CHAPTER 3

Materials and Methods

3.1 Study on the Prevalence of *Echinostoma revolutum* in Snails in Chiang Mai Province

3.1.1 Equipments and chemicals

1) Stereo and compound microscopes with ocular micrometer
2) Staining jar
3) Glass slides and cover slips
4) Scissors, needle, forceps and brush
5) Beakers and Petri dishes
6) 0.85% Normal saline solution
7) 5% formalin
8) Ethyl alcohol: 10%, 20%, 30%, 50%, 70%, 85% and 95%
9) Buthyl alcohol
10) Xylene
11) Permount
12) Aceto-camine
13) Animals serving as experimental hosts: chicks (*Gallus gallus domesticus*) and hamster (*Mesocricetus auratus*)

3.1.2 Methods for study on the prevalence of *Echinostoma revolutum* in snails

1) Study area

Snails were collected in Chiang Mai province (18° 47.414′ N, 98° 59.219′ E), during November 2011 to October 2012. The study areas were selected and occupied over ten districts of Chiang Mai province; Doi Saket, Hang Dong, Mae On, Mae Rim, Mae Taeng, Muang Chiang Mai, San Kamphaeng, San Pa Tong,

Saraphi and San Sai (Figure 3.1 and Figure 3.2). Freshwater snails were collected in both standing and running water bodies such as the pond, rice paddy, irrigation canal, stream and river. The number of investigated snails, the habitat type and coordinates of particular localities were recorded and also detailed in Table 3.1.

2) Snail sampling and identification

Snail samples were collected using a stratified random sampling approach. The snails were collected from diverse habitats by handpicking and scooping methods. At least two different habitats were investigated in each of the districts. Live snails from each area were placed in separate containers with water from the same habitat and transported to the laboratory. Live snails were kept at the laboratory in aquaria containing dechlorinated tap water and aerated at room temperature until they were examined. Snail species were identified according to the morphological features described by Brandt (1974).

3) Collection of the metacarcariae

The larval trematode infections in snails were studied by crushing methods. They were isolated using a sharp pin, gently covered with a cover slip, and observed under a light microscope. The presence of a head collar with 37 spines was highly indicative of *E. revolutum* group. The numbers of metacercariae were recorded. The data of prevalence and intensity were compared among three seasons (November 2011-February 2012 = cool, March 2012-June 2012 = hot-dry, July 2012-October 2012 = rainy). Data of metacercarial infections on snails were analyzed by ANOVA. Freshly isolated metacercariae were used for morphological observations and kept in normal saline at 4-5 ˚C for experimental infection to various definitive hosts.

4) Identification of metacercariae

Freshly isolated 37 collar spined metacercariae were fed to hamsters (*M. auratus*) and domestic chicks (*G. gallus domesticus*). Infected animals were sacrificed and necropsied at days 15 and 20 post infection, and adult worms were collected from their intestines. The collected worms were compressed, fixed in 5% formalin, stained with aceto-carmine, dehydrated in ascending grades of alcohols, cleared in xylene and mounted in Permount for permanent slides (Appendix E).

Freshly isolated metacercariae and permanent slides of adult worms were examined under a light microscope. The shape, size, and cyst wall structure were used for characterization of the metacercariae. For adult worms, the shape and size of the body and organs, number and arrangement of collar spines and the shape and size of eggs were employed as the morphological parameters. Morphological traits were studied and measured using the microscope Olympus equipped. Measurements and scales are in micrometers unless otherwise stated and the means are given in parentheses.

