# การพัฒนาเทคนิคเชิงโมเลกุลสำหรับการตรวจสอบการติดเชื้อของพยาธิใบไม้ในลำไส้ชนิด *Echinostoma revolutum* (Froelich, 1802) และสถานการณ์การระบาดในภาคกลางของประเทศไทย

# THE DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF INTESTINAL TREMATODE, *ECHINOSTOMA REVOLUTUM* (FROELICH, 1802) AND

SOTHORN ANUCHERNGCHAI

GRADUATE SCHOOL Srinakharinwirot University

2018

# การพัฒนาเทคนิคเชิงโมเลกุลสำหรับการตรวจสอบการติดเชื้อของพยาธิใบไม้ในลำไส้ชนิด *Echinostoma revolutum* (Froelich, 1802) และสถานการณ์การระบาดในภาคกลางของประเทศไทย

โสธร อนุเชิงชัย

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
วิทยาศาสตรมหาบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2561

# THE DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF INTESTINAL TREMATODE, *ECHINOSTOMA REVOLUTUM* (FROELICH, 1802) AND EPIDEMIOLOGICAL SITUATION IN CENTRAL THAILAND

SOTHORN ANUCHERNGCHAI

A Thesis Submitted in partial Fulfillment of Requirements

for MASTER OF SCIENCE (Biotechnology)

Faculty of Science Srinakharinwirot University

2018

THE THESIS TITLED

THE DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF INTESTINAL TREMATODE, *ECHINOSTOMA REVOLUTUM* (FROELICH, 1802) AND EPIDEMIOLOGICAL SITUATION IN CENTRAL THAILAND

BY

SOTHORN ANUCHERNGCHAI

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE IN BIOTECHNOLOGY AT SRINAKHARINWIROT UNIVERSITY

..... Dean of Graduate School

(Assoc. Prof. Dr. Chatchai Ekpanyaskul, MD.)

.....

ORAL DEFENSE COMMITTEE

..... Major-advisor

(Asst. Prof. Dr.Thapana Chontananarth)

..... Co-advisor

(Dr.Thanawan Tejangrura)

..... Chair

(Dr.Kittisak Buddhachat)

..... Committee

(Assoc. Prof. Dr.Parin Chaivisuthangkura)

..... Committee

(Assoc. Prof. Dr.Siwaporn Longyant)

| | |
|---|---|
| Title | THE DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF INTESTINAL TREMATODE, *ECHINOSTOMA REVOLUTUM* (FROELICH, 1802) AND EPIDEMIOLOGICAL SITUATION IN CENTRAL THAILAND |
| Author | SOTHORN ANUCHERNGCHAI |
| Degree | MASTER OF SCIENCE |
| Academic Year | 2018 |
| Thesis Advisor | Assistant Professor Dr. Thapana Chontananarth |

*Echinostoma revolutum* is a zoonosis-significant intestinal trematode. It is usually found in Southeast Asia (SEA), including Thailand. The results revealed that the overall prevalence of this trematode infection in intermediate hosts was 2.90%. In term of molecular studies, *Cytochrome B* (*CYTB*) was the most suitable gene to amplify DNA templates from eggs, cercarial stage, metacercarial stage and adult of *E. revolutum* with no cross-amplification to the tissue of other related trematodes and hosts. The specific fragment of the PCR product was 235 base pairs. The limit of detection of the primers was 0.85 picogram of *E. revolutum* DNA. The epidemiological study reveled the snail genera *Filopaludina*, *Lymnaea a*nd *Indoplanorbis* were intermediate hosts. In conclusion, the results of this study can be useful for a diagnosis or for epidemiological studies in intermediate or definitive hosts and effectively estimate the risk of *E. revolutum* infections.

Keyword : cytochrome B, species-specific primer, epidemic map, intermediate hosts, trematodes

# ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor and co-advisor, Asst. Prof. Thapana Chontananarth and Dr. Thanawan Tejangkura of the Faculty of Science at Srinakharinwirot University for the continuous support of my master's degree study and my research and for their patience, enthusiasm, and knowledge. Their guidance helped me in all the time, whenever I ran into a trouble spot or had a question about my research or writing.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Kittisak Buddhachat, Assoc. Prof. Dr. Parin Chaivisuthangkura and Assoc. Prof. Dr. Siwaporn Longyant. for their insightful comments and encouragement which incented me to widen my research from various perspectives.

I would like to offer my special thanks to Dr. Akapon Vaniksampanna for the motivation and support of laboratory knowledge. Without his passionate participation, this research could not have been successfully conducted.

I would also like to thank all member of the Applied Parasitology Research Laboratory who assisted me when ran into a trouble on the snail collection trips throughout 15 provinces of Thailand.

Finally, I must express my very profound gratitude to my parents and Janjura Parawat for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

SOTHORN ANUCHERNGCHAI

# TABLE OF CONTENTS

# LIST OF TABLES

Page

# LIST OF FIGURES

Page

# CHAPTER 1
# INTRODUCTION

## Background

The intestinal trematodes of genus *Echinostoma* belonging to family Echinostomatidae are significant zoonotic parasites. They can infect many kinds of birds and mammals[1, 2]. It causes echinostomiasis in humans via ingestion of raw or undercooked foods containing the infective stage called metacercaria. The common symptoms of echinostomiasis are severe abdominal pain, diarrhea, malnutrition, anorexia and fatigue[1, 3]. In some cases, the heavy infection of *Echinostoma* may lead to death due to anemia or intestinal perforation[1]. *Echinostoma* infections have been reported in Australia, America, Europe and Asia[1, 4, 5], particularly in Southeast Asia (SEA) including Cambodia, Laos-PDR, and Thailand[1, 3, 4, 6-10]. Therefore, echinostomiasis could be considered as a public health problem throughout the world.

In Thailand, the *Echinostoma* infections have been reported in both intermediate and definitive hosts[6, 7, 11]. Natural water resources and man-made irrigation systems are suitable ecological conditions for the distribution of various freshwater snails[12]. These snails have been recognized as the main intermediate hosts of *Echinostoma* species, especially, *E. revolutum*[2, 13]. In general, the morphological characterization has been used as classical method to identify trematode species. One of the unique characteristics of *Echinostoma* genus is the clearly visible collar spines at the anterior part of body[14]. *E. revolutum* possesses the large numbers of morphological appearances similar to other members of the 37 collar-spines complex (the revolutum group[15-17]). Therefore, it is difficult to precisely identify them, using only morphological data[11]. These problems can lead to misidentification and affected the accuracy of prevalence value.

To overcome the systematic problems, the use of molecular data has been dramatically increased for the taxonomic and systematics of *Echinostoma* species, especially *E. revolutum*. Several researchers have continued studying the molecular

data of *E. revolutum* for a decade. Most of them focused on studying two important mitochondrial genes; *cytochrome c oxidase* subunit 1 (*CO*1)[16, 18] and *NADH dehydrogenase* subunit 1 (*ND*1)[5, 16, 18] based on polymerase chain reaction (PCR) method. This method is more accurate and rapid than classical method. It has been widely used to identify trematode species, such as *Clonorchis sinensis*, *Metagonimus yokogawai*[19], *Haplorchis taichui*, *Opisthorchis viverrini*[20], *Centrocestus caninus* and *Stellantchasmus falcatus*[21]. However, the sequence data of *E. revolutum* and other cryptic species was unclear. Not all species in the revolutum group were clustered into the same clade[16, 22-25]. Because the problem associated with these genes, there have been no report on the molecular identification tools or species-specific primers for *E. revolutum* detection in their intermediate hosts.

In this study, three candidate genes, which contain species-specific information, were validated for their potential as the targeted gene for species identification. Two nuclear genes (*cathepsin B*: *CTSB* and *tyrosinase*: *TYR*) and one mitochondrial gene (*cytochrome B*: *CYTB*) have been selected. The suitable gene was optimized for sensitivity and specificity to discriminate *E. revolutum* from other related species. All *E. revolutum* specimens were collected from Chao-Phraya Basin, which are an importance agricultural area in the Central Thailand.

The aims of this study were divided into three parts. Firstly, to validate the novel species-specific target genes of *E. revolutum.* Secondly, the prevalences of *E. revolutum* in the study area was investigated. Thirdly, the accurate epidemiological map of *E. revolutum* was constructed. Consequently, the novel molecular data and species-specific primers obtained from this study can be used as epidemic control programs of *E. revolutum.*

# CHAPTER 2
# LITTERATURE AND REVIEWS

## Life cycle of *Echinostoma revolutum* (Froelich, 1802)

*Echinostoma revolutum* is the significant intestinal trematode[1, 2, 7, 9, 15]. The life cycle is complex and requires one or more intermediate hosts[26]. First, the fertilized eggs are passed in feces of definitive hosts[27]. The fertilized eggs developed to the embryonated eggs and miracidial stage in freshwater resources within eleven days[27]. After hatching, miracidium swim in respond to the complex macromolecule of snail mucus[28]. Then, they penetrate the snails' skin and develop into the intramolluskan stages (sporocyst, redia and cercaria)[28]. The freshwater snails belonging to family Lymnaeidae, especially *Lymnaea stagnalis* is known as an importance first intermediate host[27].

