

Molecular Phylogenetics of *Centrocestus formosanus* (Digenea: Heterophyidae) Originated from Freshwater Fish from Chiang Mai Province, Thailand

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Abstract: This study aimed to investigate the morphology and reconstruct the phylogenetic relationships of *Centrocestus formosanus* originating from 5 species of freshwater fish, i.e., *Esomus metallicus*, *Puntius brevis*, *Anabas testudineus*, *Parambassis siamensis*, and *Carassius auratus*, in Chiang Mai province, Thailand. Sequence-related amplified polymorphism (SRAP) and phylogeny based on internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome c oxidase subunit 1 (CO1) were performed. The results showed similar morphologies of adult *C. formosanus* from day 5 after infection in chicks. *C. formosanus* originated from 4 species of freshwater fish had the same number of circumoral spines on the oral sucker, except for those from *C. auratus* which revealed 34 circumoral spines. The phylogenetic tree obtained from SRAP profile and the combination of ITS2 and CO1 sequence showed similar results that were correlated with the number of circumoral spines in adult worms. Genetic variability of *C. formosanus* also occurred in different species of freshwater fish hosts. However, more details of adult worm morphologies and more sensitive genetic markers are needed to confirm the species validity of *C. formosanus* with 34 circumoral spines originating from *C. auratus* in the future.

Key words: *Centrocestus formosanus*, molecular phylogeny, freshwater fish, Chiang Mai, Thailand

INTRODUCTION

Centrocestus formosanus (Digenea: Heterophyidae) is a small intestinal trematode of fish-eating birds and mammals, including ducklings, chickens, rats, rabbits, cats, dogs, and foxes [1]. The worm was reported to be infected in humans in Taiwan, Japan, and Lao PDR [2,3]. It is distributed in many countries, i.e., Taiwan (China), Japan, the Philippines, India, Lao PDR, Mexico, and Brazil [3-5]. *Centrocestus caninus* is a closely related species to *C. formosanus*. It has been reported to infect humans in Thailand and Lao PDR [6,7]. Various species of freshwater fish act as the second intermediate host of *C. formosanus*, including *Puntius brevis*, *Cyclocheilichthys repasson*, *Osteochilus hasseltii*, and *Australoheros facetus* [2,5]. In northern Thailand, *Centrocestus* metacercariae have been highly infected

in freshwater fish, including *P. brevis*, *Esomus metallicus*, and *Anabas testudineus* [8-10]. Infection can lead to a reduction in respiratory capacity and cause fish mortality [11].

Encysted metacercariae are oval-shaped, and the larvae present an X-shaped excretory bladder. Excysted metacercariae are pyriform-shaped and have a terminal oral sucker equipped with 2 alternate rows of 32 circumoral spines. The adult stage is similar to its excysted metacercariae, except for the presence of eggs within the uterus. The number of circumoral spines is the main character for distinguishing the species belonging to the genus *Centrocestus* [1]. They are classified into 3 groups, i.e., those with 26-36 spines (*C. formosanus*, *C. yokogawai*, *C. caninus*, *C. longus*, *C. cuspidatus*, and *C. asadai*), 38-48 spines (*C. nycticoracis*, *C. kurokawai*, and *C. armatus*), and 50-60 spines (*C. polyspinus*) [6]. Among the first group, the shape of the excretory bladder is adopted as a useful key. *C. cuspidatus* has a V-shaped excretory bladder, and the remaining 5 species have an X-shaped excretory bladder [6]. However, their morphology is similar particularly in their larval stages, and counting the exact number of circumoral spines is difficult in some specimens. So, it is difficult to identify the species by standard methods.

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Currently, molecular approaches are one of the most effective and accurate methods for genetic characterization of helminths. Sequence-related amplified polymorphism (SRAP) is an efficient genetic marker system, revealing genetic variation in open reading frames among related organisms [12]. It has been used to study genetic variation in some parasites such as the cattle liver fluke, *Fasciola hepatica* [13]. However, there are many DNA regions that are useful for identification of helminths, discriminating between species with similar morphologies, and useful for phylogenetic studies, especially the internal transcribed spacer 2 (ITS2) in nuclear genes and the mitochondrial cytochrome c oxidase subunit 1 (CO1) in mitochondrial genes [14,15]. For molecular identification and phylogeny of *Centrocestus*, Mehrdana et al. [16] reported that *Centrocestus* metacercariae found in fish, *Xiphophorus malcolatus*, imported from Singapore revealed the highest identity (> 99%) with *Centrocestus* sp. originating from Iran based on ITS2 region. Chai et al. [3] reported that 18S rRNA was used to identify *C. formosanus* isolated from humans in Lao PDR, which showed 100% nucleotide identity with the U.S. isolate [3]. Later, Chontanarith et al. [14] studied the phylogeny of heterophyid flukes based on CO1 gene and reported that *C. caninus* was closely related to *Haplorchoides* sp. and *Pygidioopsis summa*. However, it has never been reported about the studies of *C. formosanus* relationships in different hosts before.

