CHAPTER 3

Materials and methods

3.1 Materials and research instruments

3.1.1 Materials and instruments for investigation the morphological study

1) Blender (Warring: Model 32BL80)
2) Binocular compound microscope (Olympus CH-2)
3) Digital cameras (Olympus DP-20) with linked monitor
4) Drawing tube (camera lucida)
5) Glass wares; Petri dish, beakers, dropper, slides, cover slides, Erlenmeyer flask, cylinder, different size bottles and staining jars
6) Hot plate/ slide warmer bench
7) Ocular and stage micrometer
8) Others; tissue paper, pencil/pen, ruler, label pad, brush, aluminum foil and plastic bags
9) Sieve mesh (20, 40, 60 and 80 µm)
10) Stereo microscope (Olympus SZ-30)
11) Surgical equipments; surgical blade, scissor, needle, forceps and aluminum/ plastic trays

3.1.2 Materials and instruments for molecular study

1) Adjustable automatic pipettes and tips
2) Autoclave
3) Digital camera with computer linked monitor (Kodak Gel Logic-100)
4) Electrophoresis

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5) Glass wares; petri dish, beakers, dropper, erlenmeyer flask, cylinder, pipettes and different size bottles
6) High speed ultrasonic centrifuge (KUBOTA)
7) Liquid nitrogen container
8) Laminar flow
9) Micro-centrifuge tubes 1.5 ml
10) Microwave oven
11) Mini-spin centrifuge
12) Mortar and pestle
13) PCR tubes 0.2 ml
14) Power supply
15) pH meter (Mettler Toledo: MP-230)
16) Refrigerator and freezer (-20°C)
17) Shaker incubator (WiseCube)
18) Thermal cycler machine (LifePro Thermal Cycler, Bior Serves Life)
19) UV transilluminator
20) Vortex mixer (Seoulin: Mylab Combispin SLFVL-2400)
21) Water bath (Julabo: Ecotem TW20)
22) Weighing machine (OHAUS: Adventure™)

3.2 Chemical reagents

3.2.1 Chemical reagents for investigation the morphology study

1) Bouin's fixative
2) Butyl alcohol
3) Ethyl alcohol 10%, 20%, 30%, 50%, 70%, 85% and 95%
4) Formalin 10%
5) Hydrochloric acid (HCl)
6) NaCl 0.85%
7) Permount (Fisher)
8) Pepsin solution 1%

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9) Staining agents (Haematoxylin and Borax carmine)
10) Xyline

3.2.2 Chemical reagents for molecular biology study

1) Absolute ethyl alcohol
2) Agarose (Vivantis, Malaysia)
3) Boric acid
4) Deoxyribonucleotide triphosphate (Vivantis, Malaysia)
5) De-ionized water
6) Distilled water
7) EDTA
8) Ethidium bromide
9) GF-1 Tissue extraction kit (Vivantis, Malaysia)
10) Isopropanol
11) Liquid nitrogen
12) Loading dye 6X
13) Magnesium sulphate ($MgSO_4$)
14) PCR buffer 10X
15) Primers (Operon Technologies, USA)
16) Protenase K (Vivantis, Malaysia)
17) Rnase ONE^{TM} Ribonuclease (Promega, USA)
18) Sodium chloride (NaCl)
19) *Taq* DNA polymerase
20) Tris base

3.3 Parasitic materials

The out group of related-species trematodes was prepared as: *Haplorchis taichui*, *Stellantchasmus* sp., *Opisthorchis viverrini*, *Haplorchoides* sp., *Fischoederius elongatus*, *Orthocoelium streptocoelium*, and *Paramphistomum epiclitum*. The metacercarial stage; *H. taichui* was collected from *Henicorhynchus siamensis*,

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Stellantchasmus sp. from *Dermogenys pusillus*, and liver fluke, *O. viverrini* from *Cyclocheilichthys armatus*. All fishes were individually digested with 1% pepsin solution for metacercariae collection and then force-fed to experimental hosts; dwarf hamsters and sacrificed 3 days post-infection (PI). Excepted, *O. viverrini* metacercariae were sacrificed 30 days post-infection (PI).

Adult worm of *Haplorchoides* sp. was derived from intestine of *Hemibagrus filamentus*, whereas 3 paramphistomes; *Fischoederius elongatus*, *Orthocoelium streptocoelium*, and *Paramphistomum epiclitum* were obtained from rumen of water buffalo (*Bubalus bubalis*). All adult worms were kept in 1.5 ml micro-centrifuge tube, stored in -20°C until used in DNA extraction for molecular detection.

