Research Article

The Prevalence of Human Intestinal Fluke Infections, *Haplorchis taichui*, in Thiarid Snails and Cyprinid Fish in Bo Kluea District and Pua District, Nan Province, Thailand

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Abstract

Traditionally, people in the Nan Province of Thailand eat raw fish, exposing them to a high risk of getting infected by fish-borne trematodes. The monitoring of helminthiasis among those people showed a high rate of infections by the intestinal fluke *Haplorchis taichui,* suggesting that also an epidemiologic study (of the epidemiology) of the intermediate hosts of this flat worm would be useful. In this study freshwater gastropods of thiarids and cyprinid fish (possible intermediate hosts) were collected around Bo Kluea and Pua District from April 2012 to January 2013. Both snails and fish were identified by morphology and their infections were examined by cercarial shedding and compressing. Cercariae and metacercariae of *H. taichui* were identified by morphology using 0.5 % neutral red staining. In addition a polymerase chain reaction of the internal transcribed spacer gene (ITS) was applied to the same samples. Among the three thiarid species present were *Melanoides tuberculata, Mieniplotia (= Thiara or Plotia) scabra* and *Tarebia granifera* only the latter species was infected with cercariae, with an infection rate or prevalence of infection of 6.61 % (115/1,740). In the 10 species of cyprinid fish (viz. *Barbodes schwanenfeldi, Garra cambodgiensis, Hypsibarbus salweenensis, Mystacoleucus marginatus, Osparus pulchellus, Poropuntius deauratus, P. normani, Scaphiodonicthys acanthopterus, Systomus orphoides* and *S. stolitzkaenus)*, metacercariae were found in all of them. Interestingly, *B. schwanenfeldi, M. marginatus, P. deauratus, P. normani and S. orphoides* had very high infection rates. Both morphometric and molecular identification confirmed those cercariae and metacercariae to represent the heterophyid *Haplorchis taichui.* Therefore, this study showed the high prevalence of this parasite both in its intermediate hosts as well as in humans. In order to prevent human infections, *H. taichui* has to be controlled.

Keywords*:* Fish-borne trematode; *Haplorchis taichui;* Intermediate host, Prevalence of infection

Introduction

Haplorchiasis is the major public health of Southeast Asia (Chai et al., 2005; WHO, 1995). It is caused by the minute intestinal fluke, *Haplorchis taichui* Nishigori, 1924. *H. taichui* was found in Thailand, Lao PDR, Vietnam and Philippines (Waikagul et al., 2002; De et al., 2003; Belizario et al., 2004; Sohn et al., 2014). Both human and fish-eating animals serve as the definitive hosts of *H. taichui* (Le et al., 2006). The adultworm live in the intestine of the host and produce eggs. The eggs pass through the water and are ingested by freshwater snails (of the family Thiaridae) and then develop into miracidia, sporocysts, rediae and finally cercariae. The cercariae could swim and penetrate cyprinid fish and subsequently develop to their infective stage; the metacercariae. Humans can get infection by consumption of raw or improperly cooked cyprinid fish containing metacercariae (Kumchoo et al., 2005).

The situation of haplorchiasis might be underestimated. Because the eggs of *Haplorchis* sp. hardly differentiates from the liver fluke egg, *Opisthorchis viverrini. H. taichui* had a complex life cycle as same as that of *O. viverrini* but different in species of the first intermediate host. There was a report that 63.11% of people in the north of Thailand were affected with haplorchiasis (Radomyos et al., 1998). In Chaloemprakiat District of Nan Province, *H. taichui* was found in school children and villagers during 2002-2004 (Waikagul et al., 2008). In Bo Kluea District of Nan Province, the infection rate of humans with *H. taichui* was ranging from 2.4 – 27.59 % (Wijit et al., 2009). The first diagnosis under light microscope was identified as *O. viverrini.* But in a later study with the Kato Katz technique, adult patients were found to be infected with *H. taichui*. Thus, Thai Princess Sirindhorn Debaratanasuda created the project to reduce the infection in Bo Kluea District. Because of traditional dishes in North and Northeast Thailand (e.g. lab pla), were made from raw cyprinid fish. So, these people risked to get infection in everyday life (Sukontason et al., 1999). In some cases, patients complained of abdominal pain, and diarrhea (Watthanakulpanich et al., 2010).