So, this work aimed to study the morphological details and phylogenetic relationships of adult *C. formosanus* obtained from 5 species of freshwater fish in Chiang Mai province, Thailand by using SRAP PCR and phylogeny based on ITS2 and mCO1 regions.

MATERIALS AND METHODS

Worm sample preparation

C. formosanus metacercariae were collected from 5 freshwater fish species; flying barb (*Esomus metallicus*), golden little barb (*Puntius brevis*), climbing perch (*Anabas testudineus*), long-fin mojarra (*Parambassis siamensis*), and crucian carp (*Carassius auratus*) in Mae Tang, San Sai, and Meuang district, Chiang Mai province, Thailand.

Seven trematode species at the adult stage were prepared from the metacercarial stage: *Centrocestus* from 5 freshwater fish, *Stellantchasmus falcatus* from green backed (*Mugil dussumieri*), and *Stellantchasmus* sp. from wrestling half-beak (*Dermogynus pusillus*). All metacercariae were force-fed to the experimen-

tal host (1-day-old chick). For the other parasites, adult worms of *Haplorchis taichui* were collected from humans, *Haplorchoides* sp. from yellow cat fish (*Hemibagus filamentus*), and the giant liver fluke, *Fasciola gigantica*, was obtained from a cow (*Bos taurus*). All specimens were rinsed several times with tap water and then frozen at -20°C immediately for DNA extraction.

Morphological observation of *Centrocestus formosanus* adult

C. formosanus adults were obtained from chicks 5 days after infection, fixed in 4% formalin, slightly flattened under a coverslip, and prepared for permanent slides. All permanent slides were photographed and then identified based on Yamaguti [17].

DNA extraction

Genomic DNA was extracted by using Chelex solution. In brief, 150 µl of 5% Chelex (Fluka) solution containing 10 µl of proteinase K (Sigma, Poole, UK) at a concentration of 20 mg/ml was added to approximately 20 mg of the trematode tissue. It was then heated at 55°C for 1 hr, followed by gentle vortexing and heating at 95°C for 30 min, again followed by gentle vortexing. The mixture was centrifuged at 13,000 g for 1 min. The supernatant was collected and stored at -20°C until it was used.

SRAP PCR

Ten different primer combinations (5 forward primers and 5 reverse primers) were used to perform DNA fingerprints from different species of adult parasites. SRAP PCR reaction was carried out in a final volume of 20 µl. The reactions were performed in a Thermal Cycler machine (Little Genius, Bioer Technology, Minato-ku, Tokyo, Japan). PCR protocols were indicated as follows: 5 min of initial denaturation at 94°C, followed by 10 cycles of 3 steps: 1 min of denaturation at 94°C, 1 min of annealing at 35°C, 1 min of extension at 72°C, followed by 35 cycles with annealing temperature being increased to 50°C, with a final extension at 72°C for 5 min. PCR products were separated on 1.4% TBE agarose gel electrophoresis stained with DNA-Dye NonTox (Applchem, Darmstadt, Germany) and photographed with a Sony digital camera (DSC-WX50).

Amplification of ITS2 region and CO1 gene

The ITS2 regions were amplified by using the primers, for-

ward 3S (5'-GGT ACC GGT GGA TCA CTC GGC TCG TG-3') and reverse BD2 (5'-TAT GCT TAA ATT CAG CGG GT-3'). 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 57°C, and 1 min extension at 72°C, and a final extension at 72°C for 7 min.

The CO1 genes were amplified by using the primers, JB3 (5'-TTT TTT GGG CAT CCT GAC GTT TAT-3') as the forward primer and JB4.5 (5'-TAA AGA AAG AAC ATA ATG AAA ATG-3') as the reverse primer. The PCR conditions were as follows: 3 min initial denaturation at 95°C, followed by 40 cycles of 1 min DNA denaturation at 95°C, 1 min primer annealing at 50°C, 1 min extension at 72°C, and a final extension at 72°C for 7 min.