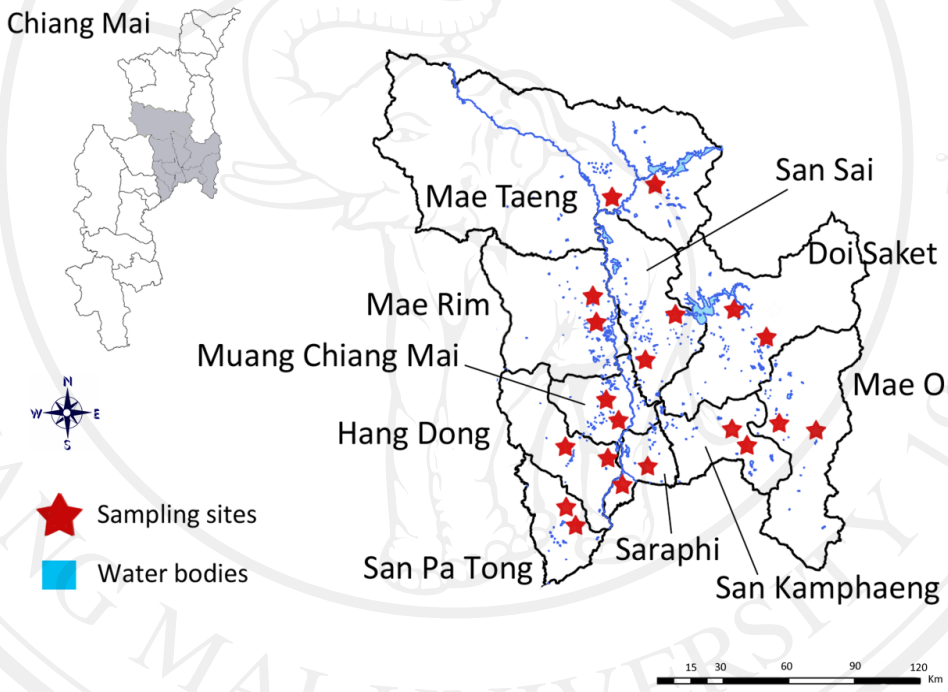

Figure 3.1 Map of study areas showing water bodies and snail sampling sites in ten districts of Chiang Mai province.