The prevalence of *H. taichui* in the intermediate hosts both thiarid snail and cyprinid fish were reported in many studies. Those studies showed that the larvae of *H. taichui* were distributed in many area of Thailand particularly in the north of Thailand.

In the past, the trematode samples were identified based on the morphology. Almost the decade, the molecular identifications of this species of trematodes were developed in many studies (Chuboon et al., 2009; Chontananarth and Wongsawad, 2010; Chontananarth et al., 2014). Both nuclear genes and mitochondrial genes were used to design the specific primers. The internal transcribed spacer genes I and II were used to discriminate between trematode families Heterophyidae and Opisthorchiidae (Sato et al., 2009).

According to the previous study of *H. taichui* in humans, the prevalence of *H. taichui* in the intermediate hosts (both the first and the second intermediate hosts) were investigated in this study. The morphometric and molecular identification were applied to identify the obtained trematode larvae to confirm the prevalence of *H. taichui* infectionsin Bo Kleua and Pua District, Nan Province, Thailand.

Materials and Methods

Snail collection and examination of cercarial infections

In this study the counts per unit of time method was performed for snail collections (Olivier and Schneiderman, 1956). Thiarid snails were collected from 4 localities in Bo Kluea District (Mang River 1: E 101º 09' 6.1" N 19º 7' 44.1", Mang River 2: E 101º 09' 12.6" N 19º 7' 55.6". Mang River 3: E 101º 09' 21.4" N 19º 8' 48.0", Waa River: E 101º 12' 13.2" N 19º 11' 30.4") and 2 localities in Pua District (Khwang Stream: E 100º 56' 0.1" N 19º 10' 29.8", Fay Chao: E 100º 57' 45.4" N 19º 10' 42.8"), during April 2012 to January 2013 (Figure 1).

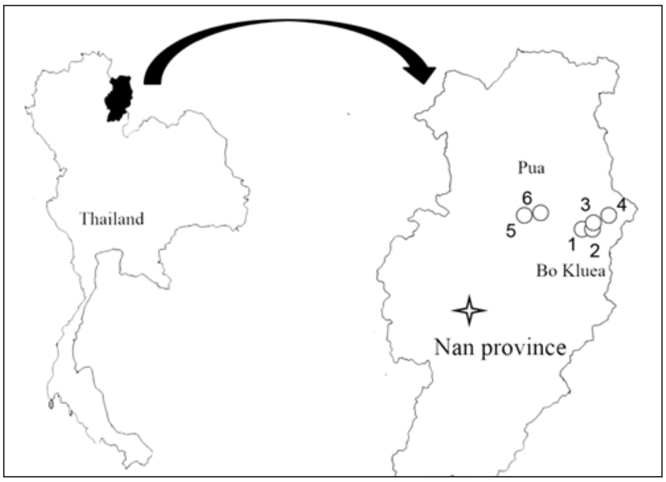

Figure 1 Map showed the collected locations in Bo Kluea and Pua Districts, Nan province, Thailand. (modified from www.thai.wikipedia.org) (see detail in materials and methods)

All snail samples were identified based on their shell morphologies according to Brandt's criteria (Brandt, 1974)and investigated the trematode infections by shedding and crushing methods (Krailas et al., 2003). Each snail was isolated in a plastic container with dechlorinatedtap-water. Cercariae were collected from the water and rediae were investigated from snail's tissues. The cercariae and rediae were stained with 0.5% neutral red and studied the morphologies under the light microscope. The individual metacercaria was drawn with a *camera lucida*. The cercariae were preserved in 95% ethanol for DNA analysis.

Fish collection and metacercarial morphology

Cyprinid fish were collected by local tool (Hoom) during April 2012 to January 2013 from 2 localities, Mang River 3 and Khwang Stream. The investigation of metacercarial infections was done by compression method. The collected fish were identified based on their morphologies according to Vidthayanon's criteria (Vidthayanon, 2005) and examined for trematode infections using the compression method. The metacercariae were identified based on their encysted and excysted morphologies. The numbers of metacercariae of each species were measured and recorded their sizes. The excysted metacercariae were stained with 0.5% neutral red and study the morphology. The individual metacercaria was drawn with a *camera lucida*. The metacercariae were preserved in 95% ethanol for DNA analysis.

