed by application of tactile pressure. Infested snails were dissected and the morphometric characters of sporocyst and redia stages were recorded.

The eggs were operculated, oval shaped, yellowish brown in colour with lattice design on the shell surface. The ciliated miracidi, was the infective stage to snails. The sporocyst was sac like and the redia was tubular and curved. The cercaria had an oral and a ventral sucker, two eye spots and a tail. The encysted metacercaria was oval in shape with 'X-shaped excretory bladder'. Excysted metacercaria was elongated and narrower at the anterior end being similar to adults. The adult worm was small and oral sucker was surrounded by 32 circumoral spines arranged in two alternated rows. Acetabulum located in the middle of the body. The excretory bladder opened to a terminal excretory pore. The morphology and morphometric characters of the life cycle stages of the *Centrocestus* sp. that infests Koi carp at Rambadagalle and Ginigathhena ornamental fish breeding centres were compared with published literature and the parasite was identified as *Centrocestus formosanus* (Nishigori, 1924), which has not been documented in Sri Lanka earlier.

Keywords: *Centrocestus formosanus*; Koi carp; *Melanoides tuberculata*; life cycle, morphology.

INTRODUCTION

The digenean parasite *Centrocestus* sp. belongs to the Family Heterophyidae, which was considered to be native to Asia (Salgado-Maldonado *et al.,* 1995; Scholz and Salgado-Maldonado, 1998). According to Mitchell *et al.* (2000), it is widely distributed throughout the world such as South and Southeast Asian countries and the United States. It is an important etiological agent that causes gill lesions and respiratory difficulties in infested fish leading to significant economic losses in the aquaculture industry (Mitchell *et al.,* 2005). Further, it is a zoonotic pathogen and humans get the infestation by ingestion of raw or partially cooked fish (Chai *et al.,* 2013).

The occurrence of *Centrocestus* sp. was recorded as a parasite in both food and ornamental fish species in Sri Lanka (Balasuriya, 1988; Sajeewani and Hettiarachchi, 1998; Hettiarachchi, 2002; Thilakarathne *et al.,* 2003; Wimalawickrama and Pathiratna 2005; Sumuduni *et al.,* 2014). Although the infestations of *Centrocestus* sp. is common in Sri Lanka, species identification has not been documented. Balasuriya (1988) had stated that metacercarial stage of *Centrocestus* sp. that infest food fish in certain areas of Sri Lanka (Dambulla, Polonnaruwa, Muruthawela, Udawalawa) was *C. armatus.* His conclusions was based on morphometric characters of the parasitic stage found on fish; and the complete life cycle of the parasite had not been studied and documented under local conditions.

Fish breeding centres at Rambodagalle and Ginigathhena in Sri Lanka are breeding and rearing more than 50 ornamental fish varieties and distributing throughout the country. Parasitic infestations directly affect the survival and quality of fish and thereby reducing the income of the above centres. Identification of parasitic species with their life cycle is essential in employing effective control methods with correct biosecurity measures and better management practices.

This study was planned to identify the species of the digenean gill parasite, *Centrocestus* sp. that infest Koi carp (*Cyprinus carpio*) at Rambadagalle and Ginigathhena ornamental fish breeding centres in Sri Lanka using morphology and morphometric characters of life cycle stages of the parasite.

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MATERIALS AND METHODS

Infested snails and quarantined Koi carps

The first intermediate host, the snail, *Melanoides tuberculata* (Fig. 1) was collected from earthen ponds of ornamental fish breeding centres in Rambadagalle (GPS points: 7° 51'N; longitude-80° 50'E, North western province) and Ginigathhena (GPS points: 7° 00'N; longitude-80° 49'E, Central province). Quarantined, 45 days old Koi carps (treated with 1ppm Trichlorofon and 2.5ppm $KMnO_4$) were collected from Ornamental Fish Breeding and Training Centre, NAQDA, Rambadagalle.

Collection of cercariae

M. tuberculata snails that were infested with the parasite *Centrocestus* sp. were induced to release cercariae by exposing to sunlight. Cercariae were identified following the methodology described by Ritossa *et al.* (2013) and Sumuduni *et al.* (2018) and were used to infest healthy, 45 days old Koi carps.