Site 1.1: Luang Nuea | Site 1.2: Talat Yai

Site 2.1: Nong Khwai | Site 2.2: San Phak Wan

Site 3.1: Mae On Klang | Site 3.2: Ban Sahakon

Site 4.1: San Pong | Site 4.2: Muang Kaeow

Figure 3.2

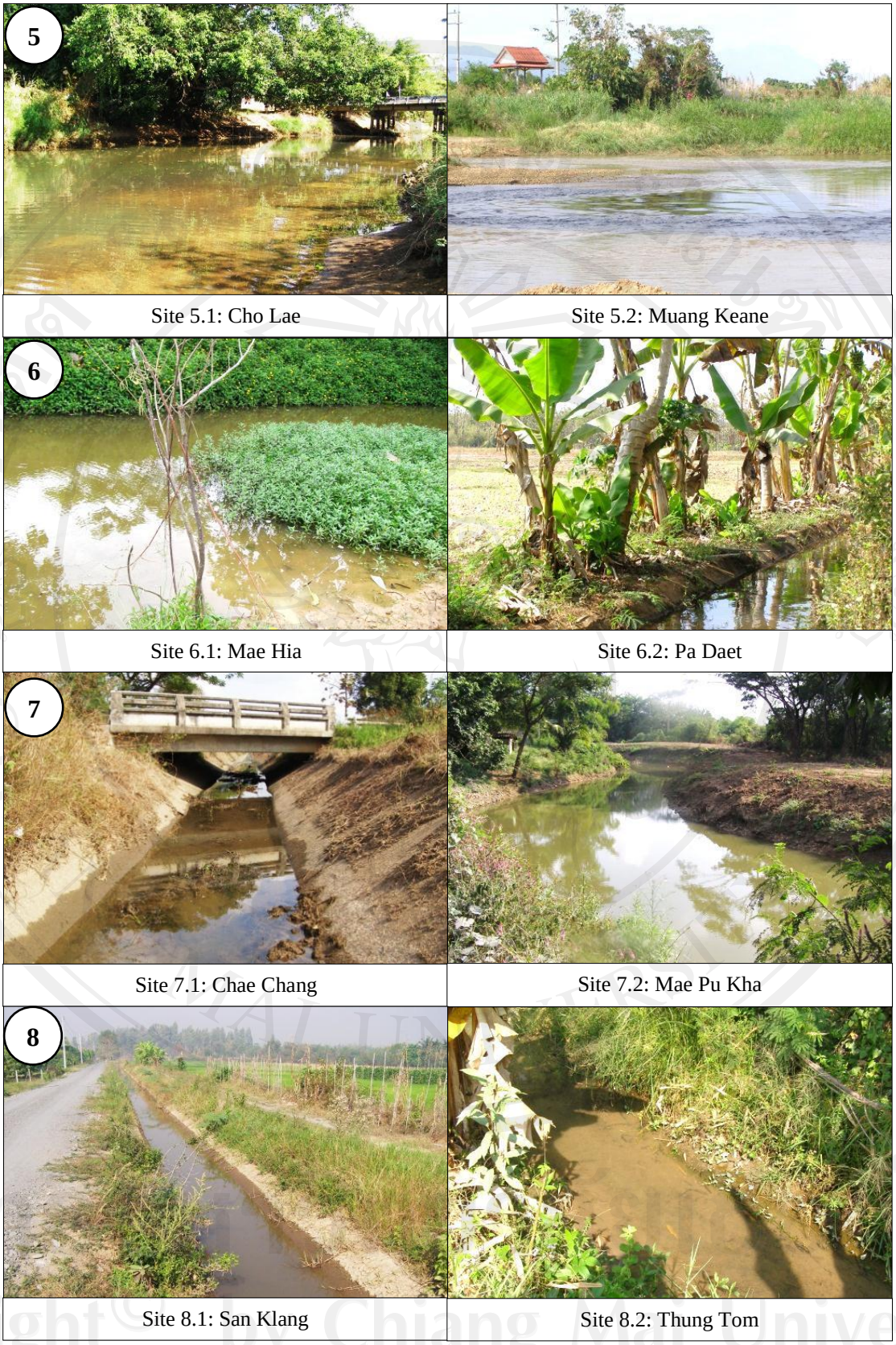

Figure 3.2

Figure 3.2 Study areas for collecting of snails in Chiang Mai province:

(1) Doi Saket District

(2) Hang Dong District

(3) Mae On District

(4) Mae Rim District

(5) Mae Taeng District

(6) Muang Chiang Mai District

(7) San Kamphaeng District

(8) San Pa Tong District

(9) Saraphi District

(10) San Sai District

Table 3.1 List of snail collected localities including habitat types observed

Sampling sites		Coordinates		Habitat type[a]	N[b]
District	Site name				
Doi Saket (DS)	Luang Nuea	18º 55′ 0.04″ N	99º 07′ 43.2″ E	RP, IC, P	548
	Talat Yai	18º 49′ 02.5″ N	99º 07′ 24.5″ E	S, P	473
Hang Dong (HD)	Nong Khwai	18º 43′ 38.6″ N	98º 55′ 48.6″ E	RP, IC	587
	San Phak Wan	18º 42′ 08.6″ N	98º 57′ 58.7″ E	IC, P	316
Mae On (MO)	Mae On Klang	18º 45′ 30.2″ N	99º 14′ 44.2″ E	RP, IC, R	447
	Ban Sahakon	18º 44′ 31.3″ N	99º 12′ 24.6″ E	IC, S	459
Mae Rim (MR)	San Pong	18º 56′ 36.2″ N	98º 56′ 46.1″ E	RP, IC	358
	Muang Kaeow	18º 52′ 25.5″ N	98º 58′ 23.5″ E	RP, P	591
Mae Taeng (MT)	Cho Lae	19º 09′ 09.1″ N	99º 01′ 00.5″ E	RP, S	812
	Muang Keane	19º 08′ 42.4″ N	99º 00′ 27.5″ E	RP, R	442
Muang Chiang Mai (MCM)	Mae Hia	18º 45′ 45.0″ N	98º 56′ 07.5″ E	P	378
	Pa Daet	18º 44′ 40.0″ N	98º 58′ 50.5″ E	IC, S	497
San Kamphaeng (SK)	Chae Chang	18º 43′ 40.4″ N	99º 08′ 15.6″ E	RP, IC	695
	Mae Pu Kha	18º 45′ 28.0″ N	99º 05′ 53.2″ E	R	418
San Pa Tong (ST)	San Klang	18º 40′ 03.5″ N	98º 53′ 04.1″ E	RP, IC, P	712
	Thung Tom	18º 37′ 34.0″ N	98º 55′ 00.5″ E	RP, P	482
Saraphi (SP)	Chumphu	18º 43′ 12.5″ N	99º 03′ 30.5″ E	RP, IC, P	654
	Nong Phueng	18º 43′ 27.5″ N	99º 02′ 01.5″ E	RP, P	519
San Sai (SS)	San Sai Luang	18º 50′ 08.8″ N	99º 02′ 32.3″ E	IC, P	493
	Muang Len	18º 53′ 27.2″ N	99º 04′ 53.4″ E	RP, S	811

Note: [a]RP: rice paddy; IC: irrigation canal; S: stream; R: river; P: pond and [b]N: number of snails examined.

