CHAPTER 3

MATERIALS AND METHODS

1. Equipments

1.1 Light microscope with the ocular micrometer

1.2 GPS (Garmin eTrex Venture^{R} HC)

1.3 Camera

1.4 Slide and cover glass

1.5 Staining jar

1.6 Beakers, Petri disks, needles, forceps, and brush

1.7 Blender

1.8 Sieve tube and shaking water bath

1.9 Autoclave

1.10 Freezer

1.11 Microwave oven

1.12 Mini-spin centrifuge

1.13 Thermal cycler machine (Bio-RAD)

1.14 High speed centrifuge

1.15 1.5 ml microcentrifuge tube and 0.2 ml PCR tube

1.16 Electrophoresis

1.17 Adjustable automatic pipettes and tips

1.18 UV transilluminitor and computer linked monitor (Gel Logic 100)

1.19 Others; tissue paper, pencil, pen, label and plastic bag

2. Chemical reagents

2.1 2% formalin

2.2 0.85% NaCl

2.3 1.0% pepsin solution

2.4 Ethyl alcohol series (10%, 20%, 30%, 50%, 70% 85%, 90%)

2.5 Butyl alcohol

2.6 Xylene

2.7 Permount

2.8 0.5% neutral red

2.9 Delafield's haematoxylin

2.10 Acetone orcine

2.11 Commercial GF-1 DNA extraction kit

2.12 Agarose gel (Vivantis, malaysia)

2.13 10X PCR buffer

2.14 *Taq* DNA polymerase

2.15 Magnesium Chloride ($MgCl_2$)

2.16 Primers

2.17 Tris base

2.18 EDTA

2.19 Boric acid

2.20 Sodium Hydroxide (NaOH)

2.216 Ethidium bromine

3.Parasite specimens

3.1 Heterophyid trematodes specimens and experimental hosts

Five species of heterophyid trematodes, including *Haplorchis taichui*, *H. pumilio*, *Centrocestus caninus*, *Stellantchasmus falcatus*, and *Haplorchoides* sp. were collected from northern Thailand. Four species of metacercarial stage of heterophyid trematodes were collected from freshwater fishes, comprising, *H. taichui* from Siamese mud carp (*Henicorhynchus siamensis*), *H. pumilio* from Moonlight gourami (*Trichogaster microlepis*), *C. caninus* from Golden fish (*Carassius auratus*), and *S. falcatus* from Half-beak (*Dermogenys pusillus*). Metacercariae were collected from various fish species, using the digestion technique with 1% pepsin solution (Srisawangwong *et al.*, 1997), and then were force-fed to 1-day old-chicks (*Gallus gallus domesticus*). Whereas, adult stage of *Haplorchoides* sp. were collected from Bagrid catfish (*Hemibagrus filamentus*).

3.2 Other trematode specimens

Adult stage of the other trematode samples namely, *Ganeo tigrinus* were collected from frog (*Limnonectes limnocharis*), *Prostorchigenes majeedi* from house lizard (*Hemidactylus frenatus*), while giant liver fluke, *Fasciola gigantica* and two rumen cow flukes, *Fischoederius elongatus* and *Orthocoelium streptocoelium* were recovered in cow (*Bos taurus*).

4. Study sites

The freshwater snails were collected from 64 sampling sites of 12 provinces in northern Thailand including, Chiang Rai, Chiang Mai, Lamphun, Lampang, Phayao, Mae Hong Son, Phrae, Nan, Sukhothai, Tak, Uttaradit, and Phitsanulok provinces (Table 3-1, Figure 3-1). They were selected on areas of public health problems with trematode infections. The coordinates for each sites were recorded using the global positioning system using Garmin eTrex Venture^{R} HC.