The cercarial stage might escapes from snail within 30 days after miracidium infected, or it might encysts to metacercarial stage in the same snail individual[27]. Several freshwater snails serve as the second intermediate hosts, such as *Bithynia*, *Filopaludina*, *Lymnaea* and *Physa*[7, 15, 27]. When cercarial stage penetrated to hosts, it encysts at the pericardial sac (Figure 1)[7]. The metacercaria (infective stage) can survive in snails up to 18 months after infection. After definitive hosts ingest the undercooked snails contained with a metacercaria and excyst in duodenum. Then, metacercariae develop to adult stage within 12 days in the intestine (Figure 2).

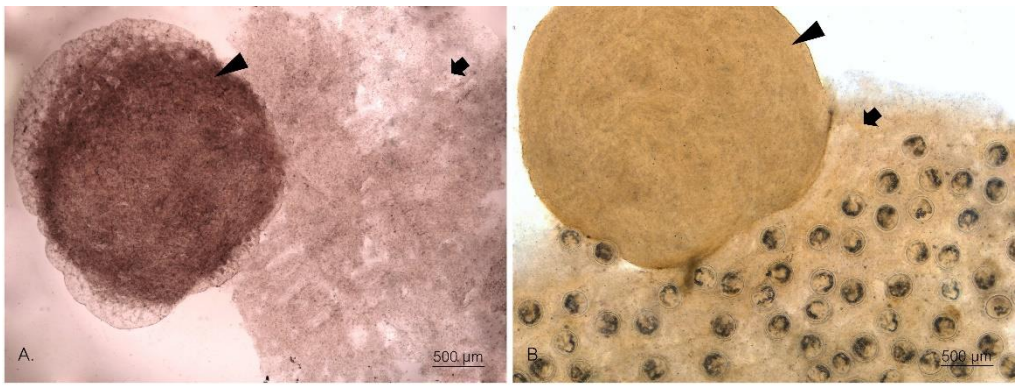



FIGURE 1 The morphological appearance of the pericardial sac of *Filopaludina* snails; A: The pericardial sac of uninfected snail. B: The pericardial sac of *E. revolutum* infected snail. The pericardial sac (solid arrow), the heart of snails (arrowhead)

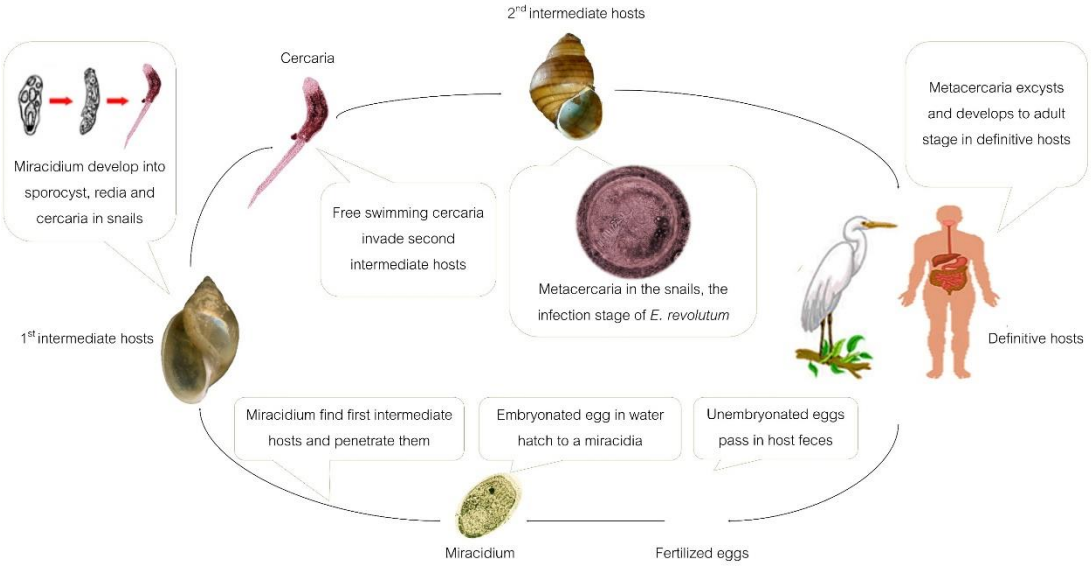



FIGURE 2 The life cycle of *E. revolutum*

Source: Modified from Centers for Disease Control (CDC). (2016 May 3). Laboratory identification of parasites of public health concerns (DPDx): Echinostomiasis (*Echinostoma* spp.). Retrieved Feb 21, 2017, from: URL: https://www.cdc.gov /dpdx/echinostomiasis

## Characterization of *Echinostoma revolutum*

### Egg stage

The yellowish egg has an operculum at one end and a wrinkly knob at the abopercular end. It is oval shape, approximately 88 – 118 × 61 – 75 µm in size. The eggs usually appear in definitive hosts feces after 10 – 13 days of infection[27]. The eggs can be fully-developed to miracidial stage and hatch within nine days, but they usually hatched after 10 – 12 days[27] (Figure 3).

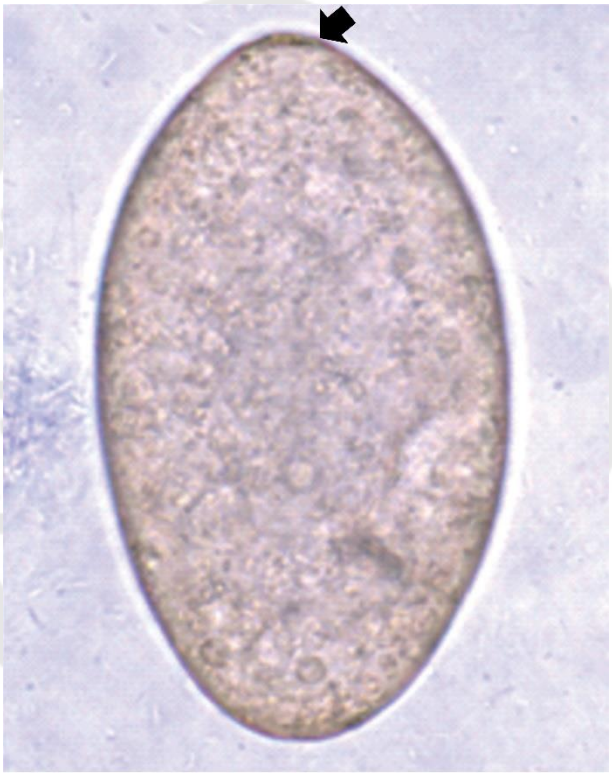


FIGURE 3 Egg of *E. revolutum* in the feces of an infected hamster and operculum of the egg (solid arrow)

Source: Modified from Chai; et al. (2011). *Echinostoma revolutum*: metacercariae in *Filopaludina* snails from Nam Dinh province, Vietnam, and adults from experimental hamsters. The Korean Journal of Parasitology. 49(4): 449-455.

**Miracidial stage**

The miracidial stage size is approximately 65 – 90 × 46 – 70 μm, broad anterior and become gradually narrower to posterior blunt end. The tegumental surface covers with numerous cilia, arranged in four rows on epidermal plates. The miracidial stage have two pairs of dark-brown eyespots. The flame cells situate at the left postero-ventral and right antero-dorsal. They connect to dorso-laterally and ventro-laterally excretory ducts, which open between third and fourth rows of epidermal plates[27].

**Intramolluskan stages**

The intramolluscan stages consist of sporocyst, redia and cercaria. The sac-like sporocyst developed from miracidial stage within ventricular cavity of snail cardiac. It develops from 162 × 85 μm to 790 × 416 μm within 10 days[27]. Birth pore is invisible.

Rediae are an elongated, approximately 517 – 2,910 × 94 – 370 μm in size[27]. Rediae are emerged from the cleft opening near the base of the sporocyst[27]. An anterior mouth, pharynx and sac-like gut are clearly visible. This stage contains numerous cercariae that can infects the second intermediate hosts. Redia is more active than sporocyst. It usually presents in cardiac cavity, but it might migrate to haemolymph and other visceral organ of snails[28].

Cercarial stage of *E. revolutum* has a collar head that surrounded with 37 collar spines, an arrangement 5-6-15-6-5 (5 corner spines, 6 lateral spines on each side and 15 spines in two-alternating row), the same as adult stage[14, 15]. The anterior part of body is covered with tiny spines. The size of body is 265 – 315 × 128 – 154 μm[15, 27]. The collar head is 130 – 150 μm in width[27]. It has the rows of dark excretory granules on both sides of body. The bifurcated caeca locate at the anterior of ventral sucker and terminated near the posterior end of body. The ventral sucker is 60 – 90 μm in diameter[27]. It locates at the posterior to mid-body. The long cylinder-shaped tail is 400 – 600 × 40 – 60 μm[29]. It has three ventral, two dorsal, and two ventro-lateral arranged on surface[27, 29]. The illustration of all intramolluscan stages are shown in Figure 4.