Infestation of healthy Koi carps with cercariae and observation of metacercariae

Fifty healthy Koi carp fish were individually placed in separate beakers and 200 cercariae were introduced to each beaker to infest the fish. After after two hours, fish were stocked into well aerated tanks and one-week after the experimental infestation the gills of Koi carps were examined under microscope to observe the development of metacercarial cysts.

Collection of sporocyst and redia life stages from the first intermediate host

Few infested snails were dissected, and squash preparations of the internal organs were observed under the microscope to identify the developmental stages of *Centrocestus* sp. inside the snail. The larval stages obtained from the snails were mounted on slides using a single staining procedure. Some quiescent stages were examined without staining and were identified using the morphological descriptions given by Hernandez *et al.* (2003).

Experimental infestation for obtaining adult parasites, eggs and miracidia

An avian (two weeks old male chicken) and a mammal (three months old male Wistar albino rat obtained from the Medical Research Institute, Colombo) were selected as definitive hosts to observe the adult parasite. The above animals were kept in cages and provided with standard laboratory feed and water *ad libitum*. Further, they were treated with Drontal plus® (a broad spectrum anthelmintic containing praziquantel, pyrantel and febantel) to clear intestinal parasitism and were kept for one week for acclimatization prior to the experimental infestation.

The chicken and the rat were fed continuously with fish experimentally infested with metacercariae of *Centrocestus* sp. once a day for a period of one week.Their faeces were checked for eggs laid by adult. At the fourth week from the end of experimental infestation, due to the presence of eggs in faeces, the chicken were euthanized using chloroform and the adult parasites were collected from the small intestine by necropsy. The rat was not euthanized as it excreted only a few eggs with the faeces. The adult parasites were mounted on slides using the single staining procedure with Borax Carmine and were identified using morphological and morphometric features (Hernandez *et al.,* 2003; Han *et al.,* 2008).

The eggs of the parasite were kept in beakers containing filtered river water and observed daily under microscope for the presence of miracidia, and the morphological features with morphometry of the miracidiae were recorded. Morphological and morphometric features of each life cycle stage of *Centrocestus* sp. recorded during the present study were then compared with the available literature to identify the species status of the parasite.

Ethical approval

Ethical clearance was obtained from the Institute of Biology, Sri Lanka to conduct the research (Registration No: ERC IOBSL136 11 15).

Figure 1: A photograph of *M. tuberculata*.

RESULTS

The life-cycle stages of the *Centrocestus* sp. encountered in the study were same as those documented in the general life-cycle of digeneans. These life-cycle stages are described below.

Sporocyst stage recovered from the snail, *M. tuberculata*

Sporocyst stage is sac like and measured 410±6µm in length (mean±standard deviation) and 135±5µm in width (Fig.2).

Redia stage recovered from the snail

The mature rediae (532 ± 73 µm x 133.2 ± 9 µm) were tubular and mostly curved (Fig. 3A) and lappets (ambulatory appendages) were absent in the mature redia (Fig. 3B). Young redia showed some movements using the locomotory appendages (Fig. 3A). The mouth opened at the anterior end and followed by an oval muscular pharynx leading to a short gut (Fig. 4B). A birth pore was present laterally at the level of the gut. The cercariae were found anteriorly in mature redia while the germinal balls and germinal cells were found posteriorly (Fig. 4A).

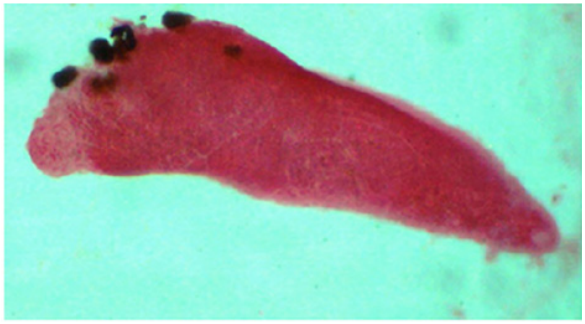

Figure 2: Sporocyst stage recovered from the snail *M. tuberculata* stained with Borax Carmine (x100).