3.2 Study on the Life History of *Echinostoma revolutum*

3.2.1 Equipment and chemicals

1) Aquarium, tanks and air pumps
2) Gauzes
3) Centrifuge
4) Passture pipete
5) Absolute ethyl alcohol
6) 1% iodine solution
7) 5%, 10% formalin
8) Diethyl ether
9) 0.85% normal saline solution
10) Animals serving experimental hosts; Chick (*G. gallus domesticus*), Snails (*Lymnaea auricularia rubiginosa*, *Filopaludina doliaris* and *F. martensi martensi*)

3.2.1 Methods for study on the life history of *Echinostoma revolutum*

1) Definitive host infections

Metacercariae of *E. revolutum* were collected from naturally infected snails *Filopaludina* spp. (*F. doliaris*, *F. sumatrensis polygramma* and *F. martensi martensi*) which are common and widely spread in Chiang Mai Province. The *E. revolutum* metacercariae in snails were collected by crushing methods. They were isolated using a sharp pin, gently covered with a cover slip, and then collected for the metacercarial stage under a light microscope. Freshly isolated metacercariae were used for experimental infection to definitive host.

Three day old chicks (*G. gallus domesticus*), 60 in number weighing between 48-60 g were used as the experimental definitive hosts (Figure 3.3). Chicks were purchased from Faculty of Animal Science and Technology, Maejo University, Chiang Mai Province. Control animals were check for negative. Fifty encysted metacercariae were orally forced fed to each chick.

The chicks were sacrificed daily at days 1-60 post-infection (PI) by excess diethyl ether for examination of the parasite. The digestive tract roughly divided into the esophagus, crop, stomach (proventiculus and gizzard), small intestine (duodenum, jejunum and ilium), caecum, large intestine and cloaca, longitudinally with a pair of scissors and placed in 0.85% NaCl for worm recovery. In small intestine, duodenum was cut in the area of the duodenal loop, jejumum was cut at level with the remnant of the yolk sac, and ilium was cut at the caeca (ileo-caeco-rectal junction). Worms were collected and examined under a stereo microscope.

The numbers recovered were recorded to determine the infectivity and worm recovery. The recovered worms were washed in 0.85% NaCl and fixed in 5% formalin under a cover slip pressure for permanent slide preparation. The development and the morphological details of the worms were observed from living specimens and permanent slides under a light microscope.

2) Fecundity of the worm

Data on fecundity were determined by uterine egg counts (UEC) and number of eggs per gram feces (EPG). Fecal samples were examined to determine the EPG. The chick feces were collected separately, incubated at 60˚C for 24 hours, weighed and fixed in 10% formalin before examination. Chick feces were checked daily by a modification of the formalin-ether concentration technique (MFECT) (Elkins *et al*., 1991). Briefly, the MFECT involves filtering 1 g of chick feces through two gauze layers**,** then washed and centrifuged with formalin and ether (7 ml of 10% formalin: 3 ml of ether). The feces were mixed with 1% iodine solution on glass slides and then observed under a compound microscope. Three replicated samples per chick were observed to determine the EPG. The correlation was used to quantify the association between the worm recovery and UEC, and/or EPG. In addition, the correlation of UEC per worm (UEC/worm) and EPG per worm (EPG/worm) was determined.

3) Observations of egg development

Mature unembryonated eggs were purified from chick feces by filtration through a series of graded sieves, then observed under a stereo microscope. The eggs were washed in 0.85% NaCl several times and then incubated in multi-well

plate cultures containing 2 ml of distilled water. The eggs were incubated at room temperature (25-28°C) with ambient room lightning for at least 2 weeks to observe the final hatch to obtain eggs with fully developed miracidia. Egg development was observed under a compound microscope from live eggs prepared as wet mounts. Egg development was monitored daily. Developmental stages were photographed using a compound microscope (OLYMPUS DP20, Olympus).

After miracidium hatched, the life spans of miracidiam were determined by observing specimens placed in vials with 2 ml of dechlorinated water. The miracidia were examined every hour to determine their viability and were considered dead or moribund when they were inactive or moving slowly at the bottom of the vials.