Agarose gel electrophoresis with DNA-Dye NonTox staining was used to visualize ITS2 and CO1 products under UV light. All ITS2 and CO1 PCR products were purified and subjected to sequencing.

Data analysis

SRAP fragments were scored for presence (the presence of the specific allele, code "1") or absence (the absence of the specific allele, code "0") in each sample. The distance matrix and dendrogram were constructed using phylogeny inference package (PHYLIP) version 3.695. A phylogenetic tree was constructed based on the combined data set of all primers, using the unweighted pair group method with arithmetic averages (UPGMA).

For the ITS2 and CO1 sequences, the sequences were aligned using ClustalW, then both genes were combined together. The phylogenetic tree was reconstructed using MEGA version 6.0 based on neighbor-joining (NJ) and maximum-likelihood (ML) methods. The statistics supported for the branches were tested using 1,000 bootstrap replicates.

RESULTS

Morphological characteristics of *Centrocestus formosanus* adults

C. formosanus obtained from the chick, 5 days after infection with metacercarial stage from 5 species of freshwater fish, were mostly similar in their morphology with the original description of this species (Fig. 1A, B). It can be described as body pyriform-shaped with broad posterior half and covered with scale-like tegumental spines from the anterior to the posterior end. Prepharynx present. Pharynx well developed. Esophagus

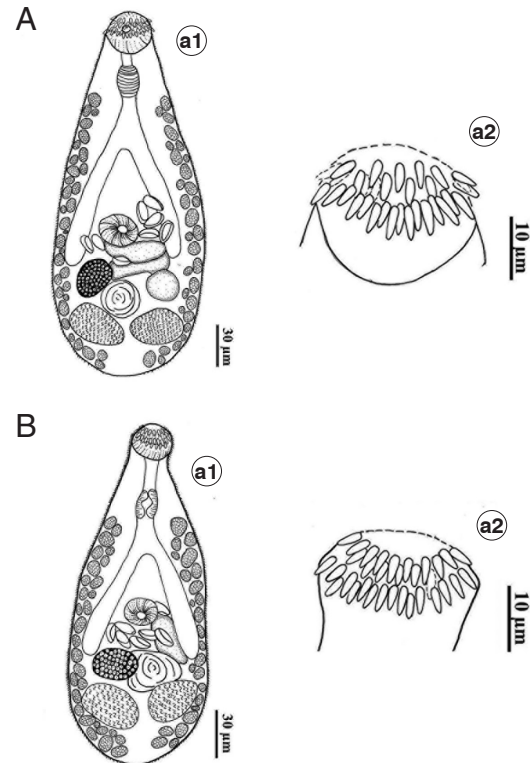


Fig. 1. The morphology of adult *Centrocestus formosanus* with 32 and 34 circumoral spines. (A) *C. formosanus* originated from *Puntius brevis* (a1) showing 32 spines arranged in 2 alternate rows around its oral sucker (a2). (B) *C. formosanus* originated from *Carassius auratus* (b1) showing 34 spines arranged in 2 alternate rows around its oral sucker (b2).

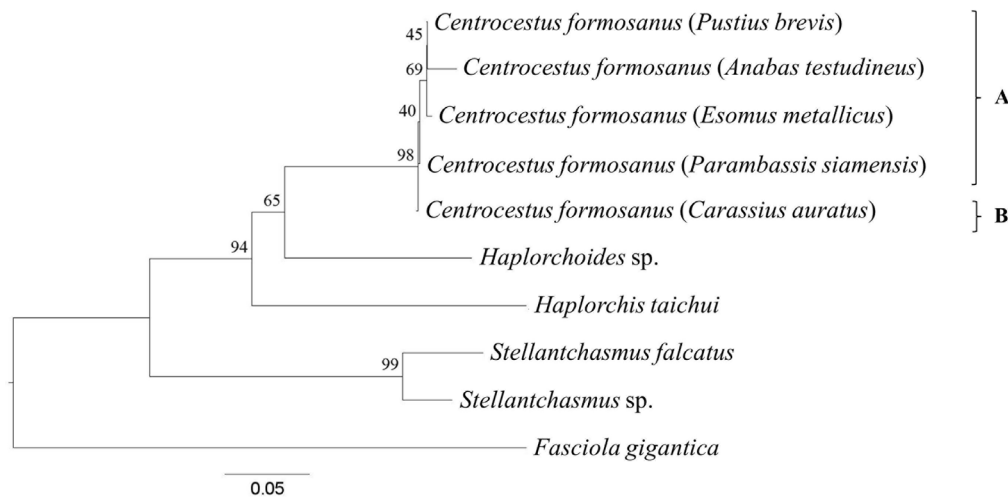
short. Ceca large, bifurcated about midway between oral and ventral suckers, terminated slightly in front of ovary. Ventral sucker smaller than oral sucker and located in the middle of body. Ovary oval, located on right side of posterior half of body. Testes oval, opposite in posterior body. Eggs oval, operculum distinct. Excretory bladder X-shaped.