3.4 Morphological study of adult worms

3.4.1 The light microscope observation (LM)

Adult worms of *F. gigantica* from 3 slaughterhouses in Doi Saket, San Pa Tong, and Mueang districts, Chiang Mai Province were prepared permanent slides for morphological investigation**.** The flukes were rinsed by tap water several times, compressed and fixed in 5% formalin, stained with Delafield's haematoxylin or borax carmine, dehydrated in an alcohol series, cleared in xylol, and mounted in permount. The permanent slides of flukes were measured, drawn, and photographed.

3.4.2 The scanning electron microscope (SEM)

The flukes were washed several times in distilled water and then fixed in 2.5% glutaraldehyde in PBS buffer (pH 7.4) at 4 °C for 24 hours. The specimens were washed several times with the PBS buffer, and post-fixed in 1% osmium tetroxide in PBS buffer at 4 °C for 24 hours, dehydrated in

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grading alcohol series. All specimens were dried in a critical point dryer (HCP-2, Hitachi, Tokyo, Japan), and mounted on aluminum stubs and coated with gold in an ion-sputtering apparatus, SPI-Model sputter coater for 4 minutes before observed by SEM (JEOL JSM-5400) with operation magnitude of 15 kilovolt (kV).

3.5 The prevalence of adult worms infection

Adult worms of *F. gigantica* were collected in liver and gallbladder of cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in slaughterhouses from Doi Saket, San Pa Tong, and Mueang districts of Chiang Mai Province. The flukes were removed from liver and gallbladder**,** and counted for the prevalence calculation. Prevalence (%) was calculated according to the percent of infected cattle divided by total number of cattle examined.

3.6 The prevalence of cercarial infection of snail intermediate hosts in natural sampling sites

3.6.1 Sampling sites for snail intermediate hosts collection

Seven sampling sites in Chiang Mai Province as Chiang Dao, Phrao, Mae Taeng, Mae Rim, Chom Thong, Hod, and Doi Tao districts were designed for the collection of snail specimens. The details and coordinates of sampling sites were depicted on figure 3.1-3.8.

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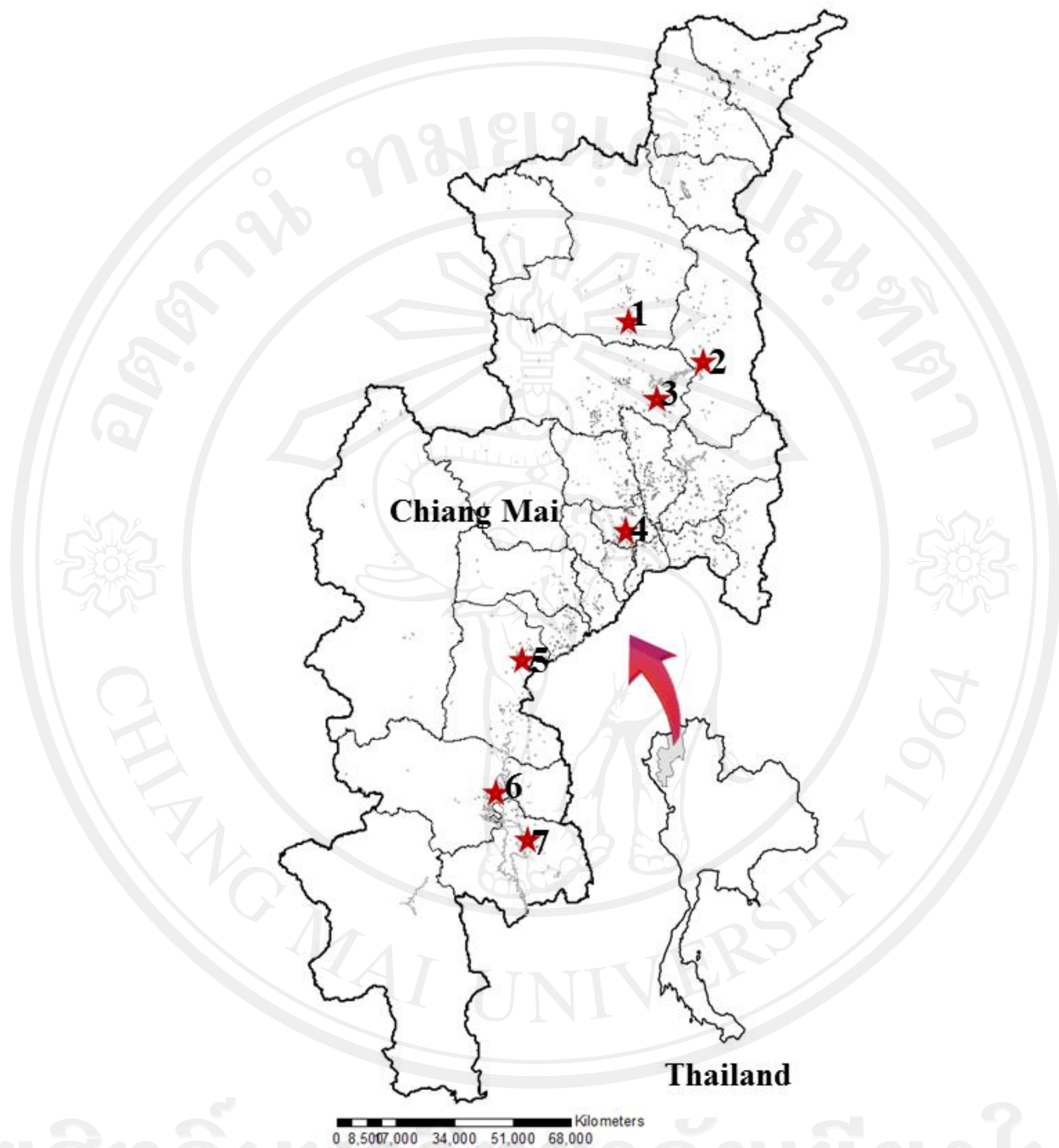

