Prevalence of *Centrocestus formosanus* Metacercariae in Ornamental Fish from Chiang Mai, Thailand, with Molecular Approach Using ITS2

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Abstract: The prevalence of *Centrocestus formosanus* metacercariae was investigated in ornamental fish purchased from a pet shop in Chiang Mai, Thailand, including *Carassius auratus* (goldfish), *Cyprinus carpio* (Koi), *Poecilia latipinna* (Sailfin Molly), *Danio rerio* (Zebrafish), and *Puntigrus tetrazona* (Tiger barb), and *Puntius brevis*, *Puntius gonionotus*, *Puntius meiacanthus*, *Cyclocheilichthys armatus*, *Anabas testudineus*, and *Hemicorhynchus siamensis*. The prevalence of *C. formosanus* metacercariae was determined by the molecular approach using ITS2. The prevalence was found in *C. auratus* (83.3%), and the highest intensity was noted in *C. carpio* (70.8 metacercariae/fish). The most important morphological character was the presence of 32-34 circumoral spines on the oral sucker. The phylogenetic studies using the rRNA ITS2 region revealed that all the specimens of *C. formosanus* in this study were grouped together with *C. formosanus* in GenBank database. This is the first report on ornamental fish, *C. carpio*, *P. latipinna*, *D. rerio*, and *P. tetrazona*, taking the role of second intermediate hosts of *C. formosanus* in Thailand. Prevention and control of metacercarial infection in ornamental fish is urgently needed.

Key words: *Centrocestus formosanus*, ornamental fish, *Carassius auratus*, ITS2, Chiang Mai

*Centrocestus formosanus* (Nishigori, 1924) (Heterophyidae) was described from Taiwan and now known to distribute widely in Asia [1]. The adult worm lives in the intestine of fish-eating birds and mammals [2-4]. Human cases infected with *C. formosanus* were reported in Lao PDR and Vietnam [4,5]. Pleurolophocercous cercariae are shed from the thiarid snail (*Melanoides tuberculata*) [1,6]. The metacercariae are found on the gill of several freshwater fish species, such as *Puntius brevis*, *Hampala dispar*, *Puntius goniomus*, *Puntius meiacanthus*, *Cyclocheilichthys armatus*, *Anabas testudineus*, and *Hemicorhynchus siamensis* [7-9]. In addition, the ornamental fish were reported to be infected with *C. formosanus* [2]. The parasite infection causes a problem in fish culture and leads to a reduction of fish production in aquaculture [1,10]. The presence of metacercariae on the gills of fish could be one of the reasons for the death of fish [2]. In Thailand, 2 species of *Centrocestus*, including *C. formosanus* and *C. caninus*, were reported; however, *C. caninus* is regarded as a synonym of *C. formosanus* [4].

The adult stage of this fluke was found in humans from Chiang Mai and Chiang Rai Provinces, northern Thailand [11,12]. The metacercariae were found in several species of freshwater fish [7,13-17]. However, there were few studies on metacercarial infection in ornamental fish species. Thus, the objective of this study was to determine the prevalence and species identification of *Centrocestus* in ornamental fish purchased from a pet shop in Chiang Mai Province, northern Thailand through morphological and molecular studies.

Total 150 ornamental fish, including 30 *Carassius auratus*, 30 *Cyprinus carpio*, 30 *Poecilia latipinna*, 30 *Danio rerio*, and 30 *Puntigrus tetrazona*, were collected from a pet shop in the Mueang District, Chiang Mai Province, northern Thailand during May-June 2016. The metacercariae were investigated on the gill of fish under a stereomicroscope, and then all the mature metacercariae (with X-shape excretory bladder) were collected.

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for further studies. Some of the metacercariae were fed to chicks (1-day old), and after 7 days the adult stages were collected from their small intestines. Some other metacercariae were used for molecular studies. The adult stage was used to make a permanent slide according to Boonchot et al. [18]. All specimens were fixed with 4% formalin, stained with hematoxylin, dehydrated in alcohol series, and finally mounted in permount.

