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Original paper

Further contribution to the life history of *Centrocestus formosanus* (Nishigori, 1924) Price, 1932 (Trematoda: Heterophyidae) with special reference to a new first intermediate host from the South Western Ghats, India

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ABSTRACT. *Centrocestus formosanus* is a zoonotic small invasive heterophyid fluke with worldwide distribution. Its three-host life cycle requires a thiarid snail as first intermediate host, fishes as second intermediate hosts and piscivorous birds and mammals as definitive hosts for completion. As far as is known, the only first intermediate host being utilized, globally, by this parasite is the snail, *Melanoides tuberculata*. In the present study, life cycle stages of *C. formosanus* were recovered naturally and successfully tested in the laboratory. Study also adds a new host to its life cycle, the snail *Bithynia* (*Digoniostoma*) *pulchella,* as the first intermediate host with natural infection. Parasitological descriptors (prevalence, mean intensity and mean abundance) of infection were measured for each host. Being an invasive parasite, addition of a new host to *C. formosanus* life cycle has notable ecological and evolutionary significance.

Keywords: *Centrocestus formosanus*, life cycle, Western Ghats, snail hosts, fish hosts, infection statistics

Introduction

Centrocestus formosanus (Nishigori, 1924) Price, 1932 is a small heterophyid fluke, described for the first time in Taiwan (Nishigori, 1924) and thereafter from many parts of the globe, including Asia [1–6], Europe [1–8], North and South America [9–13]. *Centrocestus formosanus* is a generalist parasite with a broad range of fish hosts and wide distribution in brackish and marine water [5,8,14–16]. Studies clearly show that the parasite causes severe gill lesions, cartilage proliferation around the cysts, respiratory disorders and eventually leads to death of infected fishes [17–19]. These pathological conditions are of health and economic concerns on the wild and farmed (edible and ornamental) fishes [20,21]. Furthermore, human infections can occur through consumption of raw or improperly cooked fish containing metacercariae [22].

Similar to other digenetic trematodes, *C. formosanus* has a complex life cycle with three hosts. Eggs produced by adult *C. formosanus* hatch into miracidia which uses a thiarid snail as their first intermediate host to develop into cercariae. Subsequently, free-swimming cercariae encyst specifically in the gills of second intermediate fish hosts where they develop into metacercariae. Piscivorous birds and mammals, ingesting the infected fish, complete the lifecycle [13,23,24].

As far as is known, the only first intermediate host of *C. formosanus* is *Melanoides tuberculata* (Müller, 1774) all over the world [13,24]. The present study, reports a new first intermediate host, the freshwater snail, *Bithynia* (*Digoniostoma*) *pulchella* (Benson, 1836). Life cycle stages of the parasites obtained from natural infection were experimentally tested in the laboratory. Data on parasitological descriptors (prevalence, mean intensity and mean abundance) of infection were analysed. Any study on *C. formosanus* infection has importance in the fields of veterinary, public health, aquaculture and food safety.

Materials and Methods

Survey of intermediate hosts and evaluation of parasitic infection

The fishes were collected from freshwater bodies in 31 different localities of the Wayanad region (11°27’00”N and 11°58’35”N; 75°47’50”E and 76°26’35”E), in the Western Ghats, India (Fig. 1) for a period of three years (March 2017–February 2020). They were brought alive to the laboratory, maintained in glass aquaria (105×45×45cm; water depth 30 cm), fed with fish meals and examined for parasites. The freshwater fishes, *Schistura semiarmata*, *Haludaria fasciata* and *Barilius gatensis*, collected from the streams in Vythiri, Thirunelli and Valavayal regions were infected with metacercaria of *C. formosanus*. The freshwater snail *B.* (*D.*) *pulchella* collected from the same habitat from which an infected fish has been found (Valavayal) during the same period were brought to the laboratory, washed in running water, and maintained in separate beakers containing chlorine-free water. The snails were exposed to sunlight and monitored regularly for cercariae. The pleurolophocercous cercariae emerged were studied live. The quantitative analyses of parasitological descriptors (prevalence, mean intensity and mean abundance) of infection were carried out using the free software accessible on the Quantitative Parasitology Web portal [25] (https://www2.univet.hu qpweb/qp10/index.php).