Figure 3.1 Map of 7 sampling sites were designed for the snail intermediate hosts collection in Chiang Mai Province; 1: Chiang Dao district, 2: Phrao district, 3: Mae Taeng district, 4: Mae Rim district, 5: Chom Thong district, 6: Hod district, 7: Doi Tao district

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Figure 3.2 Sampling site 1: Ping River located at Chiang Dao district of Chiang Mai Province (19º17'05.8" N, 098º58'20.9" E)

Figure 3.3 Sampling site 2: Mae Ngad River located at Phrao district of Chiang Mai Province (19º04'23.7" N, 098º51'28.9" E)

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Figure 3.4 Sampling site 3: Mae Taeng River located at Mae Taeng district of Chiang Mai Province (19º08'45.0" N, 099º02'13.8" E)

Figure 3.5 Sampling site 4: Ping River located at Mae Rim district of Chiang Mai Province (18º54'07.4" N, 098º58'36.7" E)

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Figure 3.6 Sampling site 5: Ping River located at Chom Thong district of Chiang Mai Province (18º24'21.1" N, 098º42'07.1" E)

Figure 3.7 Sampling site 6: Mae Cham River located at Hod district of Chiang Mai Province (18°12'7.40" N, 098°36'52.23" E)

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Figure 3.8 Sampling site 7: Doi Tao Reservoir located at Doi Tao district of Chiang Mai Province (17°57'18.32" N, 098°39'18.09" E)

3.6.2 Method for cercarial stages collection from snails

The snails were collected from 7 sampling sites in Chiang Mai Province, during August to September 2012 by handing method. The cercarial stage infection in all collected snails was investigated by crushing method. Crushed-snails were placed into Petri dish, poured a few amount of distilled water and observed under stereo microscope, and identified based on morphological characters. The prevalence of cercarial infection was also calculated. Presented cercariae were kept into 1.5 ml micro-centrifuge tube, stored in -20°C until used in DNA extraction for molecular detection.

3.7 Life history study

3.7.1 Collection of *F. gigantica* eggs

Eggs of *F. gigantica* were recovered from bile in gallbladder of the cows (*Bos taurus*) or water buffaloes (*Bubalus bubalis*). The eggs were washed

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several times with dechlorinated tap water, and collected under a stereo microscope.

3.7.2 Hatching of miracidium from eggs

1,000 eggs of *F. gigantica* were placed in multiple-well plates containing dechlorinated tap water, and incubated at room temperature in natural light and darkness to allow the development of miracidium. The changes of eggs were determined dairy under a stereo microscope, until the miracidium were hatched from eggs.

3.7.3 Infection of miracidium in snails

The snail intermediate hosts were used experimental infection as *Lymnaea auricularia rubiginosa* (Fig. 3.9). These snails were derived from laboratory culture in clay pot with de-chlorinated tap water and supplied with fresh lettuce leaves for feeding the snails and continuous aeration. One mouth old of F1 generation of snails were used experimental infection.

One hundred non-parasitized snails (F1) were placed in clay pots with a holding capacity of 2 liter of dechlorinated tap water, after that 1,000 miracidium were placed into clay pots. The exposed- snails in each clay pot was supplied with fresh lettuce leaves for feeding the snails and continuous aeration. The exposed-snails were daily crushed and dissected for larval stage determine.