4) The first intermediate host infections

Freshwater snails (*L. auricularia rubiginosa, F. doliaris* and *F. martensi martensi*) (Figure 3.4) were obtained from laboratory culture for more than 2 generations to confirm negative control before subjected to the experimental infections. All snail used in experimental infection were reared in a climate chamber under appropriate constant conditions of temperature, humidity and light, and fed lettuce. Experimental infections of the first intermediate host snail with miracidium were taken after complete hatching of the eggs. Incubation procedures were carried by placing the latter together with various snail species in multiwell plate containing water. Snails were kept for 6 hours in these multiwell plates with containing numerous miracidium. The exposed hosts were investigated for the intramolluscan stages; sporocysts, rediae (mother redia and daughter redia) and cercariae, by dissection and some specimens were fixed with 5% formalin under a cover slip pressure for permanent slide preparation. Snails were crushed after 1day up to 90 days to find the intramolluscan stages. The life spans of cercariae were determined by observing specimens placed in vials with 5 ml of dechlorinated water, twenty four times per day until die.

5) Second intermediate host infections

Free mature cercariae were in turn obtained after emergence or dissection from the first intermediate hosts. Experimental infection of the second intermediate

host snails with cercariae were carried out by placing cercariae together with various other snails (*L. auricularia rubiginosa*, *F. doliaris* and *F. martensi martensi*) in a multiwell plates containing dechlorinated water for 6 hours and then transferred to an aerated aquarium. Thirty infected snails were observed daily for 15 days after infection to examine encysted metacercariae. The experimentally infected snails were investigated for the encysted metacercariae by dissection. Developmental changes were recorded. Some specimens were permanently mounted after staining in aceto-carmine.

6) Morphology investigation

Specimens (eggs, miracidium, sporocysts, rediae, cercariae and metacercariae) were fixed in 5% formalin under a cover slip pressure, stained with aceto-camine or borax-carmine, dehydrated in alcohol series, cleared in xylene, and mounted in Permount (Appendix E). The specimens were photographed, measured and illustrated under compound microscopy for morphological studies. Morphological traits were studied and measured using the microscope Olympus equipped. Measurements and scales are in micrometers unless otherwise stated and the means are given in parentheses. Drawings were made with the aid of a camera lucida. Fresh living adults and some larval stages were studies for the surface ultrastructure by scanning electron microscopy (SEM).

7) Study on the surface ultrastructure of some larval stages and adults, *E. revolutum*

1. Equipments, materials and chemicals

1) Scanning electron microscope (SEM) model JEOL-JSM2001LV
2) Sputtering coater
3) Critical Point Dryer
4) Stubs and carbon sticky tape
5) 2.5% glutaraldehyde
6) 1% osmium tetroxide
7) 0.1 M phosphate buffer (pH 7.3)
8) 0.85% NaCl

9) Ethyl alcohol serial solution: 10%. 20%, 30%, 50%, 70%, 85%, 95% and absolute

10) Acetone

2. Studying the tegumental surface of *E. revolutum*

To observe the surface ultrastructure, some of the larval stages and adult worms were rinsed several times in saline and fixed in 2.5% (w/v) glutaraldehyde at 4°C for 24 hours, washed several times in phosphate buffer and post fixed with 1% osmium tetroxide for 3 hours. Subsequently, they were dehydrated in a graded alcohol series, then transferred into acetone, and finally dried in a critical-point dryer. The specimens were mounted on stubs and then coated with gold. The specimens were observed and photographed using a JEOL JSM-5400LV scanning electron microscope (Appendix E).

Figure 3.3 The experimental definitive host, chick (*G. gallus domesticus*).