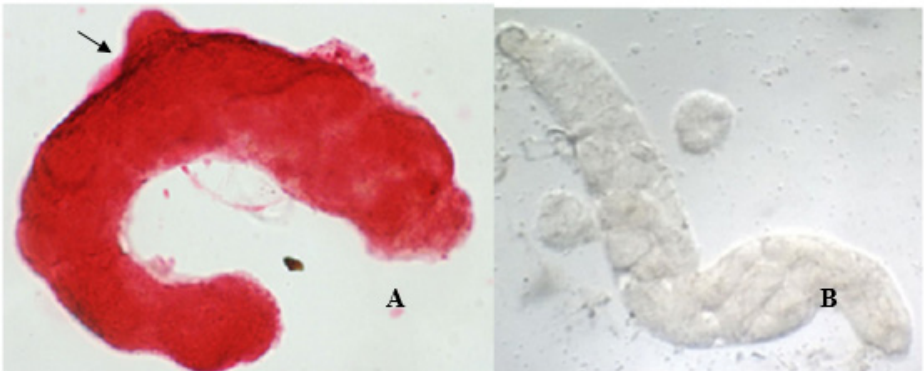

Figure 3: Redia found in the snail *M. tuberculata*; A – Young redia (x 100; stained with Borax Carmine) with ambulatory appendages (arrow); B - Mature redia (x100). Note the absence of locomotory appendages or lappets.

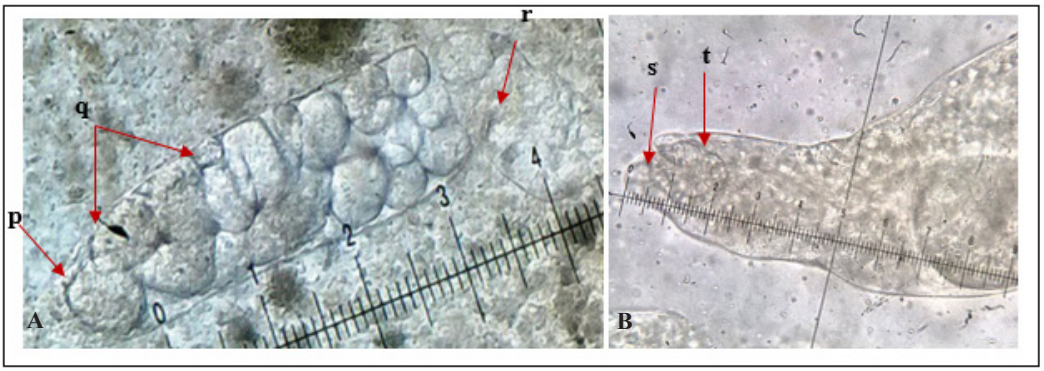

Figure 4: A - Redia found in the snail (*M. tuberculata*) with cercariae (p), germ balls (q), and germ cell deposited area (r). B- Anterior end of the redia with mouth (s) and the pharynx (t; x100).

Cercaria stage emerged from the snail

Cercaria 263.07 ± 16 µm in total length was heart shaped and the body (79.92 ± 14 x 26.64 ± 7 µm) was armed with short scale like spines while the tail (183 ± 15 x 22 ± 4 µm) lacked spines. It showed active swimming behaviour just after emerging from the snail. The oral sucker, 36 ± 2 µm in mean length, was terminal and equipped with nine small hooklets in two rows, 4 anteriorly and 5 posteriorly. Short, muscular pharynx just behind the oral sucker was followed by a thin long oesophagus. Behind the pharynx and towards the sides existed a pair of rectangular ocular spots containing brown pigments. Penetration glands were laterally arranged anteriorly at both sides of the body. The ventral sucker (40-46 µm) was located near the centre of the body.

The metacercaria stage obtained by experimental infestation of Koi carps

The metacercariae encysted in the gill filaments of the fish. Cysts were oval in shape and measured 246.42 ± 36x 164.6 ± 13 µm (Fig. 6A and 6B). Metacercarial cyst had a thin inner wall and a thick outer wall (Fig. 6C). The outer wall was fibrous and of host origin. Cyst caused an extensive proliferation of gill filament epithelium, distortion of the gill cartilage and normal gill architecture, and loss of the respiratory epithelium in heavy infestion. The metacercaria could be released from the cysts in the gills by applying a gentle pressure on the cover slip (Fig. 6D).

The morphology of the excysted metacercaria was similar to the adult flukes except for the absence of eggs (Fig. 7). A crown of 32 circumoral spines was present in the anterior region of the body (Fig. 6B) while an 'X-shaped excretory vesicle' with dark granules was observed at the posterior end of the body.