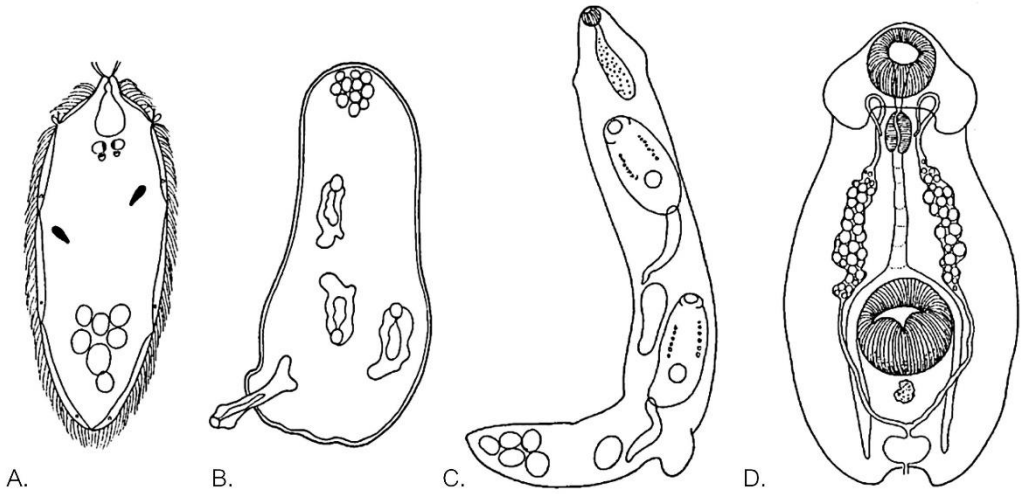



FIGURE 4 The illustration of intramolluskan stages of *E. revolutum*; A: Miracidial stage; B: Sporocyst contains rediae. C: Redia contains cercariae. D: Cercaria without tail.

Source: Modified from Kanev (1994). Lifecycle, delimitation and redescription of *E. revolutum* (Froelich, 1802) (Trematoda: Echinostomatidae). Systematic Parasitology 28(2): 125 - 144.

**Metacercarial stage**

The metacercarial cyst is spherical shape. The diameter is ranging from 170 – 220 μm[15]. The cyst wall consists of two-layers, thick outer wall and thin inner opaque wall. Large excretory granules are contained within 2 descending canals of the main excretory bladder[15]. Thirty-seven collar spines are clearly visible around their collar head and arranged in two alternating rows. Metacercariae are usually found in the pericardial sac of freshwater snails[7] (Figure 5).

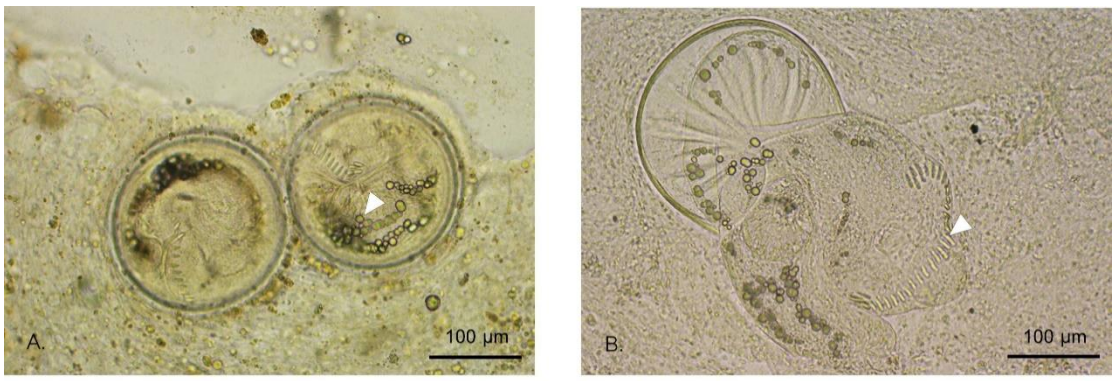



FIGURE 5 The metacercariae of *E. revolutum*; A: The living metacercariae in the pericardial sac of snail and the excretory granules (white arrowhead), B: The excysted metacercaria show 37 collar spines (white arrowhead) in their collar head.

**Adult stage**

The adult stage of *E. revolutum* displays twelve unique characteristics. The well-developed head crown is surrounded by distinct 37 collar spines. The pattern of the collar spines comprising five corner spines (3 oral and 2 aboral), six lateral spines in both sides and 15 other spines in two alternating rows. The body is elongated and flattened, ranging from 4.68 × 0.98 mm[30] in human and 5.0 – 7.2 mm × 0.8 – 1.3 mm in hamsters[15]. A huge ventral ridge is clearly visible near the ventral sucker (Figure 6A). The anterior part of tegumental surface arms with numerous tiny spines and become sparse toward posterior part of body[15].

The spherical oral sucker locates at subterminal of anterior part. The well-developed muscular pharynx is clearly visible, while prepharynx is very shot or absent. Intestine is bifurcated near its ventral sucker. It is elongated toward posterior of body and end near the terminal of body. The large acetabulum is spherical shape and locates at the two fourth of body (Figure 6B). The round ovary locates at the posterior to ventral sucker. The moderate loop of uterus appears among the ovary and first testis. Two testes are tandem, initiated at posterior of uterus (Figure 6A). The unarmed muscular cirrus is well-developed within cirrus sac, which is located at the upper of the ventral

sucker. The excretory pore appears at the ventro-terminal of the posterior part of body[15].


FIGURE 6 Photographs of *E. revolutum*; A: Adult stage recovering from experimental hamster. B: Scanning electron micrograph showing the elongated body with a distinct head collar, oral and ventral suckers.

**Current epidemiological reports of *Echinostoma revolutum***

*Echinostoma revolutum* has been a public health problem in many countries of Asia[1, 3, 4, 6, 26]. The first report in human was found in Taiwan[30]. A few cases of human infection were discovered in China, Indonesia, and Thailand until 1994[1]. The human case of *E. revolutum* infection occurred again in 2007[1]. The school children living near Tonle Sap Lake, Pursat province in Cambodia were infected with high prevalence of *E. revolutum*[1]. Although no report about human case since 2007, the outbreak of this trematode in their intermediate and definitive hosts have been reported in various countries of Europe and Asia. In France, the infection of larval stage of *E. revolutum* in *Galba truncatula* (Gastropoda, Lymnaeidae) was reported with a moderate prevalence[31]. In Korea, the first report of *E. revolutum* in the feral cats was discovered in 2005[32]. This trematode was reported as gastrointestinal helminths in *Banaraja* fowls in India[33].

In Thailand, the infection of *E. revolutum* have been reported in both intermediate and definitive hosts in various regions. At the northeastern, it infected in the domestic ducks of Khon Kaen, Nakhon Ratchasima, Roi Et and Maha Sarakham provinces with the prevalence of 10.30, 7.10, 61.80 and 75.00%, respectively[34]. At the northern, *E. revolutum* have been reported with high prevalence in many species of freshwater snails such as *Clea helena* (5.10%), *Eyriesia eyriesi* (12.50%), *Bithynia funiculata* (44.00%), *B. siamensis* (8.20%), *Filopaludina dorliaris* (58.70%), *F. martensi* (38.50%) and *F. sumatrensis polygramma* (41.00%)[7]. Table 1 shows the prevalence of *E. revolutum* in several areas of Thailand.

TABLE 1 The prevalence of *Echinostoma revolutum* in Thailand

| Provinces | Prevalences (%) | Hosts | Developmental stages |
|---|---|---|---|
| Sukhothai | 5.60 | Domestic ducks | Adult |
| Phitsanulok | 41.70 | Domestic ducks | Adult |
| Phichit | 16.70 | Domestic ducks | Adult |
| Nakhon Sawan | 50.00 | Domestic ducks | Adult |
| Lop Buri | 85.70 | Domestic ducks | Adult |
| Ayutthaya | 76.70 | Domestic ducks | Adult |
| Pathum Thani | 75.00 | Domestic ducks | Adult |
| Nakhon Ratchasima | 7.10 | Domestic ducks | Adult |
| Roi Et | 61.80 | Domestic ducks | Adult |
| Maha Sarakham | 75.00 | Domestic ducks | Adult |
| Khon Kaen | 10.30 | Domestic ducks | Adult |
| Chiang Mai | 5.10 | *C. helena* | Metacercaria |
| | 12.50 | *E. eyries* | Metacercaria |
| | 44.00 | *B. funiculate* | Metacercaria |
| | 8.20 | *B. siamensis* | Metacercaria |
| | 58.70 | *F. dorliaris* | Metacercaria |
| | 38.50 | *F. martensi* | Metacercaria |
| | 41.00 | *F. sumatrensis* | Metacercaria |
| Lamphun | 40.89 | *F. dorliaris* | Metacercaria |
| | 36.27 | *F. martensi* | Metacercaria |

**Identification of *E. revolutum***

The classical method to distinguish *E. revolutum* from other members in *Echinostoma* genus is the number of collar spines. In the past, more than 30 species have been described in the revolutum group. At present, only 12 species, consisted of *E. revolutum*[13, 15, 26]. *E. cinetorchis*[26], *E. echinatum*[15], *E. caproni*[13, 15], *E. deserticum*[35], *E. friedi*, *E. jurini*, *E. luisreyi*, *E. miyagawai*, *E. paraensei*, *E. parvocirrus* and *E. trivolvis*[13] are considered to be valid[13, 17, 26]. Although all members of this group have 37 collar spines, there are some different features in their morphologies, life cycle and geographical distributions to separate each species in this group. Their different features were described in Table 2.