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Figure 3.9 The experimental snail intermediate host; *Lymnaea auricularia rubiginosa*

The cercarial-shedding from snail intermediate host was investigated. One snail was placed in a culture plate containing distilled water. Sheded-cercariae were investigated daily under a stereo microscope.

3.7.4 Encystation of metacercaria on rice plants

Rice plants (*Oryza sativa*) were derived from culturing in laboratory for a mouth. After that rice plants were washed with tap water several time and then placed in the pot containing with 2 liter of dechlorinated tap water. Crushed-mature cercariae were placed into rice plant pot (Fig. 3.10) for cercarial encystment to metacercariae. Rice plants were investigated for the presence of metacercariae by using a stereo microscope.

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Figure 3.10 Rice plant pot for used to encystmentation of metacercaria

3.7.5 Morphological study of larval stages

The obtained larval stages were compressed and fixed in 5% formalin and stained with Delafield's haematoxylin or borax carmine, dehydrated in an alcohol series, cleared in xylol, and mounted in permount. The permanent slides of larval stages were measured, drawn, and photographed for the morphological investigation**.**

3.7.6 Incidences and worm recovery rate in experimental hosts

One mouth old F1 generation of albino mice (*Mus musculus*) and dwarf hamsters (*Phodopus campbelli*) (Fig. 3.11) derived from breeding in the laboratory were used as the experimental hosts. Sixteen of both hosts were individually force fed with 30 metacercariae and then were sacrificed every 3 days post-infection (PI). The intestine and liver of both hosts were separately removed. The adult worms from intestine and liver were

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dissected, collected and counted under a stereo microscope for incidences and worm recovery rate determine. The incidence (%) was calculated according to the percent of number of the new hosts infected divided by total number of uninfected host were used.

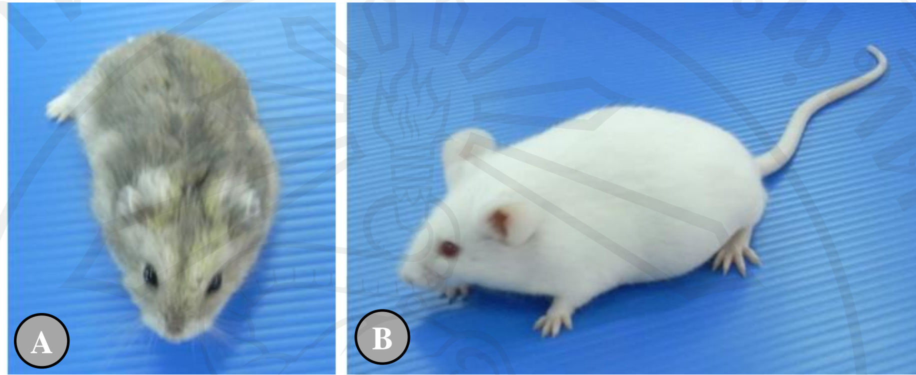

Figure 3.11 The experimental hosts; (A) *Phodopus campbelli* and (B) *Mus musculus* were used for worm recovery and maturity of adult worms study

3.7.7 Maturation of adult worms in experimental definitive host; *P. campbelli* and *Mus musculus*

The maturity of adult trematodes were investigated, adult flukes were compressed and fixed in 5% formalin, stained with borax camine, dehydrated in an alcohol series, cleared in xylol, and mounted in permount. Their size of body, oral sucker, ventral sucker, and development of genital pore, caeca, testes and ovary were also observed.

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3.8 Molecular biological study

3.8.1 Development of specific primer

1) Total genomic DNA extraction

Genomic DNA of all parasites were extracted and purified from adult worms, using the GF-1 Tissue extraction kit (Vivantis, Malaysia), according to the manufacturer's instructions. All extracted genomic DNA were diluted to a working concentration of 50 ng /μl and stored at -20 °C until used.

2) HAT–RAPD PCR

All extracted-genomic DNA was detected by HAT-RAPD PCR using arbitrary primers to compare DNA profiles**.** Sixteen commercially available arbitrary 10–mer primers (Operon technology, USA) were used individually for HAT– RAPD PCR. The nucleotide sequences of each primer were demonstrated in table 3.1. The reaction of HAT-RAPD PCR was carried out in a final volume of 20 μL, with common PCR composition. The reactions were performed in a Thermal cycler machine (LifePro Thermal Cycler, Bior Serves Life) and PCR conditions performed as follows: 1 cycle of 94 °C for 2 minutes, 35 cycles of 94 °C for 30 seconds, 48 °C for 45 seconds, 72 °C for 1 minutes and 1 cycle of final extension at 72 °C for 7 minutes. PCR products were separated on 1.4% TBE agarose gel electrophoresis, stained with ethidium bromide, and photographed with a Kodak Digital Camera Gel Logic 100.