Molecular identification

Genomic DNA from the cercariae and metacercariae were extracted using the DNeasy blood and animal tissue kit (QIAGEN, Germany). PCR of the internal transcribed spacer II was performed with the following primers:

ITS2 regions:ITS2-F: 5'-CTT GAACGC ACA TTG CGG CCA TGG G-3'

ITS2-R: 5'-GCG GGT AAT CACGTC TGA GCC GAG G-3'

The reactions were performed in 50-µl volumes with final concentrations of 5 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 U Taq polymerase, and 1 mM of the primers. The DNA samples were initially denatured at 94°C for 4 min followed by 35 cycles that consisted of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and elongation at 72°C for 2 min following Sato (2009). Then, the PCR products were loaded onto 1% agarose gels for electrophoresis.

PCR products of ITS2 were purified and sequences by Bioservice unit, Thailand. The sequences were analysed by MEGA 5.0 program (Tamura et al., 2011). The phylogenetic tree was constructed by ClustalW for alignment and Neighbor Joining method with 1000 bootstraps. For the construction of the ITS2 phylogenetic tree, 6 sequences of *H. taichui* from this study, 3 sequences of *H. pumilio* were used. From the out group, one sequence of *O. felinius* (EF 688142) and 2 sequences of *O. viverrini* (AY 584735 and this study) were incorporated to the tree.

Results

The infections of *Haplorchis taichui* in thiarid snails

A total of 1,740 snails were collected. Based on shell morphology, there were three species of thiarid snails, *Melanoides tuberculata, Mieniplotia (= Thiara or Plotia) scabra and Tarebia granifera. T. granifera* was the dominant species and it was only susceptible to *H. taichui,* with infection rates being 7.98% (115/1,440). Five locations were found snail infections, they were Mang River 1, Mang River 2, Mang River 3, Waa River and Khwang Stream. The infection rates were 1.78%, 26.27%, 31.08%, 41.18% and 0.59%, respectively.

Cercarial morphology

The cercariae of *H. taichui* were oval shape with yellow. Eye spot and pharynx were present. There were seven pairs of penetration glands, typical character of H. taichui. The ducts of penetration glands opened at the end of anterior part of body. Excretory bladder was round. Tail was long with lateral finfold and dorso-ventral finfold. Flame cell was not observed (Figure 2).

Measurement of cercariae

Body: 32-58 µm (av. 47 µm) × 63-113 µm (av. 86 µm)

Oral sucker: 7-22 µm (av. 14 µm) × 10-20 µm (av. 18 µm)

Pharynx: 2.5-10 µm (av. 5 µm) × 5-13 µm (av. 8 µm)

Ventral sucker: 10-18 µm (av. 14 µm) × 10-13 µm (av. 12 µm)

Excretory bladder: 15-25 µm (av. 18 µm) × 10-18 µm (av. 15 µm)

Tail: 10-23µm (av. 15µm) × 260-430 µm (av. 328 µm)

The infections of *Haplorchis taichui* in cyprinid fish

A total of 10 species of cyprinid fish were collected, viz. *Barbodes schwanenfeldi,Garra cambodgiensis, Hypsibarbus salweenensis, Mystacoleucus marginatus, Osparus pulchellus, Poropuntius deauratus, Poropuntius normani, Scaphiodonicthy sacanthopterus, Systomus orphoides* and *Systomus stolitzkaenus.* The infections in fish were observed from both Mang River 3 and Khwang Stream. The seasonal infection rates were shown in Table 2.

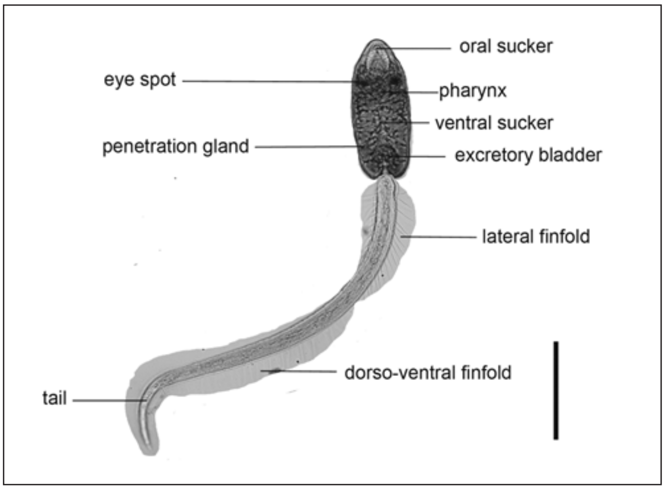

Figure 2 Image of *Haplorchis taichui* cercaria, staining with 0.5% neutral red (scale bar = 100 µm)