Morphological studies of developmental stages

Cercariae from naturally infected snails were studied with or without vital staining (neutral red) under a Nikon ECLIPSE Ni-U (Nikon, Japan) phase contrast research microscope. A few infected snails were later crushed and examined for intra-molluscan stages of the parasite. Rediae from the digestive glands were studied live with or without vital staining. The freshwater fishes, *S. semiarmata*, *H. fasciata* and *B. gatensis*, were dissected out in physiological saline (0.75% NaCl solution) under a Labomed (Luxeo 4Z) stereozoom microscope and the metacercariae were collected from gills. The metacercariae were transferred to a Petri dish containing saline and live parasites (both unstained and neutral red-stained) were observed under Nikon ECLIPSE Ni-U phase contrast research microscopeto study the morphological characteristics. Permanent whole mounts were prepared by fixing them in 5% formalin under slight cover glass pressure and staining with acetocarmine following Cantwell [26].

Experimental infection studies

Five of each newborn (infection free) freshwater fishes, *Pethia punctata* and *H. fasciata*, collected from paddy fields, where *C. formosanus* infection was never reported, were exposed to newly emerged cercariae and examined at intervals to observe metacercarial development.

Five eight-day-old, antihelminth-treated chicks brought from the poultry farm were used for experimental infection studies. After seven days, infective encysted metacercariae collected from infected fishes were fed to chicks. The development of adult flukes was traced by examining the experimental birds. Adult parasites recovered were studied alive, without or with vital staining using neutral red. Permanent preparations were made by fixing the parasites in 5% formalin under a slight cover-glass pressure and staining in acetocarmine following the procedure outlined by Cantwell [26].

Measurements, sketches and photographs

TheNikon Y-TV55 camera attached to the ECLIPSE Ni-U, Nikon, Japan phase contrast research microscope was used to take photographs. The trematodes were measured using the Nikon NIS-Elements imaging software attached to the above microscope. All measurements were in micrometers (μm), as range followed by mean in parentheses. Descriptions are based on the measurements of a minimum of ten specimens. Illustrations were made using the Nikon Y-IDT drawing tube attached to the Nikon ECLIPSE Ni-U microscope, and the details were added free-hand from observations made on live specimens.

Results

Natural infection with developing stages

The freshwater fishes, *S. semiarmata*, *H. fasciata* and *B. gatensis*, were naturally infected with metacercariae of *C. formosanus*. The fishes were heavily infected with cysts of *C. formosanus* in their gills. The parasitological descriptors of infection varied with hosts (Tab. 1). Highest values for prevalence and mean abundance of infection were recorded in *S. semiarmata* while the intensity of infection was higher in *B. gatensis*. The lowest values

Table 1. Quantitative descriptors of *C. formosanus* infection in fish hosts

Hosts	Prevalence		Mean intensity		Mean abundance		Aggregation indices (Variance/mean ratio)	Poulin's discrepancy index	
		CI		CI		CI			CI
Schistura semiarmata	0.143	0.004–0.579	40	*	5.71	0–11.4	40	0.75	0.25–0.75
Haludaria fasciata	0.009	0.002–0.026	44.7	3–85	0.4	0.0179–1.92	115.12	0.993	0.988–0.994
Barilius gatensis	0.021	0.001–0.111	81	*	1.69	0–5.06	81	0.959	0.857–0.959

* There's only 1 infected host in the sample, Confidence Interval cannot be calculated, CI – Confidence interval

for these parasitological descriptors were recorded in *H. fasciata.*

Twenty three out of 230 (10%) snails, *B.* (*D.*) *pulchella* collected from Valavayal were infected with pleurolophocercous cercaria of *C. formosanus*.

Experimental infection studies

Five immature cysts of *C. formosanus* were obtained from two *P. punctata* after 15 days. On the 29th day of post-infection eighteen cysts were obtained from four *H. fasciata*. Thus, six out of ten fishes were infected with this parasite; therefore, the total prevalence of infection was 60.00%. The adult parasites were recovered through experimental infection of five, 15-day-old chicks with metacercariae obtained from natural infections. Chicks were fed with metacercariae infected gills collected from the freshwater fish *H. fasciata* and 47 adult *C. formosanus* were recovered from the intestines of chicks one week after exposure. The prevalence and mean intensity of infection were 100% and 9.4, respectively.

Developing stages of C. formosanus

Redia (Fig. 2a): hepatopancreas of the host, *B.* (*D.*) *pulchella,* was heavily infected with redia of *C. formosanus*. Slight brownish C-shaped redia has well developed pharynx and short intestine; measured 505.01–1396.96×125.44–216.88 (1042.26×169.79). On an average, seven cercariae and 22 germ balls were found inside each redia.