There was only 1 character of *C. formosanus* that showed difference between adult flukes originated from 5 species of fish. That is the number of circumoral spines surrounding the oral sucker of *C. formosanus*. The adults originated from *C. auratus* differed from the worms originated from the 4 remaining fish species. Those from 4 freshwater fish species (*Esomus metallicus*, *Puntius brevis*, *Anabas testudineus*, and *Parambassis siamensis*) had their oral sucker with 2 alternate rows of 32 circumoral spines (Fig. 1A); however, 34 circumoral spines were found in worms from *C. auratus* (Fig. 1B). The measurements of *C. formosanus* isolated from 5 species of fish were varying and overlapped. Their measurements are shown in Table 1.

Table 1. Measurements of *Centrocestus formosanus* adults recovered in the small intestines of chicks at day 5 post infection

		<i>Esomus metallicus</i> (n=5)	<i>Puntius brevis</i> (n=5)	<i>Anabas testudineus</i> (n=5)	<i>Parambassis siamensis</i> (n=5)	<i>Carassius auratus</i> (n=5)
No. of oral spines		32	32	32	32	34
Body	L	610-750 (670)	590-710 (632)	540-900 (660)	432-640 (537.3)	600-750 (668)
	W	270-350 (298)	240-320 (276)	220-290 (252)	196-270 (237)	200-290 (259)
Oral sucker	L	57.5-72.5 (66.5)	57.5-80 (69.5)	55-65 (61.5)	42-65 (72.5)	60-80 (70)
	W	62.5-87.5 (78.5)	65-80 (72.5)	65-82.5 (72.5)	58-72.5 (64.3)	52.5-82.5 (76)
Ventral sucker	L	50-75 (60)	47.5-60 (54)	50-62.5 (56.9)	44-55 (49.7)	52.5-57.5 (55.5)
	W	62.5-70 (64)	52.5-62.5 (58)	55-72.5 (61.9)	50-62.5 (58.3)	55-70 (65.5)
Prepharynx		17.5-82.5 (53)	35-50 (44)	7.5-62.5 (36)	22-82.5 (44)	17.5-32.5 (26.5)
Pharynx	L	37.5-62.5 (51.5)	35-62.5 (47)	45-55 (51)	40-50 (46.7)	45-57.5 (49.5)
	W	37.5-52.5 (47)	35-45 (40.5)	25-50 (40)	32-52.5 (41.5)	37.5-50 (44)
Esophagus		50-100 (64.5)	30-80 (54.5)	30-62.5 (45)	20-50 (31.7)	32.5-62.5 (51.5)
Ovary	L	67.5-100 (82)	55-90 (79)	50-100 (71.9)	40-67.5 (52.5)	62.5-87.5 (76.5)
	W	62.5-137.5 (103.5)	70-150 (99.5)	75-125 (96.9)	66-82.5 (73.3)	65-150 (105.5)
Right testis	L	100-120 (110.5)	70-100 (85)	65-120 (85)	68-100 (84.3)	57.5-92.5 (76)
	W	62.5-157.5 (121)	92.5-155 (119.5)	82.5-112.5 (96.25)	88-125 (107.7)	100-137.5 (120.5)
Left testis	L	87.5-137.5 (103.5)	75-100 (84.5)	62.5-87.5 (71.9)	60-87.5 (74.2)	67.5-125 (87)
	W	50-142.5 (108.5)	87.5-120 (87.5)	87.5-105 (95)	80-120 (100)	82.5-125 (103.5)
Egg	L	32.5-40 (38)	37.5-40 (39)	37.5-42.5 (41)	34-42.5 (38.8)	40-47.5 (43.5)
	W	22.5-22.5 (22.5)	17.5-25 (20.5)	20-22.5 (21)	20-25 (22.5)	20-20 (20)

Unit (μm), range (mean), L=length, W=width.