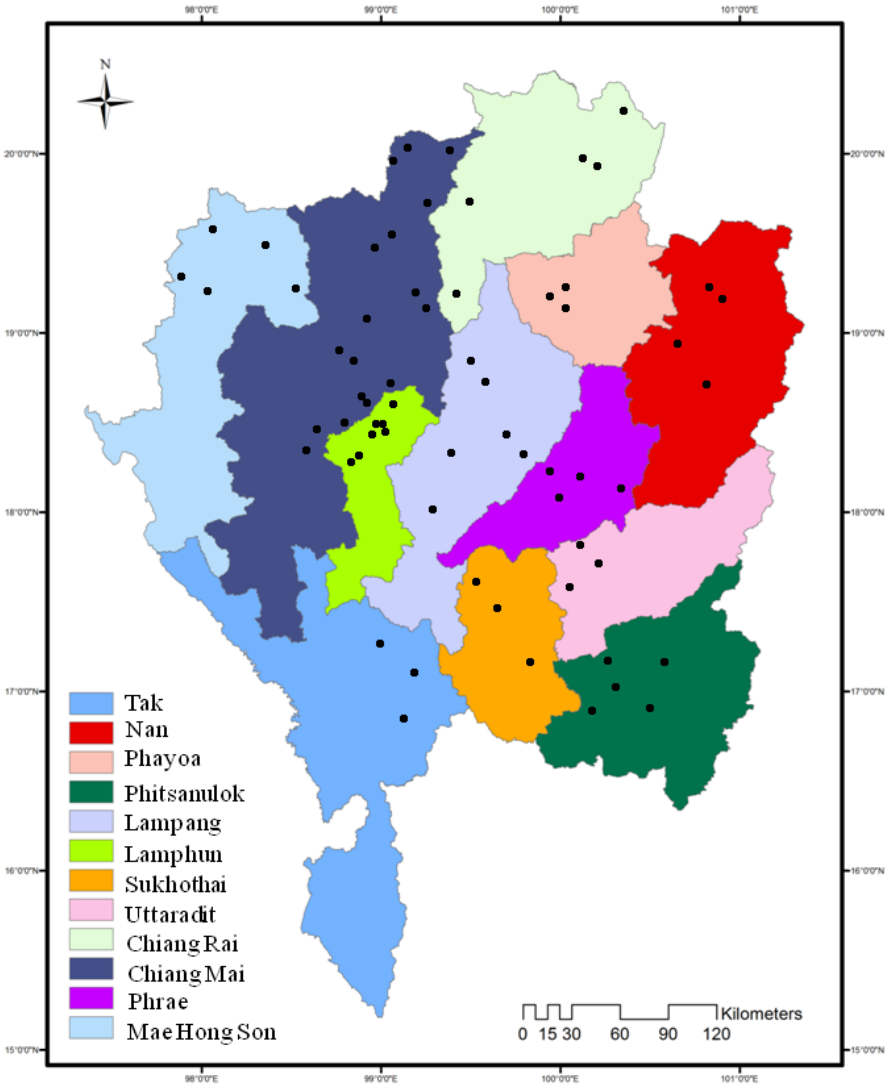

Figure 3-1. Map of the 64 sampling sites of northern Thailand, (●) where samples of snails were examined.

5. Diversity of freshwater snails

The snail specimens were collected by satisfy random method during April 2010 – June 2012. After collection, the snails were identified using Mandahl-Barth (1962), Brandt's taxonomic key (1974), and Ellen *et al*., (2008). The Species diversity index of freshwater snails found from each collecting sites will be evaluated by using the Shanon-Weiner Index.

6. Cercarial infections

For the cercarial infection investigation, each individual of snail specimen was crushed to examine under light microscope to investigate cercarial infection. The alive cercariae were vitally stained with 0.5% neutral red and identified according to morphology described by Rue, (1957), Schell (1970), Olsen, 1974, and Ito (1980). In addition, the cercarial specimens were stained with Delafield's haematoxylin or acetone orcine, dehydrated in an ethyl alcohol series, cleared with xylene, and mounted in permount. With camera lucida, illustrations were made to record information for a morphological characteristic study. The cercariae were identified at the family level and in some cases, the identification of the genus was possible.

Table 3-1. Description of collecting sites where snail collections were performed during April 2010 – June 2012 from 12 provinces, northern Thailand.