Cercaria (Fig. 2b): cercaria showed circular movements by curving body ventrally and rests for a while after vigorous movements. 'Head' kept down during resting. Creep on slides using its oral sucker. Slight brownish cercarial body measured 217.50–343.48 (294.42)×134.75–188.09 (180.04). Tail terminal; measured 250.67–396.67 (326.33) ×43.40–64.47 (57.89), provided with lateral fins up to 4/5th from proximal end and dorsal fin in the last

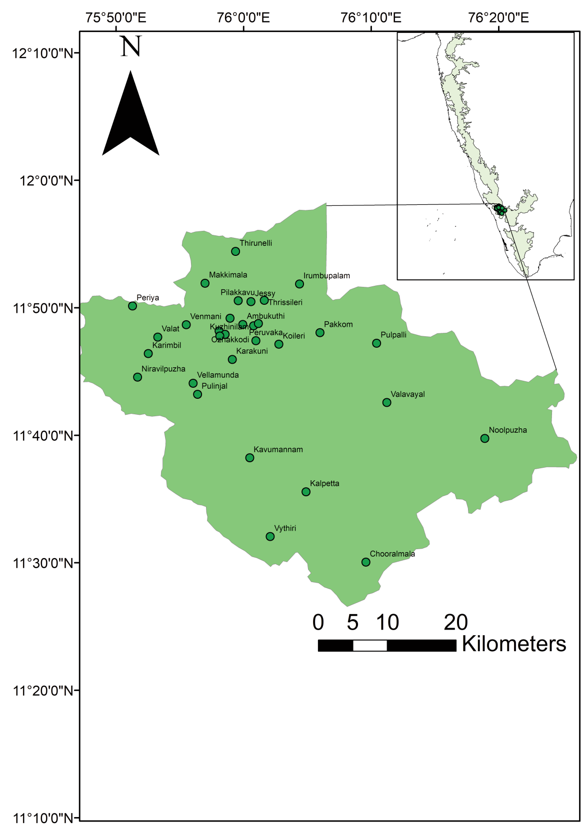

Figure 1. Study area. Locations of specimen collections in the Wayanad region of the Western Ghats

1/5th portion. Oral sucker terminal, measured 71.98–87.97 (79.90)×34.56–51.05 (46.65); provided with spines. Sub terminal mouth continues to pharynx and esophagus. Esophagus bifurcates to form caeca 46.09–69.9 (56.39)×8.28–13.76 (11.18). Pigmented eye spot positioned beside oral sucker, one on either side. Seven pairs of penetration glands arranged in two groups alongside pharynx. Triangular genital primordia above excretory vesicle. Thick-walled excretory vesicle positioned at the posterior end of body.

Metacercaria (Fig. 3): cyst oval, transparent, double layered. Body flask-shaped, spinose,