**Fig. 2.** Cladogram constructed based on SRAP profile using UPGMA method. The adult worms of clade 'A' had 32 circumoral spines, whereas those of clade 'B' had 34 circumoral spines.

The phylogenetic tree based on SRAP profile

Ten SRAP primer combinations showed better polymorphisms of 10 representative trematodes: ME1/EM1, ME2/EM3, ME3/EM4, ME4/EM5, ME1/EM3, ME2/EM1, ME4/EM6, ME5/EM4, ME3/EM6, and ME4/EM1. These primers were chosen for phylogenetic relationship construction. UPGMA phylogram analysis based on SRAP profile revealed a monophyletic group of *C. formosanus*. They were divided into 2 main groups. The

first group was *C. formosanus* originating from *E. metallicus*, *P. brevis*, *A. testudineus*, and *P. siamensis* (clade A, Fig. 2). The second one was those isolated from *C. auratus* (clade B, Fig. 2). Phylogenetic trees based on the combination of ITS2 and CO1 sequence

Phylogenetic trees were reconstructed based on a character method (ML) and a distance method (NJ) with bootstrap values of 1,000 replicates. Both methods showed the similar to

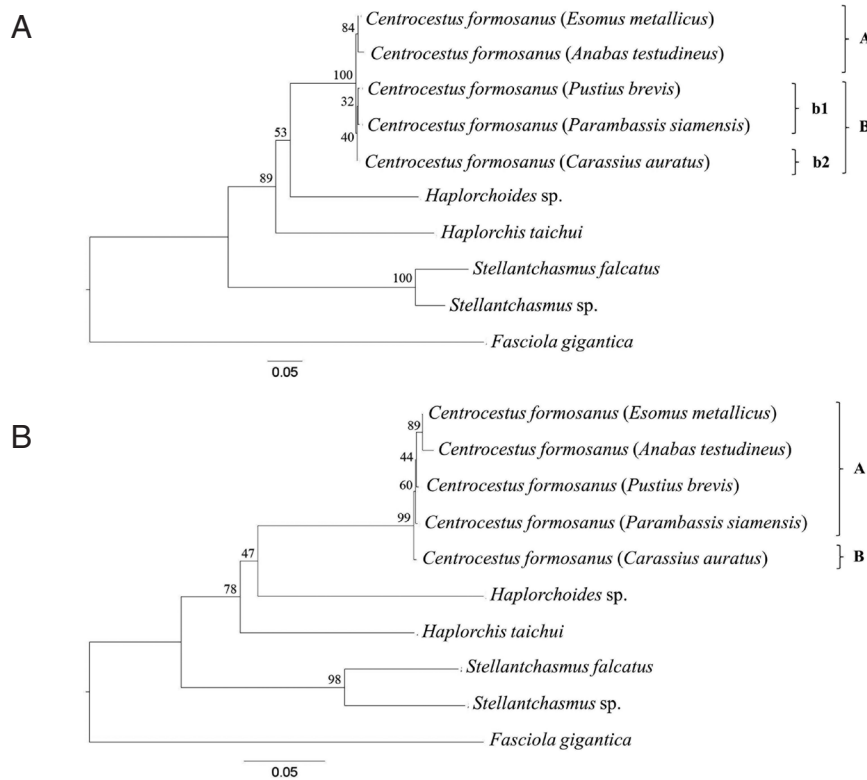


Fig. 3. Cladogram constructed based on the combination of ITS2 and CO1 sequence with 1,000 bootstrap replicates. (A) Maximum-likelihood (ML) method. The adult worms of clade 'A' and clade 'B b1' had 32 circumoral spines, whereas those of clade 'B b2' had 34 circumoral spines. (B) Neighbor-joining (NJ) method. The adult worms of clade 'A' had 32 circumoral spines, whereas those of clade 'B' had 34 circumoral spines.

polology that the *Centrocestus* group was monophyly, as shown in Fig. 3. In ML tree, *C. formosanus* was separated into 2 groups: *C. formosanus* originating from *E. metallicus* and *A. testudineus* (clade A, Fig. 3A), and *C. formosanus* originating from *P. brevis*, *P. siamensis*, and *C. auratus* (clade B, Fig. 3A) with low bootstrap values. In NJ tree, *C. formosanus* isolated from *C. auratus* was separated from the 4 remaining specimens with low bootstrap values (clade B, Fig. 3B).