Sampling sites	Locality	Georeference coordinates	
CM-01	Baan Pha Lao, Mae Taeng district	19°6' 37.317"N	99° 4' 33.439"E
CM-02	Bua Tong waterfall, Mae Taeng	19°5'54.30"N	99°4'52.87"E
CM-03	Huai Maekajan, Mae Rim district	19°3'05.23"N	98°9'40.617"E
CM-04	Huai Mae Kuet, Chiang Dao district	19°22'51.51"N	98°58'2.88"E
CM-05	Chiang Dao district	19°22'57.149"N	98° 58'5.699"E
CM-06	Baan Wan, Hang Dong district	18°42'27.00"N	98°56'5.68"E
CM-07	Baan Tawai, Hang Dong (irrigation	18°41'10.25"N	98°56'16.57"E
CM-08	Baan Thung Siao, San Pa Tong district	18°33'59.54"N	98°52'5.61"E
CM-09	Baan Mai, Chom Thong district	18°27'0.40"N	98°42'40.99"E
CM-10	Baan Luang, Chom Thong district	18°28'30.72"N	98°39'3.97"E
CM-11	Tambol Doi LO, Doi Lo district	18°28'36.25"N	98°47'45.54"E
CM-12	Tambol Yang Noeng, Salaphee district	18°42'18.55"N	99° 2'16.62"E
CM-13	Baan Luang, Chom Thong district	18°27'2.91"N	98°40'27.99"E
CM-14	Baan Pha Taek, Doi Saket district	18°55'4.56"N	99° 7'44.04"E
CM- 15	BaanLuaen Nuea, Doi Saket district	18°55'31.46"N	99° 8'13.93"E
CM-16	Baan Luang, Mae Ai district	19°54'33.28"N	99°17'19.23"E

Table 3-1. (cont.)

Sampling sites	Locality	Georeference coordinates	
CM-17	Tambol Wiangfang, Fang district	19°55'1.26"N	99°13'11.29"E
CM-18	Tambol Pongnamron, Fang district	19°57'25.74"N	99°11'18.68"E
CM-19	Mueang district	18°44'52.47"N	98°56'57.39"E
CR-01	Mae Suai district	19°38'43.56"N	99°31'36.52"E
CR-02	Chiang Khong district	20°10'34.37"N	100°26'14.57"E
CR-03	Tabol Maekhachan, Wiang Pa Pao district	19°11'33.95"N	99°30'48.43"E
CR-04	Phaya Mengrai district	19°50'58.77"N	100° 8'59.98"E
CR-05	Huai Pa Daeng, Khun Tan district	19°49'53.74"N	100°15'31.36"E
MA-01	Pangmapa district	19°31'22.13"N	98°14'43.73"E
MA-02	Baan Huai Pa, Mueang district	19°25'27.06"N	97°59'19.84"E
MA-03	Huai Maesuya, Mueang district	19°23'47.09"N	97°56'43.15"E
MA-04	Baan Wing Tai, Pai district	19°22'45.62"N	98°25'53.12"E
MA-05	Baan Wian gtai district	19°21'21.37"N	98°26'50.40"E
LA-01	Tambol Jaeson, Mueang Pan district	18°45'56.20"N	99°14'20.90"E
LA-02	Huai Maetan, Hang Chat district	18°19'37.24"N	99°16'51.76"E
LA-03	Tambol Maewa Thoen district	17°29'35.59"N	99°11'36.93"E
LA-04	Bann Rim nam, Mueang district	18°18'27.15"N	99°31'9.29"E
LA-05	Nong Lom, Hang Chat district	18°19'13.11"N	99°20'49.97"E

Table 3-1. (cont.)

Sampling sites	Locality	Georeference coordinates
LA-06	Mae Phrik, Thoen district	17°26'43.03"N 99° 7'40.28"E
LU-01	Mueang district	18°35'3.35"N 98°59'32.47"E
LU-02	Tambol Makhuea Chae, Mueang district	18°35'31.46"N 99° 4'52.67"E
LU-03	Ban Thi district	18°38'57.15" N 99° 6'33.62"E
LU-04	Pha Sang district	18°32'32.23"N 98°56'21.32"E
NA-01	Baan Don Tan, Wiang Sa district	8°34'58.88"N 100°44'53.09"E
NA-02	Tha Wan Pha district	19° 7'13.99"N 100°48'35.27"E
NA-03	Pua district	19°10'42.86"N 100°52'31.80"E
NA-04	Pua district	19°10'13.71"N 100°56'2.83"E
PY-01	Gwarnpayao, Mueang district	19° 9'46.32"N 99°54'5.68"E
PY-02	Mueang district	19°11'40.56"N 99°53'48.02"E
PY-03	Dok KhamTai, Mueang district	19° 7'43.54"N 99°54'31.08"E
PH-01	Huai Kamin reservoir, Mueang district	18° 8'27.50"N 100°13'27.96"E
PH-02	Huai Phakum, Mueang district	18° 9'49.39"N 100°10'3.41"E
PH-03	Baan Nakuha, Mueang district	18° 7'45.33"N 100°19'4.37"E
PH-04	Rong Kwang district	18°18'22.01"N 100°16'28.32"E
PI-01	Tambol Wat Phrik, Mueang district	16°42'29.58"N 100°14'46.37"E
PI-02	Tambol Wat Bot, Wat Bot district	16°56'23.56"N 100°20'50.57"E

