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▣ BRIEF COMMUNICATION

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). The parasite species was identified by the morphology of worms as well as by a molecular approach using ITS2. The results showed that 50 (33.3%) of 150 fish examined were infected with the metacercariae. The highest 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 gonionotus, Puntius meiacanthus, Cyclocheilichthys armatus, Anabas testudineus*, and *Henicorhynchus 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℃, followed by 35 cycles of 1 min DNA denaturation at 94℃, 1 min primer annealing at 60℃, 1 min extension at 72℃, and 7 min for final extension at 72℃. 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-*

Table 1. Prevalence and intensity of infection with *Centrocestus formosanus* metacercariae in ornamental fish species from Chiang Mai, Thailand

Species of fish	No. of fish examined	No. of fish infected	Prevalence (%)	Intensity[a]
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

[a]Mean no. of metacercariae per fish.

Table 2. Measurements (range and mean) of *Centrocestus formosanus* specimens originating from *Carassius auratus* (n=10) compared with 2 previous studies

Origin (fish) Measurements (μm)	*Carassius auratus* Present study (Thailand)	*Puntius brevis* Han et al. (2008) (Lao PDR)	*Carassius auratus* Wongsawad et al. (2017) (Thailand)
No. of circumoral spines	34	32 (32-34)	34
Body length	465-552 (508.8)	245-325 (286)	600-750 (668)
Body width	176-224 (204.0)	155-220 (192)	200-290 (259)
Oral sucker length	48-58 (54.4)	45-58 (52)	60-80 (70.0)
width	62-66 (64.4)	38-50 (43)	52.5-82.5 (76.0)
Prepharynx	14-34 (22.4)	-	17.5-32.5 (26.5)
Pharynx length	36-46 (39.2)	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)	-	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. KJ630863. 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.

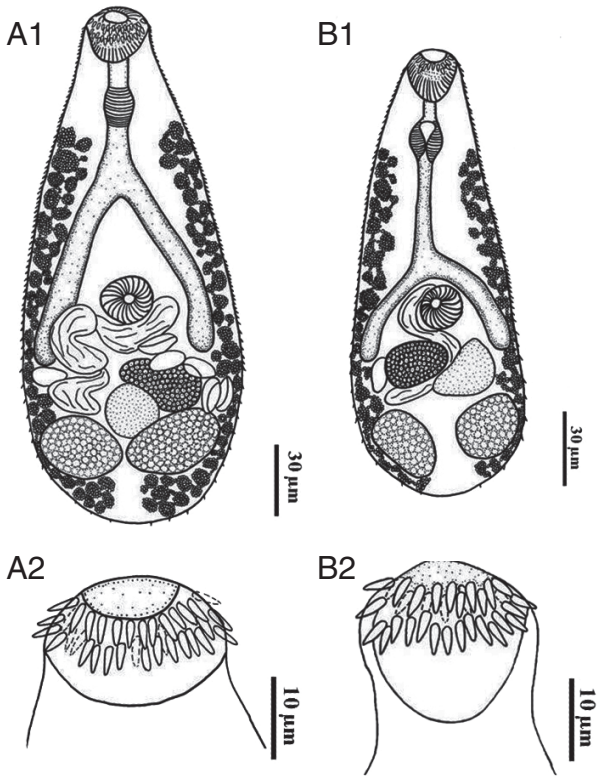

Fig. 1. Line drawing of an adult *Centrocestus formosanus* originating from *C. auratus* (A1) showing 34 circumoral spines around the oral sucker (A2) and another originating from *C. carpio* (B1) showing 34 circumoral spines around the oral sucker (B2).

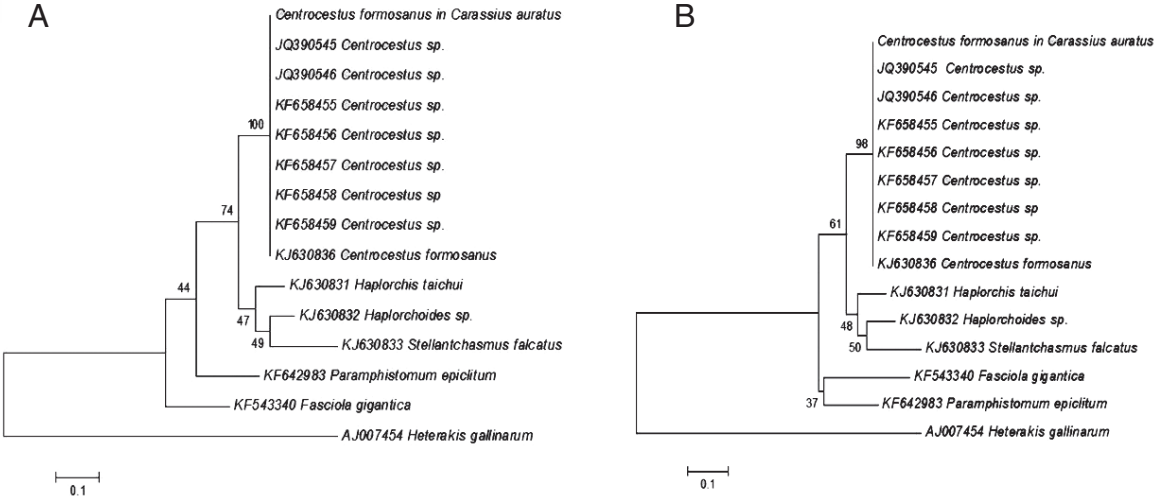

Fig. 2. Phylogenetic trees of *C. formosanus* originating from *C. auratus* in Chiang Mai, Thailand. (A) A phylogenetic tree analyzed by maximum-likelihood (ML) method using the MEGA program software version 6.0 with 1,000 bootstrap values. (B) Another tree analyzed by neighbor-joining (NJ) method using 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 *Macrognathus siamensis, P. gonionotus, P. brevis, Thynnichthys thynnoides, Puntioplites proctozysron, Esomus metallicus, A. testudineus, Parambassis siamensis*, and *Hampala macrolepidota* [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. auratus* [25,27]. In this study, the metacercariae of *C. formosanus* were found in 5 species of ornamental fish. The results elucidated 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. auratus* 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 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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