TABLE 2 The different features of the 37 collar spines in genus *Echinostoma* belonging to the revolutum group

| Species | Morphological appearances and egg size (μm.) | Larval stages (size in μm.) | 1st intermediate host (genera) | 2nd intermediate host (animals) | Definitive hosts | Geographical distribution |
|---|---|---|---|---|---|---|
| *E. revolutum*<br>synonym:<br>*E. audyi*<br>*E. paraulum*<br>*E. ivaniosi* | Cirrus sac extending to middle of acetabulum, Testes slightly lobe<br>Eggs size: 88 – 125 × 61 – 75 | Cercaria (body): 265 – 315 × 128 – 154<br>Penetration gland pores: 4 pores, Paraoesophageal gland pores: 16 – 20<br>Metacercarial cyst: 170 – 220 (diam.) | - *Lymnaea* | - Gastropods<br>- Bivalves<br>- Fish<br>- Tadpoles<br>- Turtles | - Birds<br>- Mammals<br>- Human | - Europe<br>- Asia<br>- America |
| *E. cinetorchis* | Abnormal location or disappearance of testes<br>Eggs size: 99 – 116 x 65 – 76 | Cercaria (body): 163 × 109<br>Metacercarial cyst: 139 (diam.) | - *Hippeutis*<br>- *Segmentina* | - Tadpoles<br>- Fish<br>- Gastropods | - Human<br>- Rats<br>- Mouse<br>- Dogs | - Asia |
| *E. echinatum*<br>synonym:<br>*E. barbosai*<br>*E. robustum*<br>*E. lindoense* | Deeply lobed testes, Cirrus sac never extending to middle of acetabulum<br>Egg size: 92 – 124 × 65 – 76 | Cercaria (body): 300 – 460 × 140 – 180<br>Penetration gland pores: 6 pores<br>Paraoesophageal gland pores: 60 – 64 pores<br>Metacercarial cyst: 120 – 130 (diam.) | - *Anisus*<br>- *Biomphalaria* | - Gastropods | - Birds<br>- Mammals<br>- Human | - Europe<br>- Asia<br>- America |

TABLE 2 (continued)

| Species | Morphological appearances and egg size (μm.) | Larval stages (size in μm.) | 1st intermediate host (genera) | 2nd intermediate host (animals) | Definitive hosts | Geographical distribution |
|---|---|---|---|---|---|---|
| *E. parvocirrus* | Small cirrus<br>Eggs size: 101 – 115 × 55 – 70 | Cercaria (body): 300 – 410 × 120 - 150<br>Penetration gland pores: 6 pores<br>Metacercarial cyst: 130 – 150 (diam.) | - *Biomphalaria* | - Gastropods | - Birds | - West Indies |
| *E. caproni*<br>synonym:<br>*E. liei*<br>*E. togoensis* | Two testes confluent or adjacent, Body widest at just posterior to acetabulum and attenuated to posterior end<br>Egg size: 117 × 75 | Cercaria (body): 328 × 144<br>Metacercarial cyst: 150 (diam.) | - *Biomphalaria*<br>- *Bulinus* | - Gastropods<br>- Tadpoles | - Chicks<br>- Ducks<br>- Pigeons<br>- Hamsters<br>- Rats<br>- Mouse | - Africa |
| *E. deserticum* | Parthenogenesis and Presence of 0, 1, 2 testes<br>Egg size: 58 – 74 × 36 – 46 | Cercaria (body): 306 × 133<br>Metacercarial cyst: 146 (diam.) | - *Bulinus* | - Gastropods | - Birds<br>- Mammals | - Africa |
| *E. friedi* | Cirrus sac extending to anterior margin of acetabulum, Testes typically lobe<br>Egg size: 83 – 117 × 54 – 76 | Cercaria (body): 223 – 327 × 118 – 185<br>Penetration gland pores: 6 pores<br>Metacercarial cyst: 131 – 173 (diam.) | - *Lymnaea*<br>- *Gyraulus*<br>- *Biomphalaria*<br>- *Bulinus* | - Gastropods | - Birds<br>- Mouse | - Europe |

TABLE 2 (continued)

| Species | Morphological appearances and egg size (μm.) | Larval stages (size in μm.) | 1st intermediate host (genera) | 2nd intermediate host (animals) | Definitive hosts | Geographical distribution |
|---|---|---|---|---|---|---|
| *E. trivolvis* synonym: *E. rodriguesi* | Body slightly plump, cirrus sac extending to middle of acetabulum, testes smooth, oval, or slightly irregular shape<br>Egg size: 90 – 130 × 60 – 70 | Cercaria (body): 300 – 450 × 150 – 250<br>Penetration gland pores: 6 pores<br>Paraoesophageal gland pores: 4 – 6 pores<br>Metacercarial cyst: 135 – 170 (diam.) | - *Helisoma*<br>- *Biomphalaria* | - Gastropods<br>- Mussels<br>- Planarians<br>- Tadpoles<br>- Fish<br>- Turtles | - Pigeon<br>- Ducks<br>- Chicken<br>- Hamsters<br>- Mouse<br>- Rats | - America |
| *E. jurini* | Cirrus sac extending to middle of acetabulum, testes smooth or slightly irregular in shape<br>Egg size: 96 – 132 × 72 – 88 | Cercaria (body): 327 – 445 × 159 - 254<br>Metacercarial cyst: 140 – 160 (diam.) | - *Viviparus* | - Gastropods<br>- Mussels<br>- Frogs | - Mammals | - Europe<br>- Asia |
| *E. luisreyi* | Smaller oral lateral corner spines and larger aboral lateral corner spines, excretory pore located at the dorsal of body<br>Egg size: 89 – 113 × 65 – 82 | Cercaria (body): 417 × 181<br>Metacercarial cyst: 171 (diam.) | - *Physa* | - Gastropods | - Mammals | - America |

TABLE 2 (continued)

| Species | Morphological appearances and egg size (μm.) | Larval stages (size in μm.) | 1st intermediate host (genera) | 2nd intermediate host (animals) | Definitive hosts | Geographical distribution |
|---|---|---|---|---|---|---|
| *E. miyagawai* | Very elongate body, large head collar, small ventral sucker, testes indented and subglobular, long cirrus sac extending to middle portion of acetabulum<br>Egg size: 95 × 60 | Cercaria (body): 312 – 340 × 146 – 203<br>Penetration gland pores: 6 pores<br>Paraoesophageal gland pores: 42 – 46 pores<br>Metacercarial cyst: 144 – 154 (diam.) | - *Planorbis*<br>- *Anisus*<br>- *Lymnaea* | - Gastropods | - Birds<br>- Mammals | - Europe<br>- Asia |
| *E. paraensei* | 5 – 11 of dorsalmost collar spines smaller than others<br>Egg size: 104 – 122 × 74 – 86 | Cercaria (body): 228 – 275 × 117 – 136<br>Penetration gland pores: 6 – 8 pores<br>Metacercarial cyst: 132 – 148 (diam.) | - *Biomphalaria*<br>- *Physa* | - Gastropods | - Mammals | - America |

**Alternative detection methods**

Even though the revolutum group demonstrates some different features in their morphological characteristics, the identification of each species in this group is still difficult. It requires high taxonomical skill and consumes more times due to the similarity of adult stage. To avoid taxonomic confusion, the molecular method is an alternative way that can be used to identify trematode species based on their DNA sequences. For example, the evidence of three complex species belonging to the revolutum group, *E. revolutum*, *E. miyagawai* and *E. E. jurini* in Europe were recently validated based on novel molecular data[4].