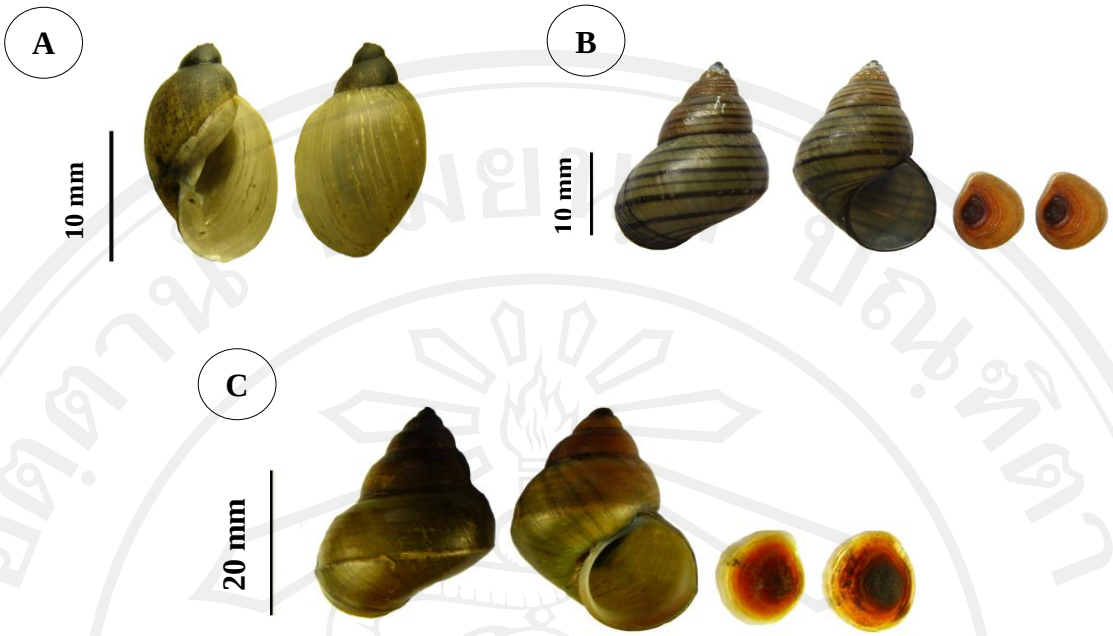

Figure 3.4 The experimental first and second intermediate host snails; **(A)** *L. auricularia rubiginosa*, **(B)** *F. doliaris*, **(C)** *F. martensi martensi*.

3.3 Molecular Identification and Phylogenetic Analysis

3.3.1 Equipments

1) Adjustable micropipette
2) Autoclave
3) Freezer
4) Hot air oven
5) Ice machine
6) Kodak-Gel logic 100 imaging system
7) Microcentrifuge tube 1.5 ml and PCR tube thin wall 0.2 dome cap
8) Microwave
9) PCR thermocycler (BIO-RAD MyCycer^{TM} thermal cycler)
10) Shaking water bath
11) UV transilluminator
12) Vortex mixer
13) White, yellow and blue tip

3.3.2 Chemicals

1) *Bst*-DNA polymerase (8 U; New England BioLabs)
2) ddH_2O (Rnase free)
3) DNeasy Tissue Kit (Qiagen, Rodgau-Juegesheim, Germany)
4) Ethidium bromide
5) GenePure LE agarose (Promega)
6) Smart 2× PCR Pre-Mix (Solgent Inc., Daejeon, Korea)
7) Tris boric EDTA buffer (TBE buffer)
8) 10X ThermoPol Reaction Buffer (New England Biolabs)
9) 0.8 M betaine
10) 6 mM MgSO4
11) 1.4 mM of each dNTPs
12) 1 kb and 100 bp ladder

3.3.3 Methods for study on molecular identification by loop-mediated isothermal amplification (LAMP)

1) Parasites and DNA extraction

Adult worms of *E. revolutum* were collected from chicks experimentally infected with metacercariae in the laboratory. Metacercariae were collected from freshwater snails, *Filopaludina doliaris*, *F. sumatrensis polygramma*, and *F. martensi martensi* as the second intermediate host of *E. revolutum* in Chiang Mai province. Several related trematodes were included as heterologous control samples for assessing the specificity of the LAMP assay, including *Echinostoma cinetorchis, E. hortense, Echinochasmus japonicus*, *Acanthoparyphium tyosenense, Fasciola gigantica, Fischoederius elongatus, Paramphistomum epiclitum* and *Orthocoelium streptocoelium*. All parasites were preserved in 70% ethanol and stored at -20°C until extraction of the genomic DNA.

Total genomic DNA from *E. revolutum* and heterologous control samples were extracted from each individual trematode specimen using the DNeasy Kit (Qiagen, Rodgau-Juegesheim, Germany). The DNA was eluted in elution buffer and stored at -20˚C until used, according to the manufacturer's instructions.