Table 1 Infection status of *Haplorchis taichui* cercariae in freshwater snails of Family Thiaridae

Localities / Thiarids	April 2012 No. of collected snails (infected snails)	Jan 2013 No. of collected snails (infected snails)	Total No.of collected snails (infected snails)
Mang River 1			
Tarebia granifera	328	11 (4) = 36.36 %	339 (4) = 1.78 %
Melanoides tuberculata	68	5	73
Mieniplotia scabra	5	2	7
Mang River 2			
Tarebia granifera	35 (15) = 42.85 %	182 (42) = 23.08 %	217 (57) = 26.27 %
Melanoides tuberculata	10	72	82
Mieniplotia scabra	5	6	11
Mang River 3			
Tarebia granifera	109 (21) = 19.27 %	139 (25) = 20.86 %	148 (46) = 31.08 %
Melanoides tuberculata	93	11	104
Mieniplotia scabra	1	10	11
Waa River			
Tarebia granifera	5 (1) = 20 %	12 (4) = 33.33 %	17 (5) = 41.18 %
Mieniplotia scabra	1	0	1
Khwang Stream			
Tarebia granifera	443	62 (3) = 4.83 %	505 (3) = 0.59 %
Melanoides tuberculata	2	3	5
Mieniplotia scabra	4	0	4
Fay Chao			
Tarebia granifera	174	40	214
Mieniplotia scabra	0	2	2

Table 2 Infection status with *Haplorchis taichui* metacercariae of freshwater fish Family Cyprinidae

Locality /Cyprinids	April 2012 No. of collected fish (infected fish)	January 2013 No. of collected fish (infected fish)	Total No. of collected fish (infected fish)
Mang River 3			
Garra cambodgiensis	24 (10) (41.67 %)	6 (2) (33.33%)	30 (12) (46.67%)
Hypsibarbus salweenensis	-	9 (6) (66.67%)	9 (6) (66.67%)
Osparus puchellus	35 (32) (91.42%)	3 (3) (100%)	38 (35) (92.41%)
Poropuntius normani	3 (3) (100%)	-	3 (3) (100%)
Scaphiodonicthys acanthopterus	8 (4) (50%)	9 (5) (55.55%)	17 (9) (52.94%)
Systomus orphoides	-	1 (1) (100%)	1 (1) (100%)
Sytsomus stolitzkaenus	-	32 (16) (50%)	32 (16) (50%)
Khwang Stream			
Barbodes schwanenfeldi	1 (1) (100%)	3 (3) (100%)	4 (4) (100%)
Mystacoleucu smarginatus	15 (15) (100%)	2 (2) (100%)	17 (17) (100%)
Poropuntius deauratus	1 (1) (100%)	-	1 (1) (100%)