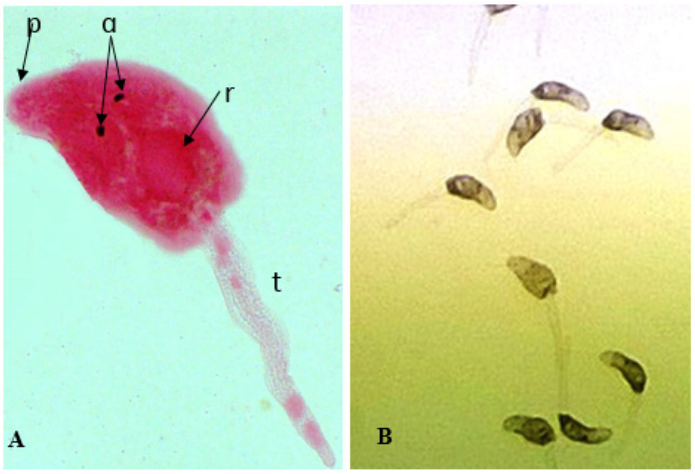

Figure 5: A -Cercaria stained with Borax Carmine (p - oral sucker, q- eye spots, r - ventral sucker, t – tail; x 100); B – Cercariae just after release from the snail *M. tuberculata* (x 40).

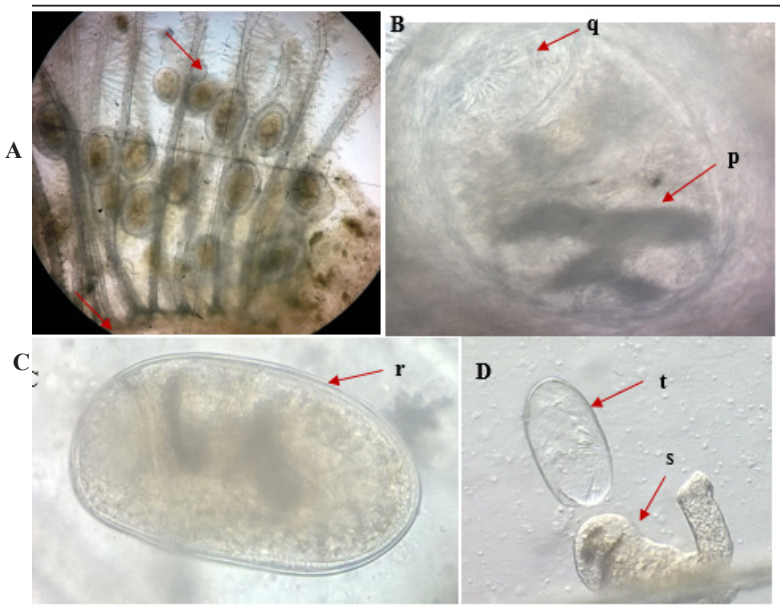

Figure 6: A - Encysted metacercariae (arrows) in the gills (x 40). B - Encysted metacercaria with 'X-shaped excretory bladder' (p) and oral spines (q) (x 100) ;C -Encysted metacercaria with thin walled cyst (r; x 100). D –Excysted metacercaria (s) and empty cyst (t; x 100).

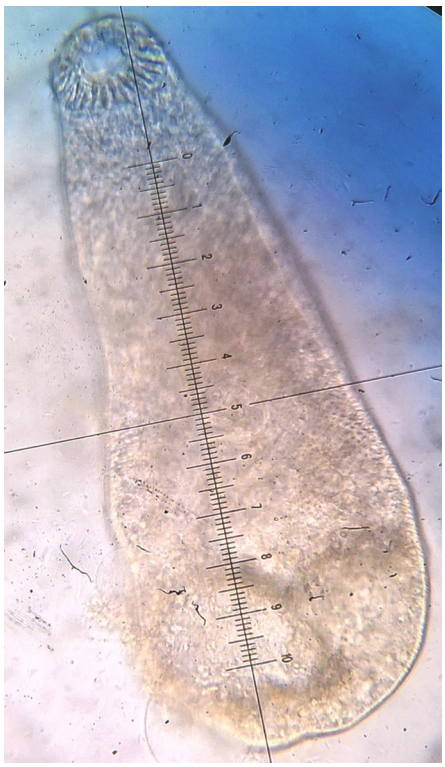

Figure 7: Excysted metacercaria (x100).