PCR is a favored method for nucleic acid amplification. It is the most valuable tool for the DNA sequence studies. This method can amplify a few copies of DNA fragments to millions or more copies of a particular DNA sequence[36, 37]. PCR reaction employs a heat-stable DNA polymerase (*Taq* polymerase) and deoxynucleotide triphosphates (dNTPs), comprising dATP, dTTP, dCTP and dGTP, to amplify PCR product. This method has been applied and used in various procedures, which are PCR-sequence specific primers, reverse transcription PCR (RT-PCR), nested PCR, multiplex PCR and real-time PCR. Numerous reports use PCR for species identification of trematodes, such as *E. caproni*[38], *E. malayanum*, *Paragonimus heterotremus*[39], *Fasciola gigantica*[40], *Opisthorchis viverrini*, *Clonorchis sinensis*, *Haplorchis taichui*, *H. pumilio* and *Stellantchasmus falcatus*[41].

**Previous molecular studies of *Echinostoma* species**

A variety of genetic markers based on DNA sequences have been developed and used effectively to identify the trematode in family Echinostomatidae, whereas a few studies reported the specific primers for diagnoses *Echinostoma* species. Recently, the fluorophore-labelled hybridization probes specific to *28S* ribosomal DNA of *E. malayanum* was developed. This probe can discriminate *E. malayanum* from two closely related species; *E. revolutum* and *Hypoderaeum conoideum*, based on the differences of melting temperatures[39]. Most of molecular studies on *Echinostoma* species were focused on variable regions for cluster group of *Echinostoma* species by

their phylogenetic relationships, particularly two important genes; *NADH dehydrogenase* subunit 1 (*ND*1) and *cytochrome c oxidase* subunit 1 (*CO*1). In 1998, Morgan and Blair used these genes to classify *Echinostoma* species from Australia[18]. They designed the molecular markers of *ND*1 and *CO*1 based on the DNA sequences of giant liver trematode, *Fasciola hepatica*[18]. At present, many studies have been using the sequence data of *ND*1 and *CO*1 as the target regions for classification and identification of *Echinostoma* species belonging to the revolutum group comprising *E. trivolvis*, *E. robustum*, *E. caproni*, *E. paraensei*, *E. friedi*, *E. miyagawai* and *E. revolutum* [16, 22, 23]. However, phylogenetic trees of *Echinostoma* species, based on the *ND*1 and *CO*1 sequences, demonstrated conflicting pattern across several continents. In Europe, the isolates of *E. revolutum* formed two separating clades with high bootstrap support[25]. Moreover, *E. revolutum* (AY168933) and *E. friedi* (AY168937) were clustered to the same clade based on *ND*1 sequences[25]. The DNA sequences of *Echinostoma* species from North America (AY222132/AY222246), previously grouped into *E. revolutum*, are re-assigned into *E. trivolvis* clade[22]. Furthermore, *E. robustum* (U58102) and *E. friedi* (AY168937) were grouped within the same monophyletic clade based on *ND*1 sequence[22]. Georgieva and colleagues reconstructed phylogenetic tree based on *ND*1 sequence, clustered *E. revolutum* (AF025832) and *E. friedi* (AJ564379) to the same clade[24].

In Southeast Asia, the molecular classification based on *CO*1 sequences of *E. revolutum* (GU324943 and GU324944) and *Echinoparyphium recurvatum* (GU324945)[23] were conflicted with the report in 2015 that classified the sequences from accession number GU324943 and GU324944 as *E. miyagawai* and GU324945 as *E. revolutum*[16, 23]. From above reports, the molecular identification based on *ND*1 and *CO*1 sequences are still insufficient to distinguish *Echinostoma* at a species level. The taxonomic studies are necessary to combine with the molecular data for clearly identify species of them.

**Candidate genes and primer designs**

For species classification based on sequence alignment programs, the result is depended on identity and similarity scores between query and subject sequences. The mutation in DNA sequences might affected the primer design. Therefore, it is necessary to review the mutation rates of nuclear and mitochondrial DNA. The overall mitochondrial DNA mutation rate of *Drosophila melanogaster* is estimated 10 folds higher than the nuclear mutation rate[42]. Moreover, mitochondrial DNA can be inherited by maternal line to the next generation and affected to their haplotypes[43]. Consequently, the target of primers must be conserved at species level.

Two nuclear and one mitochondrial genes were selected and investigated the potential as the markers for *E. revolutum* identification. For nuclear DNA, it is necessary to know the copy number of genes. The multicopy gene will give rise to the difference sequences of PCR product and affected to alignment. Therefore, single-copy nuclear genes were selected in this study[44, 45].

The length of *tyrosinase* mRNA is approximate 1,500 base pairs. It is ubiquitously distributed in almost all taxa ranging from bacteria to mammals[46]. This gene encodes tyrosinase enzyme, which involved in various functions, comprising melanization processes, primary immune responses, and protein cross-linking in eggshell sclerotization[46-48]. In the previous reports, this gene has been studies in various trematodes species. Cai and colleagues[46] studied *TYR* gene of *S. japonicum (SjTYR*), and revealed the conserved regions within the critical regions for TYR enzyme activity. These regions can be used as the target of primer design[46]. For other trematodes, the sequences of *TYR* in *Clonorchis sinensis* was studied by using degenerated primers[49]. The results showed four *TYR* sequences comprising of CsTYR1 (KC195913), CsTYR2 (KC195914) CsTYR3 (KC195915) and CsTYR4 (KC195916). These gene shared conserved amino acid residues, Cu A and Cu B (Figure 7) which are at the same positions as in *Schistosoma* species[49]. Therefore, the *TYR* gene was selected as a candidate nuclear gene due to their important function and availability of the conserved regions

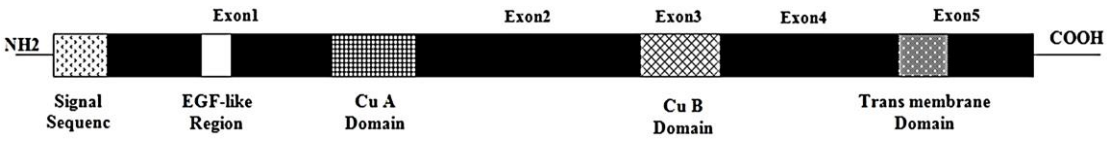



FIGURE 7 The gene mapping of *TYR* shown two conserved domains namely; Cu A domain and Cu B domain that can used as the target for DNA amplification.

Source: Kalahroudi; et al. 2014. Two novel *tyrosinase* (*TYR*) gene mutations with pathogenic impact on oculocutaneous albinism type 1 (OCA1). PloS one. 9(9): e106656

The second nuclear DNA gene is *cathepsin B* gene (*CTSB*) (approximate length 1,023 base pairs). This gene is encoded cathepsin B peptidase of parasites, which plays the important role for penetrating hosts[50]. The protein product of this gene was investigated in various species of parasites such as *Ancylostoma caninum*[51], *Schistosoma mansoni*[52], *P. westermani*[53], *C. sinensis*[54], *O. viverrini*[55], and *S. haematobium*[56]. However, the molecular data of some species are still insufficient, including *Echinostoma* species.

In metacercarial stage, CTSB peptidase is essential for excystment in the intestine of host and newly excyst juvenile uses this protein for penetrate and migrate from site to site[50]. In 2004, Meemon and colleagues studied tissue-specific expression of *CTSB* from *F. gigantica*. They used primers, which are designed from conserved regions, and successfully amplify this gene by PCR method[57]. The role of *CTSB* activity in the biology of trematodes was tested by RNA interference (RNAi) method. The result found that larval penetration to rat gut *in vitro* was reduced; and death after 14 hours[58]. Thus, *CTSB* was used as one of candidate genes in this study to investigate sequence data of *E. revolutum* and it will be helpful to molecular studies of this species in the future.

An only mitochondrial gene in this study, *cytochrome B* gene (*CYTB*), is a protein encoding gene that contains the information of cytochrome B protein. This protein is a subunit of the respiratory chain proteins. The nucleotide sequence of the *CYTB* contains species-specific information and has been used to identify species in various organisms[59]. In 2000, Parson and colleagues revealed the species identification of numerous species of vertebrates, including mammals, birds, reptiles and amphibians[59]. This gene is also used as molecular marker for species identification of parasitic protozoa, *Leishmania* species. The result showed this gene can identify *Leishmania* species at the species level[60]. Various reports applied this gene as the species specific marker for *Plasmodium* species, such as *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*[61-63]. There are various reports used this gene as molecular marker for studies of cestodes, *Echinococcus felidis*. The nested PCR amplification using primers designed from the conserved regions of *Echinococcus granulosus* mtDNA (database Accession No. AF297617) was successfully separated various *Echinococcus* to each species clades[64]. Furthermore, this gene was also used as marker gene for classifying species of other cestodes such as *Paranoplocephala arctica*[65], *Echinococcus shiquicus*[66], and *Diphyllobothrium* spp[67]. Additionally, in 2015, this gene was used to discriminate cryptic species of hairworm (*Gordius* spp.) and separate them to correct clade[68].