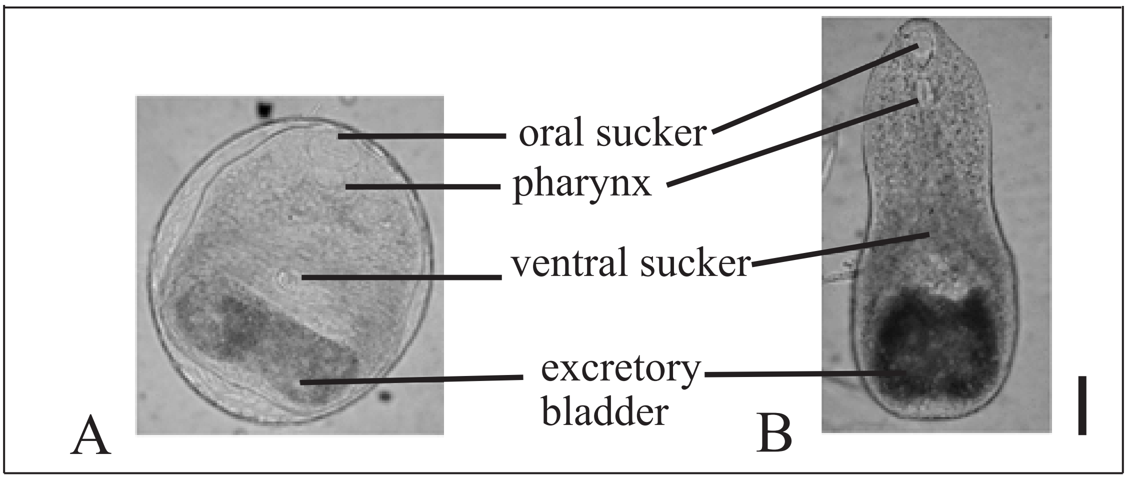

Figure 3 Images of metacercaria of *Haplorchis taichui*, staining with 0.5% neutral red (scale bar = 50 µm)

A: encysted metacercaria

B: excystedmetacercaria

Metacercarial morphology

Encysted metacercaria of *H. taichui* was round with 2 cyst walls. The inner cyst wall was thicker than outer cyst wall. Furthermore, the surface of larvae had minute spines. There were spines at oral sucker. Ventral sucker and pharynx were clearly observed. Gonotyl was present nearby ventral sucker. Esophagus was long. Only one testis was observed at the end of body. Ovary was above testis. Excretory bladder was big with black pigment (Figure 3).

Measurement of metacercaria

Encysted metacercaria: 180-280 µm (av. 214.5 µm) x 200-290µm (av. 231.6 µm)

Body: 87.5-162.5 µm (av. 142.9 µm) × 215-307.5 µm (av. 201.4µm)

Oral sucker: 40-57.5 µm (av.49.63 µm) × 32.5-45 µm (av.40.13 µm)

Ventral sucker: 27.5-50 µm (av. 37.38 µm) × 27.5-50 µm (av. 37.38 µm)

Pharynx: 17.5-30 µm (av. 23.25 µm) × 25-42.5 µm (av. 33.88 µm)

Esophagus: 60-115 µm (av. 84.5 µm)

Excretory Bladder: 100-150 µm (av. 128 µm) × 67.5-122.5 µm (av. 85.88 µm)

Testis: 37.5-62.5 µm (av. 44.25 µm) × 37.5-50 µm (av. 43.63 µm)

Ovary: 22.5-35 (av. 28.88 µm) × 25-37.5 µm (av. 28 µm)

Molecular identification

All samples had the PCR products of ITS2 gene with about 500 bp (data not shown). All of them were identified as *H. taichui*. Some sequences were aligned with *H. pumilio* and *Centrocestus formosanus* from Pasak Cholasid Dam (from Parasitology and Medical Malacology Research Unit, Silpakorn University) and adult of *O. viverrini* (TM) (kindly provided from Faculty of Tropical Medicine, Mahidol University). We also added the sequence of *H. taichui, O. viverrini* and *O. felinius* from GenBank. The result of phylogeny also showed that samples from Bo Kluea (BK) were *H. taichui* (Figure 4).

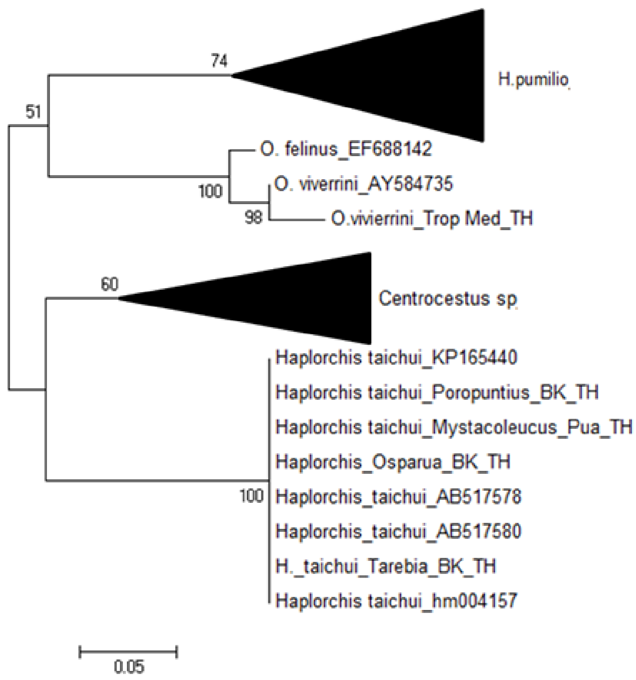

Figure 4 Phylogenetic tree of partial internal transcribe spacer II (ITS2) of *Haplorchis taichui* from Bo Kluea District, Nan Province, Thailand comparing to *H. pumilio* and *Centrocestus sp.* from Pasak Cholasid reservoir of Thailand, adult of *O. viverrini* from Faculty of Tropical Medicine, Mahidol University and *O. felinius* from Gen Bank (the names containing the trematodes and their hosts)