The adult parasite obtained by experimental infestion

The adult worm was small, pyriform and 506.54 ± 156 x 213.38 ± 34 µm in length and width (Fig. 8A) and the cuticle of the worm was covered with small scale-like spines. Oral sucker was terminal, oval in shape and surrounded by 32 circumoral spines arranged in two alternated rows (Fig. 8B). The spines of the anterior row measured 16.66 ± 1.23 x 6.66 ± 0.54 µm (length and width) while the length and width of the spines of the posterior row measured13.33 ± 1 x 5 ± 0.4 µm. The pre-pharynx was short and the muscular pharynx (49.6 ± 2.4 µmx 40.5 ± 2.23 µm) was followed by a short oesophagus. The intestinal caeca was slender and diverged outwards the acetabulum and extended to the level of the ovary. Acetabulum (46.63 ± 1.9 x 43.35 ± 2.5 µm) was located in the middle of the body. Testes were large, rounded and situated at a short distance from the posterior end of the body. The right testis was slightly larger (57.24 ± 1.94 x 42.07 ± 1.76 µm) than the left one (47.82 ± 4 x 33.34 ± 3 µm) and located on opposite sides, narrowly separated by the 'X-shaped excretory bladder' which opened to outside by terminal excretory pore.Oval shaped ovary (67± 2 x 50 ± 2 µm) was located immediately anterior to right testis. Vitellaria were extending along the lateral margin starting from the posterior side of the pharynx to posterior end of the body.

Eggs of the parasite

The eggs were oval, operculated and measured 45.6 ± 3.6 x 26.6 ± 2.2 µm. The shell surface was yellowish brown in colour and had a lattice design (Fig. 9). The findings of the current study are comparable with the published literature and are given in the Tables 1 and 2.

Miracidia of the parasite

Miracidia, the infestive stage to the snails, were obtained after keeping the eggs for 5 days in water at room temperature (28-30C). Miracidia excysted after giving a slight pressure on the egg and the ciliated and free swimming miracidia measured as 45.8 ± 3.2 x 24.5 ± 2.2 µm in length and width (Fig. 10).

Comparison of the measurements of adult and other life cycle stages with published data

The measurements of the adult stage encountered in this study were compared with the past studies on *Centrocestus* sp. (Table 1) and the measurements of the life-cycle stages were compared with that of *C. formosanus* of the previous studies (Table 2).

Considerable differences on the morphology and morphometry of the organs of the adult (Table 1) and the life cycle stages (Table 2) of *Centrocestus* sp. had been recorded from different countries. When compared with published reports, the measurements obtained for the different body parts of the adult *Centrocestus* sp. in the present study showed intermediate ranges for ovary and oral sucker, while the eggs, pharynx and body length have shown higher values as pointed out in the study of Wongsawad *et al.,* (2013). Further, the measurements of the testis and the ventral sucker showed lower length ranges (Table 1). The measurements of the life cycle stages found in the present study revealed intermediate values for the adult stage and higher values for redia, metacercaria and eggs while the measurements of the body and the eggs were consistent with Wongsawad *et al.,* 2013 (Table 2).