Recently report in 2016 recommended *CYTB* as one of the best markers for the molecular studies of trematodes[69]. For *Echinostoma* species, the complete mitochondrial genome of some species has been studied. The primers for initially *CYTB* amplification in *E. revolutum* was designed from conserved regions of *E. paraensei* (KT008005), *E. hortense* (KR062182 and NC_028010) and *E. caproni* (AP017706).

According to these previously reports, all candidate genes (*TYR, CTSB* and *CYTB*) occur on various kind of parasites. They are expressed in all stages of trematodes and play important roles in their life cycle. Consequently, these genes were selected as the candidate genes for validating the suitable gene to use as a marker for molecular and epidemiological studies of *E. revolutum.* The result was used to construct

epidemiological map of *E. revolutum* from each province within the study area to estimate the risk area of *E. revolutum* in the central Thailand. The molecular marker and epidemiological data obtain from this study is benefit for the epidemiological and molecular studies of *E. revolutum* in the large scale.

# CHAPTER 3
# MATERIALS AND METHODS

## Source of parasitic materials

### Sampling regime

The freshwater snails were collected from the paddy fields, irrigation canals and marshes in all districts of 15 provinces of Central Thailand (Figure 8). The study areas are Ang Thong, Ayutthaya, Chainat, Lopburi, Nakhon Nayok, Nakhon Pathom, Nakhon Sawan, Nonthaburi, Pathum Thani, Samut Sakhon, Samut Songkhram, Sara Buri, Sing Buri, Suphanburi and Uthai thani (Figure 9), collected following stratified random sampling method[70]. This method is best used with a population divided into distinct subgroups[70]. Each subgroup was separated as each stratum and the snail specimens were collected randomly in each stratum area. Snail specimens were classified following the taxonomic key described in Brandt (1974)[71].


FIGURE 8 Examples of collecting site; A: The pond with freshwater snails attached on the leaf or grass that sank in the water. B – D: The ecological condition which found in the collecting site comprising, pond, irrigation canal and paddy field, respectively.

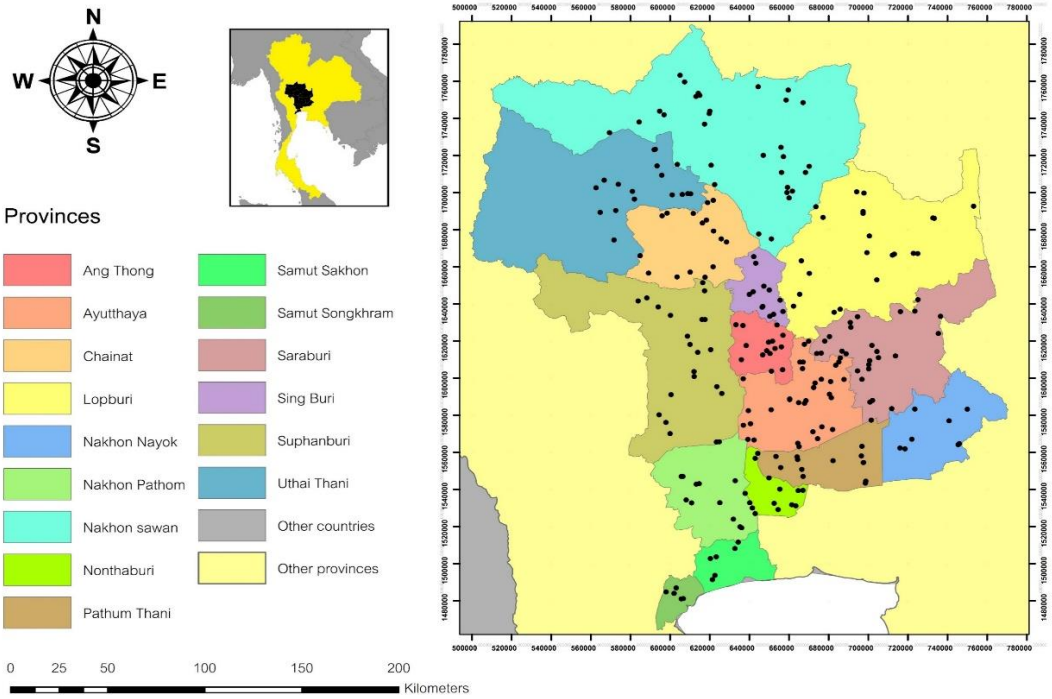



FIGURE 9 The study areas; 15 provinces of Central Thailand, the black dots indicated location of the sampling sites in this study.

**Collection of larval stage of echinostome**

The echinostome cercariae and the metacercariae were isolated from freshwater snails. The snail specimens were examined for the echinostome larval stage infection under the high magnificent stereomicroscope by crushing method[72]. After that, the echinostome cercaria were cleaned by distilled water and kept at -20°C. The metacercariae were cleaned and removed any debris or adhering mucus by normal saline solution (0.85% NaCl). The numbers of echinostome cercaria and metacercaria were recorded for each snail. Finally, living metacercariae were used for the experimental infection to obtain adult worms.

**Experimental infections and parasite recovery rate**

Sixty hamsters (*Phodopus roborovskii*) were infected with *E. revolutum* metacercariae which are collected from freshwater snails in Chao-Phraya basin, Thailand. The ratio of metacercaria and hamsters was fifty per one according to Chai 2011 protocol[15]. The metacercariae were infected directly into the stomach by force-feeding with a gavage tubes according to the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals[73]. All animals were euthanized by ether overdose at 1 to 20 days post infection (3 animals per days). The intestine of each hamster was gently removed and examined under the high magnificent stereomicroscope for investigating the presence of adult worms of *E. revolutum.*

**Fecal examination**

Fifty metacercariae of *E. revolutum* were forced-feeding to each experimental hamster. After that, 0.1 g of feces was collected after 1 day of infection and examined for the *E. revolutum* eggs by two fecal examination methods; fecal direct smear and sedimentation method with formalin-ether technique[74].

**Direct smear examination**

Fecal sample was placed on the microscope slide. Then, one drop of iodine (5 µl) was applied directly on the specimen and mixed thoroughly. Smears the feces and pushed gently around the cover glass until it flatten. The specimens were examined under light microscope.

**Formalin-ether technique**

Feces was grinded in 10 ml of formalin. Debris was removed by filtration with double layer of gauzes. Then, the solution was collected in 15 ml collection tube, centrifuged at 2,000×g, removed the supernatant and kept all sediments. Next, 10 ml of formalin was added to the tube, shaking vigorously, left it at the room temperature for 5 minutes and 3 ml of ether was added. The solution was shaked vigorously again for 1 minute and centrifuged at 2,000 ×g to separate the solution into 4 layers: ether (the top layer), debris, formalin, and sediment with eggs, respectively (Figure 10). Aqueous layers were discarded and sediment was inspected for the presence of eggs[74].

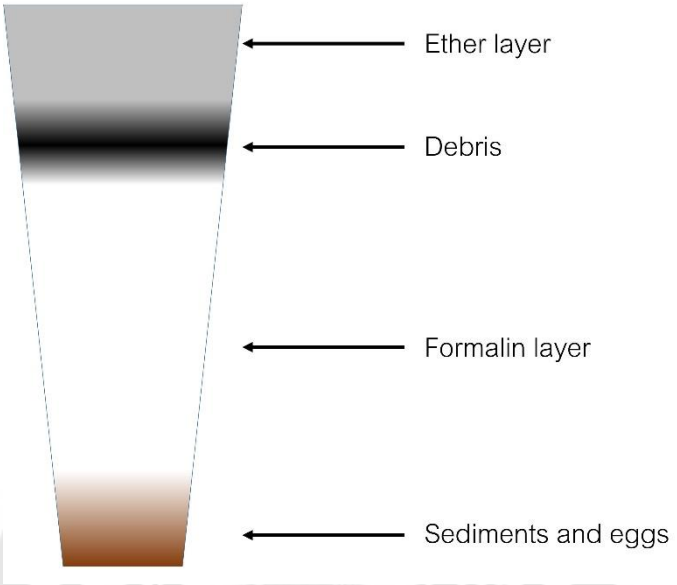



FIGURE 10 Four separated layers within the collection tube

**Morphological study of *Echinostoma revolutum***

**Permanent slide study under light microscope**

Any debris and adhering mucus remained within parasites were cleaned and removed by using Phosphate Buffered Saline (PBS). Each parasite was placed between two glass slides, wrap with nylon thread to flatten parasites and immerse in 4 % formalin for at least 10 days. After 10 days, the parasite specimens were removed from nylon thread. Then, they were transferred and kept in distilled water for 2 hours for removal of formalin in parasite tissues. After that, specimens were incubated with staining solution (hematoxylin) and left overnight. Followed by dehydration with ethanol series (10, 20, 30, 50 and 70%) and destained in acid alcohol (1% conc. HCl in 70% ethanol). Next, the specimens were washed with 70% ethanol to remove any acid. Last, the specimens were dehydrated in series of 80%, 95% and absolute ethanol, respectively. The specimens were placed in xylene for clarifying, mounted with mounting medium for the morphological identification under light microscope. The criteria for separate *E. revolutum* from other *Echinostoma* species are the body length, the head collar spines, number and arrangement of collar spines, the shape and location of testes and the present of cirrus.