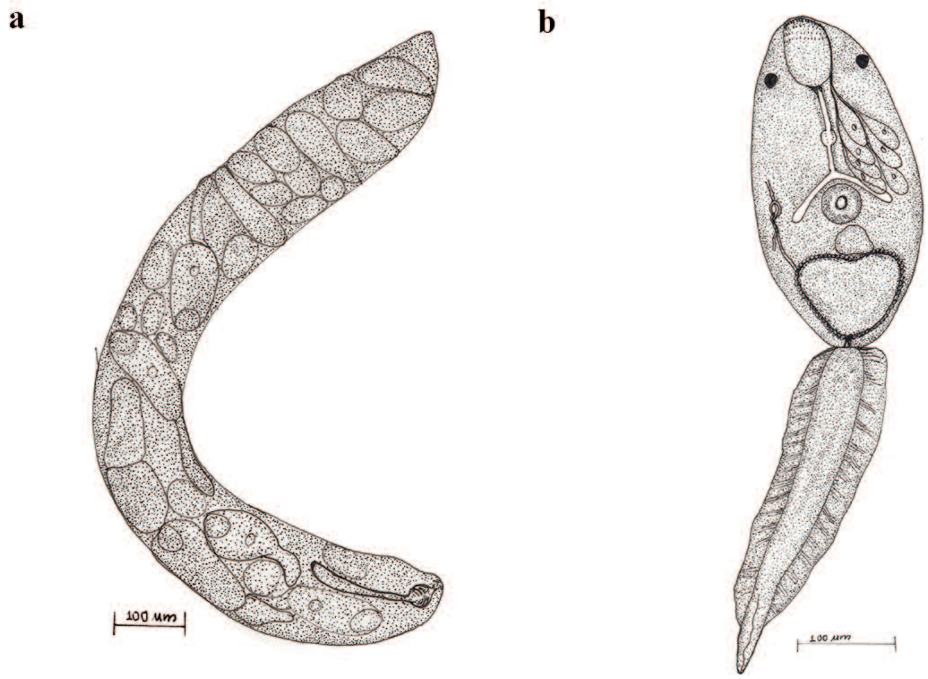

Figure 2. Redia and cercaria of *C. formosanus*. a. Redia, b. Cercaria

measured 248.67–423.99×94.24–154.42 (355.81×118.07). Body spines larger and densely distributed in the anterior part of body become progressively smaller and sparse towards posterior end. Oral sucker 37.21–47.95×37.14–49.54 (41.71×43.23), terminal, funnel-shaped, with 34 circum-oral spines arranged in two alternate rows. Ventral sucker post equatorial, oval, 20.34–38.31×30.27–44.33 (28.53×32.96) and 99.65–205.37 (161.89) distance away from oral sucker and 84.17–139.29 (117.61) distance away from posterior end. Short pre-pharynx 24.58–56.81 (38.84) leads to muscular pharynx 16.48–30.56×14.18–31.95 (26.35×25.69). Esophagus 35.10–59.37 (46.11) long. Caeca measured 91.26–133.18×7.22–11.23 (103.66×9.46), terminate anterior to the excretory bladder. Testes