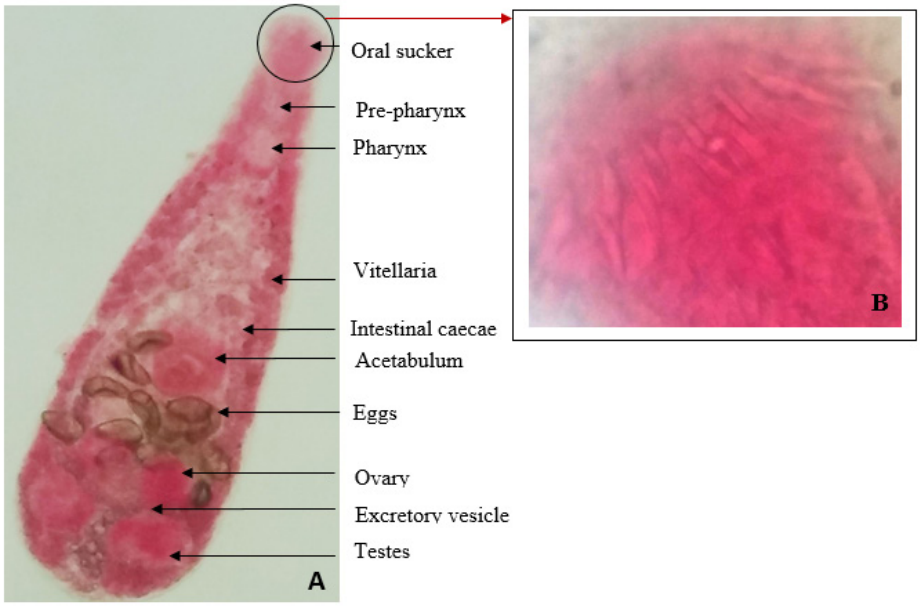

Figure 8: A - Adult of *Centrocestus* sp. stained with Borax Carmine (x100); B – Anterior end of the adult parasite with spines (arrow; x400).

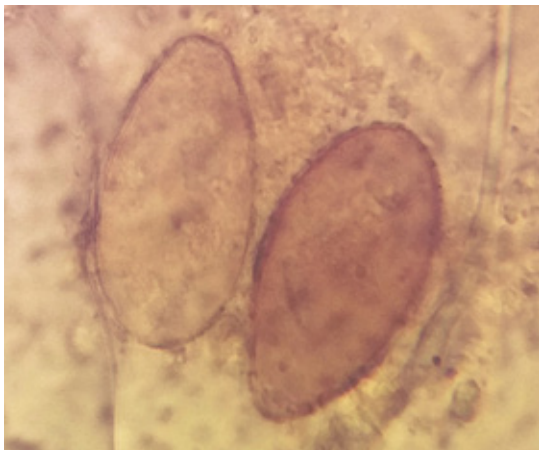

Figure 9: Eggs of the parasite *Centrocestus* sp. (x400).

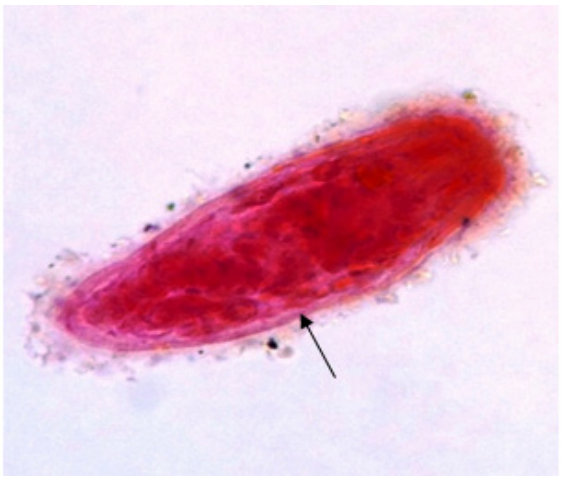

Figure 10: The ciliated (arrow) miracidium of *Centrocestus* sp. stained with Borax Carmine (x400)

Table 1: Morphometric characters of the adult stage of *Centrocestus* sp. recorded during the present study compared to published literature in micrometers (μm)