Discussion

The results showed that collected larval stages of trematodes from Bo Kluea District, Nan Province, Thailand were *Haplorchis taichui.* For morphological identification, neutral red was used to see the internal organ of cercariae and metacercariae. (Yamaguti, 1975). Moreover, we used the primer sets from Sato (2009) because they were designed for discrimination between *Opisthorchis viverrini, Clonorchis sinensis, H. taichui* and H. *pumilio.* and other heterophyids. However, the genetic variation of heterophyidtrematode was observed (Dung et al., 2013). So, we sequenced some of them and made a phylogenetic tree. Due to the same size of sequences*, H. pumilio and O. viverrini* are sister groups in the tree.

Originally, *H. taichui* was assumed to be endemic to the north of Thailand (Boonchot et al., 2005; Namue et al., 1998). Patients suffering from haplorchiasis were reported in Chiang Mai, Lampang and the Nan Province of Thailand, particularly Bo Kluea District (Namue et al., 1998; Wijit et al., 2009). The larval stages of this parasite were also found in the first intermediate host Thiarid snails and the second intermediated host, cyprinid fish from Chiang Mai, North Thailand (Boonchot et al., 2005; Kumchoo et al., 2005; Sukontason et al., 2001), which both were very abundant in the Northern area of Thailand. Those intermediate hosts were available for human transmission. From other study, cercariae of *H. taichui* were also found in *Melanoides tuberculata* from Tansawan Waterfall, Phayao Province, North of Thailand (Krailas et al., 2014). In extending, *H. taichui* cercariae were found in both Bo Kluea and Pua District, Nan Province. *H. taichui* metacercariae was found in all of cyprinid fish from those areas. Our survey showed that cyprinid fish from Mang River which was very close to South Bo Kluea Villages, where people in these area were highly infected with this species of trematode (MOPH, 2009; Wijit et al., 2009).

It was well known that *H. taichui* had the thiarid snails as the first intermediate host. From the three species of thiarid in the area, only *Tarebia granifera* was found to be infected. We still did not understand how *T. granifera* had more susceptibility than the other snails in this area. From the south of Thailand, it seemed to be that *M. tuberculata* had the highest infection rate among thiarid snails (Krailas et al., 2011). Perhaps, *H. taichui* would infect the most abundant species of each area.

In Thailand, only *Mystacoleucus marginatus* and *Systomus orphoides* were reported that they could be the second intermediate host of *H. taichui* (Boonchot et al., 2005; Rim et al., 2008). And it was confirmed by the present study that 8 species of cyprinid fish, *Barbodes schwanenfeldi, Garra cambodgiensis, Hypsibarbus salweenensis, Osparus pulchellus, Poropuntius deauratus, Poropuntius normani, Scaphiodonicthys acanthopterus* and *Systomus stolitzkaenus* were recorded as more second intermediate host of *H. taichui* in the literature.

Fishborne trematodiasis was a serious public health in Asia and Southeast Asia (Macpherson et al., 2005). Although the liver fluke, *Opisthorchis viverrini* might be a major cause of this illness, the minute intestinal fluke also posed a problem. Due to the misdiagnosis of stool examination, the prevalence of minute intestinal fluke might be underestimated (Kaewkes et al., 1991). Many studies showed that the minute intestinal flukes in the family Heterophyidae were recovery from human especially *Haplorchis taichui.* It was the most prevalent species in humans among the heterophyid trematodes (Chai et al., 2005).The present study has confirmed that *H. taichui* was prevalent in Bo Kluea and Pua Districts of Nan Province, Thailand. So, people in these areas should avoid eating the raw or undercooked cyprinid fish.

Acknowledgements

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