*C. formosanus* genomes were extracted by Chelex (Fluka, Sigma-Aldrich, St. Louis, Missouri, USA) following Caron et al. [19]. DNA products were amplified with ITS2 region. The reactions were performed in a Thermal Cycler machine (Little Genius, Bioer Technology, Tokyo, Japan). The primer combination; forward 3S (5’-GGT ACC GGT GGA TCA CTC GGC TCG TG-3’) and reverse BD2 (5’-TAT GCT TAA ATT CAG CGG GT-3’) were performed in PCR for ITS2 gene. The PCR conditions were as follows: 2 min initial denaturation at 94°C, followed by 35 cycles of 1 min DNA denaturation at 94°C, 1 min primer annealing at 60°C, 1 min extension at 72°C, and 7 min for final extension at 72°C. PCR products were tested by gel electrophoresis with DNA Dye NonTox (Applichem, Darmstadt, Germany) stain. After gel electrophoresis, PCR products were purified and sent to sequence analysis. The sequence was analyzed by ClustalW in MEGA software version 6.0 [20]. The sequence of *C. formosanus* was compared and checked by BLAST program on National Center for Biotechnology Information database for species confirmation and gathering of essential sequences for phylogenetic analysis. ITS2 sequences of *C. formosanus*, *Centrocestus* sp., *Haplorchis taichui*, *Haplorcoides* sp., *Stellantchasmus falcatus*, *Paramphistomum epiclitum*, *Fasciola gi-

| Species of fish | No. of fish examined | No. of fish infected | Prevalence (%) | Intensity |
|----------------|---------------------|---------------------|----------------|----------|
| *Carassius auratus* (Goldfish) | 30 | 25 | 83.3 | 3.3 |
| *Cyprinus carpio* (Koi) | 30 | 11 | 36.7 | 70.8 |
| *Poecilia latipinna* (Sailfin Molly) | 30 | 5 | 16.7 | 1.4 |
| *Danio rerio* (Zebrafish) | 30 | 6 | 20.0 | 5.2 |
| *Puntigrus tetrazona* (Tiger barb) | 30 | 3 | 10.0 | 2.0 |

*Mean no. of metacercariae per fish.*

| Origin (fish) | Measurements (µm) | Carassius auratus (n= 10) compared with 2 previous studies |
|---------------|--------------------|----------------------------------------------------------|
| *Carassius auratus* | Present study (Thailand) | Han et al. (2008) (Lao PDR) | Wongsawad et al. (2017) (Thailand) |
| No. of circumoral spines | 34 | 32 (32-34) | 34 |
| Body length | 465-552 (508.8) | 245-325 (266) | 600-750 (668) |
| Body width | 176-224 (204.0) | 155-220 (192) | 200-290 (259) |
| Oral sucker length | 48-58 (64.4) | 38-50 (43) | 52.5-82.5 (76.0) |
| width | 62-66 (64.4) | 52 (40.8) |
| Pharynx length | 14-34 (22.4) | 28-34 (32) | 45-57.5 (49.5) |
| width | 30-40 (34.4) | 20-30 (26) | 37.5-50 (44.0) |
| Esophagus | 26-50 (40.8) | 34 (32-34) | 32.5-62.5 (51.5) |
| Ventral sucker length | 36-46 (42.8) | 45-55 (48) | 52.5-57.5 (55.5) |
| width | 44-56 (51.2) | 33-45 (35) | 55-70 (65.5) |
| Ovary length | 46-70 (56.0) | 50-80 (60) | 62.5-87.5 (76.5) |
| width | 50-120 (81.2) | 34-46 (42) | 65-150 (105.5) |
| Right Testis length | 60-80 (68.8) | 45-93 (65) | 57.5-92.5 (76.0) |
| width | 94-110 (100.4) | 24-50 (38) | 100-137.5 (120.5) |
| Left Testis length | 48-100 (68.0) | 55-88 (66) | 67.5-125 (87.0) |
| width | 76-100 (86.0) | 30-63 (40) | 82.5-125 (103.5) |
| Egg length | 32-38 (34.8) | 30-36 (34) | 40-47.5 (43.5) |
| width | 20 | 15-19 (17) | 20-20 (20.0) |
gantica, and Heterakis gallinarum from GenBank were aligned with C. formosanus from this study. The phylogenetic tree was constructed using the MEGA software version 6.0. The data was analyzed by character analysis (maximum-likelihood) and distance analysis (neighbor-joining) with 1,000 bootstrap values.