Table 3-1. (cont.)

Sampling sites	Locality	Georeference coordinates	
PI-03	Tambol Baan Krang, Mueang district	16°58'31.44"N	100°33'30.65"E
PI-04	Tambol Tha Pho, Mueang district	16°45'52.47"N	100°12'17.44"E
PI-05	Tambol Phrom Phiram, Phrom Phiram district	16°59'10.38"N	100°11'58.41"E
SU-01	Kong Krailat district	16°57'13.24"N	99°57'28.83"E
SU-02	Si Samrong district	17° 9'59.12"N	99°51'30.93"E
SU-03	Thung Saliam district	17°18'47.26"N	99°32'6.46"E
TA-01	Sam Ngao district	17°13'9.72"N	99° 2'35.87"E
TA-02	Mueang district	16°52'31.56"N	99° 7'45.78"E
TA-03	Ban Tak district	17° 2'56.46"N	99° 4'37.18"E
UT-01	Huai Pong, Lablae district	17°38'30.14"N	100° 2'18.75"E
UT-02	Baan Ko, Mueang district	17°35'33.54"N	100° 5'58.08"E
UT-03	Tambol Ban Dan Na Kham, Mueang district	17°42'45.81"N	100° 7'36.27"E

7. DNA extraction

The genomic DNA of trematodes were extracted from parapleurolophocercous cercariae from each sampling site and adult specimen of each trematode species including *Haplorchis taichui, H. pumilio, Stellantchasmus falcatus, Centrocestus caninus, Haplorchoides* sp., *Ganeo tigrinus, Prostorchigenes majeedi, Fasciola gigantica, Fischoederius elongatus,* and *Orthocoelium streptocoelium* using a

commercial GF-1 tissue DNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions. All genomic DNA of each specimens were diluted to a concentration at 100 ng/µl with elution buffer and kept at -20 ^{o}C until used.

8. Development of a DNA specific primer of *Haplorchis taichui* and *H. pumilio*

8.1 mtCOI amplification protocol

The PCR amplification of partial mtCOI fragment of 5 heterophyid trematode species and *Ganeo tigrinus*, *Prostorchigenes majeedi*, *Fasciola gigantica*, *Fischoederius elongatus*, and *Orthocoelium streptocoelium* used by a pair of primers described by Yu *et al*. (1997). It consists of (JB3) 5′ TTTTTTGGGCATCCTGACGTTTAT 3′, as a forward primer and (JB 4,5) 5′ TAAAGAAAGAACATAATGAAAATG 3′, as a reverse primer. The PCR amplifications were carried out in a final volume of 20 µl, including 100 ng of DNA template, 50 pM of each primer (JB3 and JB4,5), 1.5 mM of $MgCl_2$, 200 µM of dNTPs, and 0.5 unit of *Taq* DNA polymerase. The amplification procedure involved an initial denaturation step at 95 ^{o}C for 3 min, then 40 cycle including denaturation at 95 ^{o}C for 1 min, primer annealing at 50 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min, and final extension at 7 ^{o}C for 7 min, respectively. PCR products were analyzed after electrophoresis separation at 100 volt for 40 min on 1.8% agarose gels stained with ethidium bromide in TBE buffer. Gels were visualized by a Kodak digital camera (GelLogic 100). The sequences were performed for checking by the BLAST program in the NCBI (National Center for Biotechnology Information) database, to confirm the PCR target. The eletropherograms of each sequence were examined for sequence

accuracy using a Sequence Scanner version 1.0 and Bioedit version 7.1. All sequences were aligned automatically using Clustal X version 2.0.