**The tegumental surface of *E. revolutum***

The specimens were carefully cleaned using PBS to avoid damage to any spine of parasite specimens. Each specimen was pre-fixed in 2.5% glutaraldehyde in PBS until used. To study with SEM, the parasite specimens were washed with PBS for 15 minutes for 3 times or until all glutaraldehyde are completely removed. They were post-fixed with osmium tetroxide for 6 hours to increase an electron scattering rate. After metal coating, washed specimens with PBS followed by rehydration with ethanol series (10, 20, 30, 50, 70, 80, 95 and absolute), critical point drying (CPD) and coated with gold particle to complete process. The coated specimens were kept in dry cabinet for further studied under SEM.

**Molecular study of *Echinostoma revolutum***

**Genomic DNA extraction**

Genomic DNA was isolated from each trematode by grinding the whole worm and extracting the DNA by using DNeasy® Blood & Tissue Kit (Qiagen®). Each trematode was placed into 1.5 ml microcentrifuge tube containing 180 µl of buffer ATL and 20 µl of protease K, and grinded into small pieces. Then, the mixture was incubated at 56 ºC until completely lysed, vortex occasionally during incubation. Two hundred microliter of buffer AL were add to the lysate, mixed immediately by vortexing and incubated at 56 ºC for 10 minutes. Then, 200 µl of absolute ethanol was added into the mixture and mixed thoroughly by vortexing, followed by transferring each suspension to DNeasy® mini spin column. The suspension was centrifuged at 11,000 rpm for 1 minute and discarded the flow-through. The column was washed with 500 µl of AW1 buffer and centrifuged at 11,000 rpm for 1 minute, discarded the flow-through. The mixture was washed again with 500 µl AW2 buffer, centrifuged at 14,000 rpm for 3 minutes and discarded the flow-through. To elute genomic DNA from the spin column, 50 µl AE buffer was added and incubated at room temp for 1 minute before centrifuged at 11,000 rpm. Genomic DNA was kept at -20 ºC.

**Species confirmation based on molecular biological method**

The genomic DNA of adult worms were amplified using two set of primers comprising *ND*1 primers (JB11: 5'-AGATTCGTAAGGGGCCTAATA-3' and JB12: 5'-ACCACTAACTAATTCACTTTC-3') and *CO*1 primers (JB3: 5'-TTTTTTGGGCATCCT GAGGTTTAT-3' and JB13: 5'-TCATGAAAACACCTT AATACC-3')[5] specific to *ND*1 and *CO*1 genes, respectively. The PCR processes comprised of 4 min at 94ºC, follow by 35 cycles of 94ºC, 47ºC and 72ºC for 1 min (*ND*1) or 94ºC, 48ºC and 72ºC for 1 min (*CO*1), then, followed with final extension at 72ºC for 10 min. Distilled water was used as negative control in all PCR protocols. All PCR products were sequenced and aligned using Basic Local Alignment Search Tools (BLAST) with sequences from NCBI database and reconstructed the maximum-likelihood tree for clustering the species of adult worm to confirm their species together with the morphological characteristics. Moreover, they were verified by the species-specific primers of *E. revolutum* based on *18s* ribosomal

DNA gene that recently report in 2019 [75]. The *E. revolutum* was used in the next experiment.

**Gene candidates for developing the species-specific primers**

Two nuclear genes (*TYR* and *CTSB*) and one mitochondrial gene (*CYTB*) were selected as candidate genes in this study. For the nuclear genes, there is no report of *Echinostoma* sequences in the database. Therefore, the primers and PCR condition were used according to the previous reports of *TYR*[49] and *CTSB*[57]. Regarding to the *CYTB* gene, the primers were newly designed in this study following the guidelines for primer designed[76] based on the sequences of *Echinostoma* species and other related species in NCBI database.

The *TYR* degenerated primers were designed based on amino acid sequences of conserved region (CuA and CuB) of the tyrosinase protein[49]. The PCR reaction for *TYR* was initited by using the degenerated primers (Tyr-deg-F and Tyr-deg-R) with the PCR condition comprised of 4 min at 94ºC, follow by 12 cycles of 40 s at 94ºC, 50 s at 48ºC and 1.5 min at 72ºC. After that, the reaction continued with 25 cycles of 40 s at 94ºC, 50 s at 60ºC and 1.5 min at 72ºC. Finally, the final extension was performed at 72ºC for 10 min[49].

For the second gene, the *CTSB* primers were designed from conserved regions of giant liver trematode, *Fasciola gigantica*[57]. This species is belonging to the same order (Echinostomida) of *E. revolutum*. Thus, these primers can be used to amplify *E. revolutum*. The PCR amplification was consisted of 35 cycles at 94, 55, 72 ºC with 1 min each step[57].

The novel primers for *Echinostoma CYTB*; namely, EchCBF (5'-TGCTGATTCATGTTTGAGGTTTGG - 3') and EchCBR (5'- AAGGGTACTCTGGATGACA AGC -3') were designed and used for initially amplified this gene. These primers were designed based on completed mitochondrial DNA sequences of the relate species *E. revolutum* in NCBI database comprising, *E. paraensei*, two *Isthmiophora hortensis* (*E. hortense*), *E. caproni* and *Echinochasmus japonicus* (KT008005, KR062182, NC_028010, AP017706 and KP844722, respectively). The primers were manually

designed following the guidelines for primer designed[76] based on these conserved sequences and their properties were analysed using OligoAnalyzer 3.1 program (https://sg.idtd na.com/calc/analyzer). The expected product size was 837 base pairs. Five adult specimens were amplified using the gradient-PCR started with pre-denaturation at 94 °C for five minutes followed by a thirty-five cycle of DNA amplification reaction (thirty seconds for denaturation at 94°C, fifty seconds for primer annealing from 51 to 61.6 °C, and for one minute at 72°C for primer extension). Then, the final extension was performed at 72°C for seven minutes. The PCR reaction contained 1.6 mM of $MgCl_2$, 0.28 µM of each primer, 50 µM of each dNTP, 0.2 Unit of *Taq* polymerase (Vivantis), 1x of PCR buffer A (Vivantis) and 8.5 ng of genomic DNA. All synthetic oligonucleotide primers were purchased from Integrated DNA Technologies (IDT®). The primers details shown in Table 3 and the detail of *cathepsin B* and *cytochrome B* sequences shown in Figure 11-12.

TABLE 3 The detail of primers used in this study

| Primer names (Target genes) | Sequences of primers | Products (bp) | References |
|---|---|---|---|
| Tyr-deg-F (*TYR*) | 5'-YNDSWTTYSYWACNTGGCAYMG-3' | 1,493-1,661 | Bae; et al., 2013[49] |
| Tyr-deg-R (*TYR*) | 5'-YCMRYCCARTCCCARTADGG-3' | | |
| Forward (*CTSB*) | 5'-TGGCCACAATGCTGGACG-3' | 1,008-1,014 | Meemon; et al. 2004[57] |
| Reverse *(CTSB)* | 5'-TCAAAGACGTGGCATTCC-3' | | |
| EchCBF | 5'-TGCTGATTCATGTTTGAGGTTTGG-3' | 837 | Designed in this study |
| EchCBR | 5'-AAGGGTACTCTGGATGACAAGC-3' | | |

A.

```
>AY227674.1 Fasciola gigantica cathepsin B (cat-B2) mRNA, complete cds
TTCATTCATACAGCTGATGCTCGTCAAGATGAACTGGTTAATCGTGTTTGCCATTATCGCCGTTGTCCAA
GCAAAACCAAACCATAAACCGCAATTCGAGGCTTTCTCCGATGAACTGATTCGATTTGTGAACGAGGAAT
CTGGTGCCAGTTGGAAAGCTGCACGGTCCACTCGATTCAGCAATGTTGACCACTTTAAACTGCACTTGGG
AGCTCTGTCTGAAACACCAGAAGAAAGAAATGCCCTGCGACCAACCATAAAACATGACATATCGAAAAAC
GATCTGCCAGAGTCTTTTGATGCTCGTTCTCAATGGCCACAATGCTGGACGATTTCGGAGATTCGGGACC
AGGCCAGTTGTGGTTCCTGTTGGGCCACGGCGGCAGCCAGTGCAATGAGTGACCGTGTGTGTATTCATTC
TAACGGTCAAATGAGACCACGTTTGGCCGCAGCAGATCCACTCAGTTGTTGCACGTACTGTGGTCAGGGG
TGCAGAGGTGGCTATCCACCGAAGGCTTGGGATTATTGGATGCGCGAGGGTATCGTGACTGGTGGTACTT
GGGAAAATCGAACCGGTTGTCAGCCCTGGATGTTTACCAAGTGTGATCACGTTGGTGACTCTCGAAAATA
TAGTCGTTGTCCTCACTACACCTATCCGACACCTCCTTGCGCAAGAGCTTGCCAAACTGGATATAATAAA
ACGTACGAGCAAGATAAGTTTTACGGAAATTCATCTTACAACGTGGGGGAACACGAGTCTTATATCATGC
AAGAGATCATGAAAAATGGTCCAGTTGAAGTGACTTTCGCCATATTCCAGGACTTCGGTGTGTACAGAAG
TGGAATTTACCATCACGTGGCCGGTAAATTCATCGGTAGACATGCCGTCCGTATGATCGGTTGGGGTGTT
GAGAATGGCGTGAATTACTGGTTGATGGCCAATTCGTGGAATGAGGAATGGGGTGAGAATGGATACTTCC
GAATGGTAAGAGGAAGAAACGAATGCGGCATCGAATCAGAAGTTGTCGCCGGAATGCCACGTCTTTGAGC
AACACGGAAATAACCTTAAAAACTCCCTCTTTGAATTGCTGTTATACAATTTGCAATACATTTCTTGCTA
ACTTACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

B.