Item		Present study (N=20)	*C. armatus* (Tanabe, 1922)	*C. formosanus* (Hernandez *et al.*, 2003) Venezuela	*C. formosanus* (Han *et al.*, 2008) Korea	*C. formosanus* (Pinto and Melo, 2010) Brazil	*C. formosanus* (Yousif *et al.*, 2016) Egypt	*C. formosanus* (Wongsawad *et al.*, 2013) Thailand
Body	Length	350 - 663	350 - 630	410 - 600	245 - 325	232 - 478	410 - 600	600 - 750
	Width	247 - 179	182 - 284	212 - 360	155 - 220	184 - 279	300 - 360	200 - 290
No.of circumoral spines		32	44	32	32-34	32	32	34
Pharynx	Length	47 - 52	37 - 54	40 - 52	28 - 34	27 - 36	35 - 40	45 - 57.5
	width	43 - 52	28 - 47	35 - 40	20 - 30	16 - 24	40 - 52	37.5 - 50
Oral sucker	Length	50 - 55	56 - 74	55 - 63	45 - 58	47 - 67	55 - 63	60 - 80
	width	44-49	61 - 76	45 - 52	38 - 50	33 - 49	45 - 52	52.5 - 82.5
Ventral sucker	Length	44 - 48	49 - 61	50 - 41	45 - 55	44 - 60	51	52.5 - 57.5
	width	40 - 46	54 - 69	32 - 61	33 - 45	41 - 55	50	55 - 70
Testis (right)	Length	55 - 60	92 - 137	70 - 100	45 - 93	33 - 55	70 - 100	57.5 - 92.5
	width	40 - 44	47 - 74	60 - 87	24 - 50	63 - 93	60 - 87	100 - 137.5
Testis (left)	Length	43 - 51	81 - 115	48 - 59	55 - 88	36 - 60	65 - 96	67.5 - 125
	width	30 - 36	37 - 74	34 - 40	30 - 63	65 - 91	60 - 82	82.5 - 125
Ovary	Length	64 - 69	59 - 74	50 - 80	40 - 52	40 - 52	75 - 97	62.5 - 87.5
	width	47 - 52	86 - 103	34 - 46	67 - 101	67 - 101	32 - 50	65 - 150
Egg	Length	42 - 49	28 - 32	21 - 40	30 - 36	32 - 37	20 - 38	40 - 47.5
	width	24 - 29	16 - 17	17 - 20	15 - 19	16 - 20	12 - 18	20 - 20

Table 2: Morphometric characters of life cycle stages of *Centrocestus* sp. recorded during the present study compared to published literature.

Stages of life-cycle	Data from the present study on *Centrocestus* sp. (Length x width) (N = 20)	Data on *C. formosanus* from published studies
Redia	533 ± 73 (460 - 700) x 133.2 ± 9 (124 - 142)	476 x 130 (Yousif *et al.,* 2016)
Cercaria-body -tail	79.92 ± 14 (94 - 66) x 26.64 ± 7 (19 - 34) 183.15 ± 15 (168 - 199) x 22 ± 4 (18 - 26)	113 - 154 x 76 -103 114 – 155 x 18 – 24 (Mitchell and Goodwin, 2004)
Metacercaria	246.42 ± 36 (210 - 283) x 164.6 ± 13 (151.6 - 178)	107 - 225 x 78-159 (Mitchell and Goodwin, 2004)
Adult	506.54 ± 156 (350 -663) x 213.28 ± 34 (179 - 247)	600 - 750 x 200 - 290 (Wongsawad *et al.,* 2013)
Eggs	45.6 ± 3.6 (42 - 50) x 26.6 ± 2.2 (24 - 29)	40 - 47.5 x 20 - 20 (Wongsawad *et al.,* 2013)

DISCUSSION

In general, *Centrocestus* sp. use fish eating mammals and birds as definitive hosts while the first and the second intermediate hosts are aquatic snails and fishes respectively. The definitive hosts get infested by the ingestion of metacercariae, which are usually encysted in the branchial tissues of the infested fishes. Upon ingestion the metacercariae are ex-cysted in the intestine of the definitive hosts due to the action of digestive enzymes. Thereafter, the young flukes occur in the intestine and develop into egg producing adults within a few days of infestation. The eggs are released into the intestinal lumen and excreted in the feces, and if the eggs reach the water, they hatch and release miracidia (Lo and Lee, 1996).

Different snail species have been identified as the first intermediate host and among them *M. tuberculata* has been found to be the most common host for *Centrocestus* sp. in many countries (Muller, 1774; Mitchell *et al.,* 2005; Pinto and Melo, 2010). Snails are infested by either eating the eggs or by the penetration of miracidia and thereafter the miracidia reach the digestive gland of the snail and transform into the sporocyst stage. Sporocyst asexually produces redia that give birth to a thousand-fold of cercariae, the infestive stage to fish. The free swimming cercariae, which are released into the water by the infested snails, actively penetrate the gills of the fish and encyst as metacercariae in the branchial tissues (Mitchell and Goodwin, 2004).

Compared to the information derived from past literature, the parasite that infest Koi carps at the two ornamental fish breeding centers was identified by the present study as *Centrocestus formosanus* (Nishigori, 1924) and its complete life cycle has also been described in the current study. This is the first time that the sac like sporocyst of *C. formosanus* is described and it giving birth to redia is reported. Cercariae, germ balls, birth pore and lappets were observed in both mature and immature rediae. The observed morphologies of the rediae of different maturity stages are similar to findings reported for *C. formosanus* by Hernandez *et al.,* (2003) and Yousif *et al*., (2016).