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3) Fragment screening and sequencing of HAT–RAPD fragments

The *F. gigantica* specific fragment was obtained from HAT-RAPD profiles and purified from agarose gel, using the GF-1 Gel DNA Recovery Kit (Viviantis) according to the manufacturer's instructions. The purified DNA was subjected for sequencing directly by without cloned and transformed at 1st Base Company, Limited, Malaysia.

Table 3.1 Nucleotide sequences of each arbitrary primer were individually used for HAT- RAPD PCR.

Primers	Sequences 5´-3´
OPA-01	TGCCGAGCTG
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPN-02	ACCAGGGGCA
OPN-03	GGTACTCCCC
OPN-04	GACCGACCCA
OPN-05	ACTGAACGCC
OPN-06	GAGACGCACA
OPN-07	CAGCCCAGAG
OPN-08	ACCTCAGCTA
OPN-09	TGCCGGCTTG
OPN-10	ACAACTGGGG
OPP-11	AACGCGTCGG

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4) Designing and synthesizing of *F. gigantica*-specific primers

F.gigantica-specific primers (both forward and reverse) were designed, based on sequence data of serotype fragments, selected from HAT-RAPD profiles using primer-BLAST software (ncbi.nlm.nih.gov) and were also synthesized at BioDesign Company, Limited, Thailand Science Park, Pathumthani Province.

5) The specificity of *F. gigantica*-specific primers

Two pairs of designed primers were individually tested for specificity with all parasite DNA samples. The optimum PCR conditions for *F. gigantica*-specific detection were also determined by varying the total amount of $MgCl_2$ and optimizing the annealing temperature until each of the specific PCR products will be clearly generated and no any of cross-reaction with other tested parasites was observed.

6) Reaction sensibility of *F. gigantica*-specific primers

The sensibility of *F. gigantica*-specific primers was determined by varying the concentration of DNA template of adult stage, which it is serially diluted in 10 folds. The initial concentration used was 50 ng and serially diluted to 5 ng, 500 pg, 50 pg and 5 pg respectively.

7) Specificity test on experimental larval stages of *F. gigantica*

All experimental larval stages of *F. gigantica* such as miracidium, sporocyst, redia, cercaria, metacercaria, were individually extracted for genomic DNA and then all genomic DNA were tested with *F. gigantica*-specific primers.

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8) Specificity on field-collected samples of larval stages

The obtained cercariae on field-collected samples from Chiang Mai Province were extracted for genomic DNA. All extracted genomic DNA of each cercarial type were individually amplified with *F. gigantica*-specific primers. The specific band was determined from DNA profiles of each cercarial type.

3.8.2 Phylogenetic study

1) Amplification of internal transcribed spacer subunit 2 (ITS-2) region

Primers to amplify the entire nuclear second internal transcribed spacer region (ITS-2) were as described by Bowles et al., (1995). The pair of primers were 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′ (3S-F) as a forward primer, and 5′-TATGCTTAAATTCAGCGGGT-3′ (BD2-R) as a reverse primer. The final volume of each amplification reaction was 20 μL, with common PCR composition. The PCR was carried out in a My cycler^{TM} Thermal Cycler (Bio-Rad, USA) with PCR conditions are as follows; 1 cycle of 94 °C for 4 minutes, 30 cycle of 94 °C for 1 minutes, 50 °C for 30 seconds, 72 °C for 45 seconds and 1 cycle of final extension at 72 °C for 7 minutes. PCR products were separated on 1.4% agarose gel electrophoresis, stained with ethidium bromide, visualized on UV trans-illuminator and photographs digitally using Kodak Gel logic. The PCR products were purified and subjected for sequencing directly by without cloned and transformed.

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2) Data analysis

ITS-2 sequence data obtained in this study were compared with ITS-2 sequences of *F. gigantica* and related-species currently available in NCBI-GenBank. All ITS-2 sequence data were aligned and trimmed to provide an equivalent sequences among each trematode species using BioEdit 5.0.6 software. Phylogenetic tree of each trematodes were performed by maximum likelihood (ML) and UPGMA method using MEGA 5.05 software (Tamura *et al*., 2007) with bootstrap 1,000 replicates. The nucleotides diversity was calculated by DNAsp (Rozas *et al*., 2003) and confirmed the distinct species according to “4x rule” as following Birky *et al.* (2010).

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