2) LAMP primers design

The *E. revolutum*-specific primers used for the LAMP assay were selected on the basis of a highly conserved region of the parasite genome. The nuclear ribosomal DNA internal transcribed spacer subunit 2 (ITS2) sequence of *E. revolutum* was retrieved from the GenBank (accession number AF067850), and was used to design six LAMP primers targeting eight conserved regions in the ITS2 sequence (Table 3.2). The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward primer (LF) and loop backward primer (LB) were designed using the Primer Explorer V4 software (http://primerexplorer.jp/e). The LAMP primers were designed and selected based on the criteria of Notomi *et al*. (2000) and Nagamine *et al*. (2002).

3) LAMP outer primer specificity tested by PCR

The specificity of the LAMP outer primers for the *E. revolutum* detection was confirmed by the conventional PCR with the outer primers F3 and B3. The outer primers were selected to specifically amplify *E. revolutum* targeting the 182 bp DNA fragment of ITS2. The PCR reaction was performed in a 30 µl reaction mixture containing Smart 2× PCR Pre-Mix (Solgent Inc., Daejeon, Korea), F3 and B3 primers (10 *p*mol each), 2 µl of DNA template and dH_2O. The reactions were performed in a Mastercycler gradient (Eppendorf) and the PCR conditions were as follows; an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation (1 min at 94°C), annealing (30 s at 55°C), and extension (45 s at 72°C), with a final extension for 7 min at 72°C. The PCR products (3 µl) were evaluated on 1% agarose gel and stained with ethidium bromide and then photographed using the UV Image system. The amplified PCR product representing *E. revolutum* was subjected to sequencing.

4) LAMP assay

The LAMP assay was carried out in a final reaction mixture of 25 µl which contained 10X ThermoPol Reaction Buffer (New England Biolabs, Beverly, MA) [20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25°C], 0.8 M betaine, 6 mM $MgSO_4$, 1.4 mM of each dNTPs, primers FIP and BIP (40 pmol each), loop primer (20 pmol each), primers F3 and B3 (5 pmol each), *Bst*-DNA polymerase (8 U; New England BioLabs), ddH_2O (Rnase free) and template DNA (2 µl). No DNA template was added in the negative control reaction. The reaction mixture was incubated at optimal temperature, 63°C for 60 min and then heated at 80°C for 3 min to terminate the reaction.

5) Detection of LAMP product

The LAMP products were visually detected by adding 1 µl of 1:10 diluted 10,000×concentration fluorescent dye SYBR Green I (Invitrogen, Carlsbad, CA) to the reaction tube, and also 3 µl of the LAMP products were electrophoresed in a 2% agarose gel and stain with ethidium bromide. The reaction tubes and stained gel visualized and photographed under exposure to UV light.

6) Specificity and sensitivity of LAMP assay

The specificity of the LAMP assay was evaluated using *E. revolutum* DNA sample (adult and metacercaria) as the positive control and other related trematodes as described above were used for heterologous control samples. A negative control (no DNA template) was included.

The sensitivity of the LAMP assay was assessed using the 10-fold dilution series of genomic DNA and comparison with conventional PCR. Serial dilutions of genomic DNA were made from 10 to 1×10^{-8} ng/µl and used as templates for LAMP and PCR assay.

7) Field sample testing by LAMP

The naturally infected snail including *Clea helena, Eyriesia eyriesi, Bithynia funiculata, B. siamensis siamensis, F. doliaris, F. sumatrensis polygramma,* and *F. martensi martensi* were collected in an area of *E. revolutum* appearance, Chiang Mai province. The snails were examined for metacercariae by the crushing method and observed under a stereomicroscope. Echinostomes metacercariae were separated based on the number of collar spines. The presence of 37 collar spines was highly indicative of the *E. revolutum* group. Metacercariae were collected and subjected to DNA extraction by using DNeasy Kit (Qiagen). The DNA samples were used as the template for evaluation of LAMP.

Table 3.2 Sequence of LAMP primers used for detection of *Echinostoma revolutum* targeting the ITS2 sequence.