DISCUSSION

In the present study, 5 species of freshwater fish, i.e., *E. metallicus*, *P. brevis*, *A. testudineus*, *P. siamensis*, and *C. auratus* were highly infected with *Centrocestus* metacercariae. The adult worms from chicks 5 days after infection were mostly similar in their morphology having 32 circumoral spines except for the host *C. auratus* from which the worm had 34 circumoral spines. The number of circumoral spines of *C. formosanus* is 32 spines in most cases [1,18]. It is also similar to a previous

study that reported 32 circumoral spines of *C. formosanus* [3-5]. This species differed from the related species; *C. caninus* which has 26-30 spines [6]. They were also different in the presence of a prepharynx in *C. formosanus*, whereas *C. caninus* has no prepharynx [6]. The number of circumoral spines was accepted as the most reliable criteria although the number varied showing a range in some species [1]. However, it has some doubts that *C. formosanus* originating from *C. auratus* revealed 34 circumoral spines in all specimens. It is different from other studies which reported 32 spines in most cases.

Only a few studies have demonstrated the molecular characterization of heterophyid trematodes, i.e., *Centrocestus*, recovered in freshwater fish in Thailand, especially in the northern region [14,19]. The molecular approach and DNA sequencing techniques have been successfully developed for identification and studies of their phylogenetic relationships. In our study, 2 molecular techniques (SRAP PCR and phylogeny) and 2 DNA regions (ITS2 and CO1 regions) were performed for studying the phylogenetic relationships among *C. formosanus* recovered

from different fish species. Our analysis revealed a monophyletic tree of *C. formosanus*. It was separated from other heterophyid trematodes and out group (*F. gigantica*) with moderate bootstrap values (Figs. 2, 3).

The trees were derived based on SRAP profile and ITS2 and CO1 sequences. All tree topologies were similar to each other (Figs. 2, 3). However, the tree from concatenate data set revealed some difference topology with the individual gene trees. Within the *C. formosanus* group, there were 2 main clades which had the same character as the circumoral spine numbers (clades 'A' and 'B'; Figs. 2, 3B). *C. formosanus* with 34 spines originated from *C. auratus* (clade 'B'; Figs. 2, 3B) was separated from *C. formosanus* with 32 spines (clade 'A'; Figs. 2, 3B). However, *C. formosanus* originated from *C. auratus* were also divided into a subclade (clade 'b2'; Fig. 3A) that is associated with the number of circumoral spines that were divided into 32 and 34 spine groups. Although, *C. formosanus* isolated from *C. auratus* (clade 'b2'; Fig. 3A) was closely related to *C. formosanus* isolated from *P. brevis* and *P. siamensis* (clade 'b1'; Fig. 3A).

In our study, 5 species of freshwater fish are divided into 3 families, including Cyprinidae, Anabantidae, and Ambassidae. *C. formosanus* isolated from *E. metallicus* (Cyprinidae) and *A. testudineus* (Anabantidae) were grouped into the same group (clade 'A'; Fig. 3A), whereas those from *P. brevis* (Cyprinidae) and *P. siamensis* (Ambassidae) were grouped together (clade 'B'; Fig. 3A). However, the worms from *C. auratus* (Cyprinidae) were separated into other group (clade 'b2'; Fig. 3A). These results agreed with the reason that intraspecific variation among specimens from different host group was low because of their functional constraints [13,20]. However, they have genetic variability within the same host group of the family Cyprinidae. For the genetic divergence of both genes, ITS2 and CO1 revealed that the distance value between *C. formosanus* with 32 and 34 spines were 0.007 (0.7%) and 0.025 (2.5%), respectively. These indicated that both *C. formosanus* groups may be different which correlated to the number of circumoral spines.

In conclusion, our study demonstrated that *C. formosanus* originating from 5 freshwater fish species were morphologically similar, except for a difference in the number of circumoral spines (32 and 34 spines). Molecular techniques were a useful tool for the study of phylogenetic relationships among *C. formosanus* originating from difference species of freshwater fish; the worms originated from *C. auratus* were genetically unique from those originated from other 4 species of freshwater fish.

However, more sensitive genetic markers are needed to confirm the species validity of a new genotype, i.e., *C. formosanus* having 34 circumoral spines originating from *C. auratus*.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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