8.2 Primer designed

From the partial mtCOI sequence data of 10 trematode species and other trematodes previously published or available in Genbank data based (http://www.ncbi.nlm.nih.gov/nucleotide/), specific forward and reverse primer were designed for *Haplorchis taichui* and *H. pumilio*. The pair of specific primers of both trematode species were designed to product amplicons of different size for accurate discrimination the PCR product on the ethidium bromide-strained agarose gel.

8.3 Sensitivity and specificity test of *Haplorchis taichui* and *H. pumilio* specific primers

Two pairs of specific primers were optimized PCR conditions. The master mix of PCR reaction were evaluated. The *H. taichui* and *H. pumilio* specific primers were tested for specificity period to perform in the molecular identification by attempting to amplify them with all 10 adult trematode species and snail tissues. For the sensitivity investigation, two-fold series dilution (100 ng/µl – 0.20 ng/µl) of DNA template of *H. taichui* and *H. pumilio* were amplified.

8.4 Development and optimizing of multiplex PCR for discrimination of *Haplorchis taichui* and *H. pumilio*

The multiplex PCR was developed in a single reaction, under high stringency condition, using concurrently both pair of specific primers (1 pair for *Haplorchis taichui* and 1 pair of *H. pumilio*). Test were done using different combatant DNA

template DNA (one or both species) and primers. The multiplex PCR conditions were exactly as those used in single species reaction. The specificity and sensitivity were tested again for accurate discrimination.

9. Molecular identification of cercarial stage of *Haplorchis taichui* and *H. pumilio*

The total genomic DNA of pleurolophocercous and parapleurolophocercous cercariae from each samples were amplified using multiplex PCR reaction for identification the *Haplorchis taichui* and *H. pumilio* infection in freshwater snail form northern Thailand. Amplification was perform in a thermocycles with the initial denaturation step at 95 ºC for 5 minutes, then 35 cycles including denaturation at 95 ºC for 45 seconds, primer annealing at 55 ºC for 45 seconds, extension at 72 ºC for 1 minutes, and final extension at 72 ºC for 7 minutes. PCR products were analyzed through 2.0% agarose gels electrophoresis separation at 50 volts for 40 minutes in TBE buffer, stained with ethidium bromide, and visualized with a Kodak digital camera (Gel Logic 100).

10. Phylogenetic relationships of heterophyid trematodes

All phylogenetic trees were generated using Mega version 5.0. All molecular data was analyzed by neighbor-joining (NJ) methods. The reliability of internal branches in both of trees was assessed using the bootstrap method, 1,000 replicates. Both methods used the Kimura two – parameter model. The five isolates of *H. taichui*, and three of *Metagonimus* spp., *S. falcatus, C. caninus, Haplorchoides* sp. were used for analyzing the phylogenetic tree (Table 3-2).

Table 3-2. List of material and sequences of mtCOI used for generated phylogenetic relationship of heterophyid trematodes.

Species of trematodes	Locations	Accession numbers
Haplorchis taichui	Chiang Mai, Thailand	KC404636 [1]
H. taichui	Thanh Hoa, Vietnam	JN809909[2]
H. taichui	Quang Tri, Vietnam	JN809893[2]
H. taichui	Ha Giang, Vietnam	JN809875[2]
H. taichui	Chumporn, Thailand	EF055885[2]
H. pumilio	Bangkok, Thailand	KF044303[1]
Centrocestus caninus	Chiang Mai, Thailand	KF044300[1]
Stellantchasmus falcatus	Chiang Mai, Thailand	KF044301[1]
Haplorchoides sp.	Chiang Mai, Thailand	KF044302[1]
Metagonimus sp.	Korea	AF096232[2]
Metagonimus yokogawai	Korea	AB470519[2]
Metagonimus takahashii	Korea	AF096231[2]
Orthocoelium streptocoelium	Chiang Mai, Thailand	KF044306[1]
Ganeo tigrinus	Chiang Mai, Thailand	KF044307[1]
Prostorchigenes majeedi	Chiang Mai, Thailand	KF044308[1]
Fasciola gigantica	Chiang Mai, Thailand	KF044304[1]

Remark: 1 = the sequence from this study

2= the sequence from GenBank databased (http://www.ncbi.nlm.nih.gov/)