The morphology and morphometry of the cercariae recorded during this study are consistent with the morphological features reported by Hernandez *et al.,* (2003) and Yousif *et al.,* (2016). It belongs to the group of pleurolophocerca, which has a heart shaped body with two prominent eyespots and a single non-forked tail. The X-shaped excretory bladder of the metacercariae is a unique feature to identify the genus *Centrocestus* and the number of spines around the oral sucker of the metacercaria vary according to the species (Chen, 1942). The morphology of the excysted metacercaria is similar to the adult except for the absence of eggs.

There are different characteristics to identify the adult of the *Centrocestus* species. Identification of the fluke mainly depends on the number of circumoral spines, form of the margin of the testes and ovary, maturity stage of egg, and the shape and form of excretory bladder. According to various authors the main characteristic that could be used to differentiate the species of *Centrocestus* is the number of spines around the oral sucker; Nishigori, (1924) has reported that *C. formosanus* has 32 spines, Tanabe (1922) has recorded 44 spines in *C. armatus* and according to Loos, (1899) *C. cuspidatus* has 36 circumoral spines. Present study has recorded about 32 circumoral spines arranged in 2 alternative rows around mouth, a large bipartite seminal vesicle, an oval, smooth ovary and an X-shaped excretory vesicle in adult fluke. Adult fluke having the same characteristics was reported by Nishigori (1924) from Northern Formosa; Chen (1942) from Hong Kong; Hernandez *et al.,* (2003) from Venezuela; Han *et al.,* (2008) from Lao PDR; Pinto and Melo (2010) from Brazil, Yousif, *et al.,* (2016) from Egypt. Many authors have used the number of spines around the oral sucker as the main character to differentiate the species of *Centrocestus* (Yousif *et al.,* 2016). According to Chen (1942); Kobayasi (1970) and Premvati and Pande (1974) adult *C. formosanus* has 32 circumoral spines.

The morphology of the egg of *Centrocestus* sp. observed during the present study is consistent with

descriptions presented by Han *et al.,* (2008) and it appears that the morphology of the eggs of *Opisthorchis viverrini* and *C. formosanus* are similar as both species have a lattice pattern on the shells of their eggs. Miracidium is the stage that infests snails and gives birth to sporocysts. This stage possesses cilia, for swimming which help to locate the first intermediate host, the snail. Miracidia did not naturally hatch during the present study which is consistent with the study of Pinto *et al.,* (2018). These authors concluded that the eggs are passively transmitted to the snails.

The slight differences in the morphometry obtained in this study compared to the studies done elsewhere could be related to the method of fixation of the parasitic stages, changes in the morphometry of cercaria body due to its movements and the variations in the morphometry of different developmental stages of redia (Hernandez *et al.,* 2003). Further, the environmental factors and the condition of the hosts may also influence the morphological features of the developmental stages (Thomas *et al.,* 2014). Although the parasite has the same origin of second intermediate and definitive host, the measurements of the adult, eggs and the internal parts of the body show different values for *C. formosanus* in the studies carried out by Wanlop *et al.,* (2017) and Wongsawad *et al.,* (2013) in Thailand.

The high abundance of the first intermediate host *M. tuberculata* due to the favourable environmental conditions in the freshwater bodies is the main factor for the high prevalence of *Centrocestus* sp. in the fish in Sri Lanka. This digenean parasite causes considerable impact on both ornamental and food fish industry in Sri Lanka, and the information on the biology of this parasite and its larval stages is important to formulate sustainable control measures.

CONCLUSION

This is the first detailed study carried out to describe the morphological features of all developmental stages of *Centrocestus* sp. that infests Koi carps in Sri Lanka. Despite the slight variations in the morphometry, it appears that the *Centrocestus* sp. reported in Koi carps at Rambadagalle and Ginigathhena fish breeding centers is *C. formasanus* based on the number of circumoral spines. Further work is necessary to document all *Centrocestus* species that could occur in cultured ornamental and food fishes as well as in wild fishes of Sri Lanka by utilizing conventional and molecular techniques.

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DECLERATION OF CONFLICT OF INTEREST

Authors declare no conflict of interest.

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