Primer	Length (bp)	Sequence (5′-3′)
F3	18	GCTTGGGTTTTGCCAGCT
B3	20	TCCACCCGTAGTCATATGCA
FIP	41	CAAGACATGGATGCGTCCGGAT - CTTGTCATGTGAGGTGCCA
BIP	40	CCATGATGGGATGTGGTGACGG - AGAAGCGCCAAACATGCT
LF	21	ACATTGGGGAAACGCCATAGA
LB	22	TGGTTTAATATGGCTATGCCCC

3.3.4 Methods for study on phylogenetic analysis

1) Amplification of internal transcribed spacer subunit 2 (ITS2) region and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene

Partial regions of the ITS2 and ND1 gene were amplified. Samples used for the analysis of both regions are summarized in Table 3.3. The primer set BD1 (5′-GTCGTAACAAGGTTCCGTA-3′) and BD2 (5'-TATGCTTAAATTCAGCGG GT-3') (Morgan and Blair, 1995) was used for ITS2, and the primer set JB11 (5′-AGATTCGTAAGGGGCCTAATA-3′) and reverse, JB12 (5′-ACCACTAACTAA TTCACTTTC-3′) (Morgan and Blair, 1997a; 1997b) was used for ND1.

PCR amplification was performed in 30 µl of reaction mixture containing 15 µl of Smart 2×PCR Pre-Mix (Solgent Inc.), 1 µl (10 pmol/µl) of each primer, 5 µl of DNA template and 8 µl of distilled water, using a Mastercycler gradient (Eppendorf). The thermocycling protocol were as follow 96° C for 1 min, followed by 35 cycles of 96° C for 30 s, 54° C for 1 min, and 72° C for 1 min, and a final extension step of 72° C for 10 min for ITS2. The cycling profile for the PCR reaction of ND1 consisted of an initial denaturing step for 2 min at 94° C, followed by 39 cycles at 94° C for 30 s, 48° C for 20 s, and 72° C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized with a 1% agarose gel containing ethidium bromide. PCR products were subjected to sequencing by SolGent, Co., Ltd. (Daejeon, Korea). The sequences obtained were checked by BLASTN search in the NCBI database to confirm the PCR target.

2) Sequences analysis

DNA sequences of ITS2 and ND1 were analyzed with MEGA (version 6.0) (Tamura *et al*., 2013). Multiple alignments were completed by ClustalW which is integrated in the MEGA program. The following is the parameters for alignment: gap opening penalty 15, gap extension penalty 6.66, and transition weight 0.5.

3) Phylogenetic analysis

Phylogenetic analysis were constructed and determined by comparing the sequences with other isolates of *E. revolutum* and other *Echinostoma* that are available in GenBank (NCBI). The distance method was employed to obtain a neighbor-joining tree (NJ) tree, and also constructed phylogenetic tree. The ITS2 sequence of *Fasciolopsis buski* (EF612481) was applied as an out group for ITS2 phylogenetic trees and used the ND1 sequence of *Fasciola gigantica* (AB603739) as the out group for ND1 phylogenetic trees. The NJ tree were calculated based on Kimura 2-parameter model with gamma rate heterogeneity (+G). DNA substitution model was selected using neighbor-joining trees with a default gamma parameter of 5. The probabilities were evaluated by the bootstrap test (1000 replicates). The relationship genetic distance within and among species groups of Southeast Asia isolates was calculated for each gene with MEGA version 6.0

Table 3.3 Summary of samples used in this study

Sample name	Geographic region	Host		Comment
		Metacercaria	Adult	
E. revolutum THA (Fm)	Thailand	*Filopaludina martensi martensi*	Experimental host: hamster	
E. revolutum THA (Fd)	Thailand	*F. dorliaris*	Experimental host: hamster	
E. revolutum THA (Fp)	Thailand	*F. sumatrensis polygramma*	Experimental host: hamster	
E. revolutum THA (Bf)	Thailand	*Bithynia funiculata*	Experimental host: hamster	
E. revolutum THA (Bs)	Thailand	*B. simensis simensis*	Experimental host: hamster	
E. revolutum THA (Ch)	Thailand	*Clea helena*	Experimental host: hamster	
E. revolutum THA (Ee)	Thailand	*Eyriesia eyriesi*	Experimental host: hamster	
E. revolutum LAO	Lao PDR		Human	
E. revolutum VNM	Vietnam	*Filopaludina* sp.	Experimental host: hamster	
E. revolutum CAM (K-1)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-2)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-3)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-4)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-5)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-6)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (K-7)	Cambodia		Human	K: Kratie Province
E. revolutum CAM (T-1)	Cambodia		Human	T: Takeo Province
E. revolutum CAM (T-2)	Cambodia		Human	T: Takeo Province