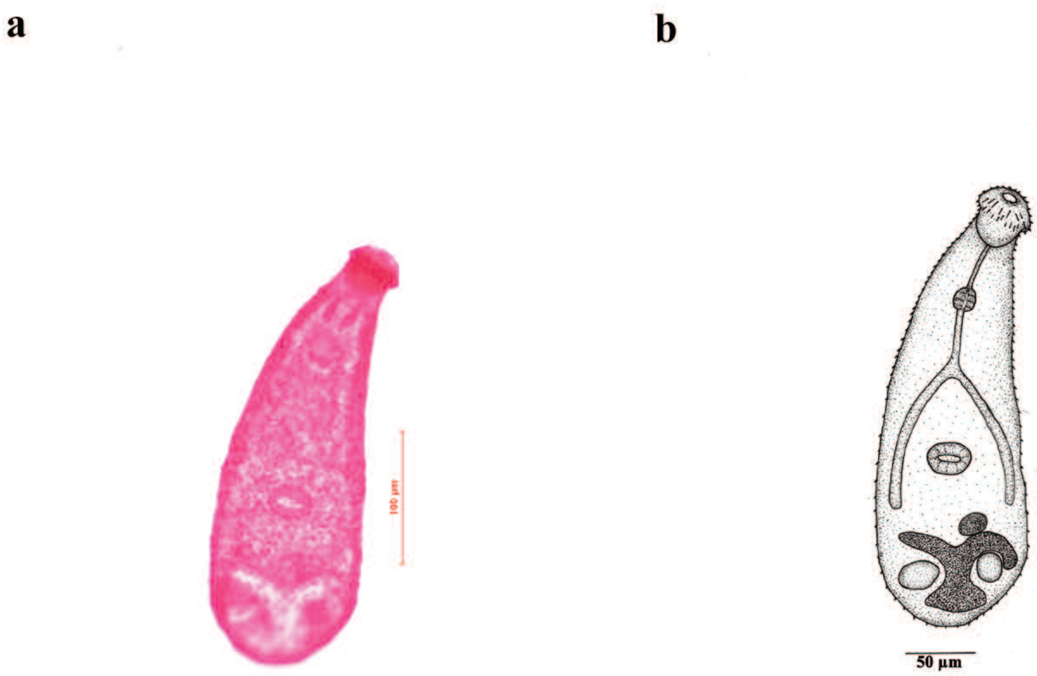

Figure 3. Metacercaria of *C. formosanus.* a. Photograph, b. Line drawing

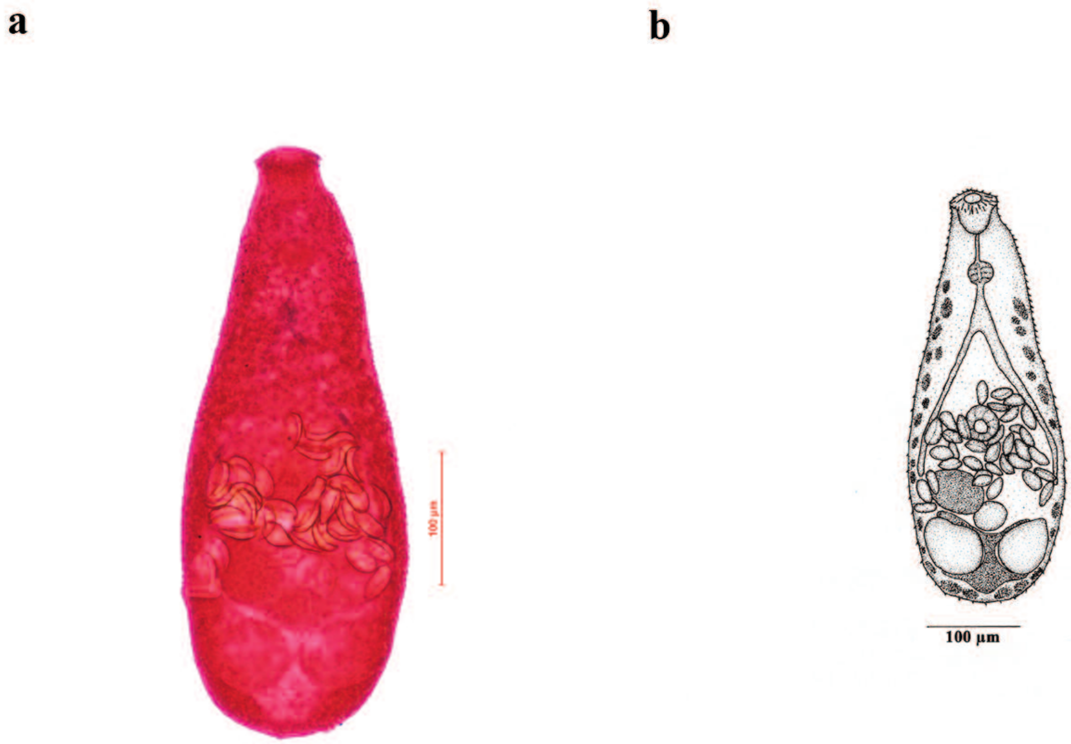

Figure 4. Adult of *C. formosanus.* a. Photograph, b. Line drawing

two, round to oval, symmetrical, enclosed by arms of X-shaped excretory bladder. Testes located side by side, measured 26.75–40.71×13.64–62.25 (34.82 ×43.68) and 16.78–42.52×24.25–65.90 (33.12 ×41.80). Ovary oval, lies just anterior to excretory bladder, measured 12.75–29.22×15.20–37.82 (21.23×26.46). Excretory bladder X-shaped, at posterior end of body, filled with excretory concretions.

Adult (Fig. 4): body flask shaped, spinose, measured 372.67–511.63×144.44–193.26 (432.71× 166.84). Body spines larger and densely distributed in the anterior part of body become progressively smaller and sparse towards posterior end. Oral sucker 40.65–50.28×41.32–53.10 (45.23×48.23), terminal, funnel-shaped, with 34 circum-oral spines arranged in two alternate rows. Ventral sucker post equatorial, oval, 27.78–43.00×30.73–42.84 (35.64 ×38.77) in size. Ventral sucker 155.84–231.03 (189.77) distance away from oral sucker. Short pre-pharynx 25.48–44.65 (33.61) leads to muscular pharynx 18.73–28.42×24.95–34.44 (23.16×29.03). Esophagus 21.66–64.43×13.31–26.31 (41.24× 17.47) in size. Caeca measured 132.83–170.66 ×8.36 –16.43 (157.00×11.16), terminate anterior to the excretory bladder. Testes two, round to oval, situated symmetrically, enclosed by arms of X-shaped excretory bladder. Testes located side by side, measured 40.39–53.28×58.52–75.39 (50.06×63.68) and 38.97–52.85×58.37–66.15 (49.32×60.97). Ovary oval, lies just anterior to excretory bladder, measured 28.84–50.98×37.33–68.77 (39.06×48.87). Uterus coiled, located between testes and intestinal bifurcation. Vitelleria form in a small group of follicles distributed at the level of pharynx to the posterior end of body. Eggs measured 24.25–31.54 ×12.66–17.97 (27.78×14.95) in size. Excretory bladder X-shaped, at posterior end of body, filled with excretory concretions.