Total 50 (33.3%) of 150 ornamental fish examined were infected with metacercariae of C. formosanus. The highest prevalence was found in C. auratus (83.3%), and the highest intensity of infection was in C. carpio (av. 70.8 metacercariae per fish). The results of other fish are shown in Table 1. The measurements of adult worms are shown in Table 2. The morphology of C. formosanus from 5 ornamental fish was almost similar. The metacercariae were oval shaped, with X-shaped excretory bladder and 32-34 circumoral spines surrounding the oral sucker. The adult flukes were pyriform shaped, and the tegument was covered with scale like spines. All adult worms originating from C. auratus possessed exclusively 34 circumoral spines (Fig. 1A1, A2), whereas those worms originating from other fish were armed with 32 spines arranged in 2 rows (Fig. 1B1, B2). They were both regarded as C. formosanus.

The BLAST results of C. formosanus originating from C. auratus of this study showed 99% similarity with 5.8S rRNA gene (partial sequence), ITS2 of C. formosanus GenBank accession no. KF630863. The phylogenetic trees were reconstructed based on a maximum-likelihood and neighbor-joining methods with bootstrap values of 1,000 replicates. The results from both methods showed that the topology is similar (Fig. 2) to that of C. formosanus originating from C. auratus [25] which was grouped with Centrocestus sp. and C. formosanus from GenBank with 100% and 98% bootstrap values, respectively.

**Fig. 1.** Line drawing of an adult Centrocestus formosanus originating from C. auratus (A1) showing 34 circumoral spines around the oral sucker (A2) and another originating from C. carpio (B1) showing 34 circumoral spines around the oral sucker (B2).

**Fig. 2.** Phylogenetic trees of C. formosanus originating from C. auratus in Chiang Mai, Thailand. (A) A phylogenetic tree analyzed by maximum-likelihood (ML) method using the MEGA program software version 6.0 with 1,000 bootstrap values. (B) Another tree analyzed by neighbor-joining (NJ) method using the MEGA program software version 6.0 with 1,000 bootstrap values.
C. formosanus metacercariae were found in ornamental fish from many countries, such as Mexico, Australia, Denmark, and Iran [21-24]. In Thailand, the metacercariae of C. formosanus have been reported in several fish species, such as Macragnosthus siamensis, P. gonionotus, P. brevis, Thynnichthys thynnoides, Puntioplites protocyrson, Esomus metallicus, A. testudineus, Parambassis siamensis, and Hampala macrolepida [14,25,26]. However, this fluke is not well known in ornamental fish in Thailand, and in Chiang Mai Province, it has been reported only in C. aurasus [25,27]. In this study, the metacercariae of C. formosanus were found in 5 species of ornamental fish. The results elicited that ornamental fish can serve as the second intermediate host for C. formosanus. In addition, C. formosanus metacercariae were found for the first time in C. carpio, D. rerio, P. latipinna and P. tetrazona in Thailand.

The species confirmation of C. formosanus was based on morphological and molecular methods. The unique character of C. formosanus is the number of circumoral spines around the oral sucker. They commonly have 32 circumoral spines arranged in 2 rows around the oral sucker [1,4,8,18,24,28], whereas Centrocestus armatus has 42-44 circumoral spines [3,29,30]. In the present study, there were 32 circumoral spines in worms originating from 4 fish species (C. carpio, P. latipinna, D. rerio, and P. tetrazona) which agreed to previous studies [1,2,24,28]. The worms originating from C. aurasus fish had exclusively 34 circumoral spines which also resembled previous studies [8,21]. Thus, our specimens of 2 different origins were both considered morphologically to be C. formosanus. Molecular studies of our specimens using ITS2 by maximum-likelihood and neighbor-joining methods revealed a high relationship with C. formosanus from GenBank. They were separated from other heterophyid flukes by maximum-likelihood and neighbor-joining methods revealed a high relationship with C. formosanus from GenBank. They were separated from other heterophyid flukes and also from P. epiclitum, F. gigantica, and H. gallinarum (out group). The results of morphological and molecular studies were accorded.

This is the first report of C. formosanus metacercariae in C. carpio, P. latipinna, D. rerio, and P. tetrazona ornamental fish. The phylogenetic trees showed high relationships of our specimens with C. formosanus from GenBank database. Infection of these fish with C. formosanus metacercariae should be prevented and controlled. It can also help to reduce the motility rate of fish before export or sell to customers.

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CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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