Discussion

Taxonomic identification of species of *Centrocestus* is mainly based on the number of circumoral spines, maturity stage of eggs, level of caecal endings, shape of excretory bladder and presence of the pre-pharynx [22–24]. *Centrocestus formosanus* has 32 circum-oral spines arranged in two alternative rows, a large bipartite seminal vesicle, an oval ovary and an X-shaped excretory vesicle [22, 23]. Morphology of parasite obtained in the present study is similar to that of *C. formosanus* with all above mentioned characters. Although morphometry is different, the present parasite is identified as *C. formosanus*. Earlier studies [23,24,27–30] pointed out that even though the morphological characteristics of *C. formosanus* recovered from different countries are the same, there are considerable differences in their morphometry.

Centrocestus formosanus requires mollusc, in the family Thiaridae in particular [24], as its first intermediate host with fishes as second intermediate, and fish-eating birds and mammals as definitive hosts. In the present study, freshwater fishes, *S. semiarmata*, *H. fasciata* and *B. gatensis*, were naturally infected with metacercaria of *C. formosanus* and *B.* (*D.*) *pulchella* with cercaria. The experimental infection of cercaria emerged from *B. (D.) pulchella* was successful in *P. punctata* and *H. fasciata* and the adult forms were reared in chicks.

It was Nishigori [27] who identified adult parasite from natural and experimental definitive hosts from Taiwan and larval stages were studied by Chen [31]. Pinto and Melo [30] confirmed *M. tuberculata* as the first intermediate host. Snail gets the infection by passive ingestion of eggs of fluke [13]. Sewell [32] reported cercaria of this trematode in India. The heterophyid trematode *C. formosanus* is native to Asia and expanded its geographical distribution to Europe, Africa and Americas [13,24].As far as is known, *M. tuberculata* is the only first intermediate host for this parasite worldwide [24]. According to Pinto et al. [13] the worldwide expansion of *C. formosanus* is probably related to the wide distribution of its molluscan intermediate host, the invasive *M. tuberculata*, which has been reported as natural first intermediate host of this digenean in both Asia and the Americas.

The present study revealed a new first intermediate host, *B.* (*D.*) *pulchella* with natural infection. Wide range of distribution and capacity to overcome environmental constraints like water scarcity [33] may be reasons to choose the available snails as first intermediate hosts. Long intra-molluscan phase of *C. formosanus* [13] also demands the longevity of hosts. Furthermore, according to previous studies [34,35] host specific parasites can take advantage of opportunities to parasitize multiple host species by switching to new hosts.

Heterogeneity among host individuals in exposure to parasites or in susceptibility to infection is thought to be the main factor generating aggregation, with properties of parasites themselves explaining some of the variability in aggregation levels observed among species [36]. The index of discrepancy measures the departure between the observed parasite distribution and a hypothetical one in which all hosts harbor equal number of parasites [36,37] and the index varies between 0 (no aggregation) and 1 (maximum aggregation). In the present study, the metacercaria of *C. formosanus* showed indices values, which corresponded to the aggregate distributions, higher than 0.7 in all fish hosts. The study of Jithila et al. [38] reported an over-dispersed aggregate distribution of different species of metacercariae in *H. fasciata* and *Pseudosphromenus cupanus* in the Western Ghats, Wayanad region. Though studies are plenty on various other aspects of freshwater fishes of the Western Ghats, studies on parasitological aspects are scanty and this study [38] was the first approximation to the infection patterns by metacercariae in this region.

Moreover, the importance of *C. formosanus* dissemination is related to its ability to infect various fish species including those with ecological or commercial and public health importance [8,22]. In fishes, the gill lesions caused by *C. formosanus* metacercariae eventually lead to respiratory failure and death and thereby affecting production in fish farms [1,8,10,17,39,40]. Besides, naturally infected freshwater fishes obtained during the present study, *S. semiarmata*, *H. fasciata*, and *B. gatensis* are endemic to the Western Ghats region [41]. Being an invasive parasite[8], addition of a new host to *C. formosanus* life cycle has notable ecological consequences and has evolutionary significance too.Thus, more studies are required to address these issues.

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