```
  1 mnwlivfaii avvqakpnhk pqfeafsdel irfvneesga swkaarstrf snvdhfklhl
 61 galsetpeer nalrptikhd iskndlpesf darsqwpqcw tiseirdqas cgsgwataaa
121 samsdrvcih sngqmrprla aadplsccty cgqgcrggyp pkawdywmre givtggtwen
181 rtgcqpwmft kcdhvgdsrk ysrcphytyp tppcaracqt gynktyeqdk fygnssynvg
241 ehesyimqei mkngpvevtf aifqdfgvyr sgiyhhvagk figrtavrmi gwgvengvny
301 wlmanswnee wgengyfrmv rgrnecgies evvagmprl
```

FIGURE 11 Amino acid and nucleotide sequences data of *CTSB.* A: The sequence data of *CTSB* designed from *F. gigantica* (AY227675); Green and yellow indicated the position of forward and reverse primers, respectively. The primers were designed within start codon (double underline) and stop codon (underline). B: The brown labeled are amino acid site in the active sites of the cathepsin B protein.

A.

>KR062182.1:718-1791 Echinostoma hortense mitochondrion, complete genome
ATGATTTTTCTTATACGTAGTAAAGTGGTTAATTTGCCTACAAACGTTTCTTTAAATTATTTGTGGTGTG
GGGGGTTCATGATAAGCTTTTTCTTGATTCTTCAAGTTGTTTCTGGCGTAATTCTCTCTCTTTTGTATGT
TGCTGATTCATGTTTGAGGTTTGGGTGTGTGTTAAGCTTCACTTCTGAAGGATTATTTTTGTGGTTGTTT
CGCTATTCTCATATATGGGGTGTAACTTTTATTTTTATTCTTTTTTTTGTACTTATGGGTCGTGCTCTCT
ATTATTCAAGGTATAGCATGTTTGGGGTTTGAAAAGTGGGGTTTGTTCTGTATTTACTGATGATGATTGA
GGCTTTTTTAAGTTATATCTTACCGTGGCATCAGATGTCGTATTGAGCGGCAACGGTTTTGACGTCTGTG
CTAAAAAGGATACCTATAGTTGGTAGTGTTCTTTATAAGTTTGTGGTAGGTGGGTTCTCTGTGACTAATG
TAACATTAGTCCGGGTTTTTTCAGCTCACGTTTGTTTGGCTTTTGTAATATTAGGTTTTAGGGTGATTCA
CTTGTTTTATCTTCATAAGGGGGGTTCTAATAATCCTCTATTTGTTTTTGGAGGGTATGGGGATGTAATT
TTGTTTCATTCTTACTATACAACTAAGGATGGGTTTGTATTAATGTGTTGGGGTGCTTTACTGTTGGTTT
GTCCAGACTTAGTGTTGGATGTTGAAAGCTATATAGAAGCAGACCCGTTAGTTAGTCCTGTTTCTATAAA
GTCTGAGTGATATTTTTTTAGCCTTTTATGCTATGCTTCGTTCTATAGAGTCAAAGGTTGGAGGGTTAAT
TCTGGTTTTAGTTTTTTTATTTGTTTTATGGTTGCCCACCTTAAAAGAATCTTGCGGCTATGATTTAGGT
CGTCAGTATGTTTTTTGATGAAGGGTTTCAATTTTTGGTTTATTGAGGTACTTGGGGGCTTGTCATCCAG
AGTACCCTTATGTAATGATAAGAAAGATCGCAAGTCTTTCGGTAATTGTACTATTGTTTATGTTAAGGGG
ATTATGGGTAATTCCTTATTCTAG

B.

```
  1 miflirsnvv nlptnvslny lwcggfmisf flilqvvspv ilsllyvads clsfgcylsf
 61 tseglflwlf pyshlwgytf ifilffvlmg ralyyssysm fgvwnvgfvl yllmmieafl
121 syilpwhqms ywaatvltsv lnsipivgsv lykfvvggfs vtnvtlvrvf sahvclgfvi
181 lgfsvihlfy lhkggsnnpl fvfggygdvi lfhsyyttkd gfvlmcwgal llvcpdlvld
241 vesyieadpl vspvsiksew yffsllcyas fysvngwsvn sgfsfficfm vahlnsilrl
301 wfsssvcflm kgfnfwfiev lgglsssvpl cndkndrksf gnctivyvkg imgnslf
```

FIGURE 12 Amino acid and nucleotide sequence data of *CYTB* of *Isthmiophora hortensis*. A: The primers of *CYTB* for *E. revolutum* were designed from *Isthmiophora hortensis* (*E. hortense*: KR062182). All primers located within conserved regions of *CYTB*. Green and yellow indicated forward and reverse primers, respectively. The primers located within start codon (double underline) and stop codon (underline). B: The brown labeled are amino acid site at the binding chain of the cytochrome B protein.

### Purification and analysis of PCR products using agarose gel electrophoresis

All amplicons were characterized on 1% agarose gel. The appropriated band was cut from agarose gel and purified using NucleoSpin® (Macherey-Nagel) according to the manufactured protocol. After purification, amplicons were verified on 1% agarose gel. The purified products were sent to sequencing service (1st BASE DNA Sequencing Services: Axil Scientific Pte Ltd, Singapore). Then, all sequencing data was aligned using BLAST program to check the correction of target genes. Criteria to select the best gene was following, 1) it must has ability to reproducible and can amplified both larval and adult stage of *E. revolutum*, 2) the sequences must have adequate nucleotide length for design and creating the species-specific primers, 3) it must show highest sensitive that can ampified low concentration of DNA. After selected the gene, the sequences of selected gene were used in the next experiment.

## Primer design for specific PCR amplification

### Primer design and optimization

The species-specific primers for *E. revolutum* were manually designed following the guidelines for primer designed[76] and checked the primer properties with OligoAnalyzer 3.1 program (https://sg.idtdna.com/calc/analyzer). These primers were designed based on specie-specific region obtained from previous experiment. The annealing temperature and time were optimized for each primer set. To optimize annealing temperature of PCR assay, the procedure was performed with gradients PCR and verified with 1% agarose gel electrophoresis. The result of ideal condition was a single band without any smear or non-specific products. The best condition obtained from this part was used in specificity and sensitivity tests of primers.

### Specificity and sensitivity test of primers

The species-specific primer set was test for specificity against hosts of *E. revolutum* and other related species of intestinal trematodes commonly found in snails (*E. cinetorchis, E. ilocanum*, *Isthmiophora hortensis* (*E. hortense*), *Echinochasmus japonicus*, *T. anastomusa*, *H. taichui* and *S. falcatus*). Moreover, the

intermediate hosts, (*L. auricularia, F. sumatrensis polygramma, F. martensi, I. exustus, B. siamensis, C. helena*), and definitive hosts *Phodopus roborovskii* (hamsters), *Gallus gallus* (domestic chicken) and *Anas platyrhynchos* (domestic duck) were also used to verify the specificity of primers.

The sensitivity test was divided into two parts. First, the primers were tested with the genomic DNA of *E. revolutum* and the mixed DNA (metacercaria of *E. revolutum*, *F. sumatrensis polygramma*). All specimens were extracted by using the same protocol as described eariler in this section. The initail concentration of DNA was 8.5 ng/µl. To investigate limit of detection of the primers, the PCR reactions were done with ten-fold serial dilutions of the template DNA. The desirable sets of primer should successfully amplify *E. revolutum* DNA at the lowest concentration with no cross-reaction with hosts or related species. The validated primer set was used for identifying the echinostome cercaria and metacercaria that were collected from the collecting sites. The result was compared with classical method (crushing and observed under microscope) to evaluate the diferrent of these methods.

**Phylogenetic relationship analysis**

To investigate the genetic variations within the obtained *E. revolutum* in the study area and reveal the relationship with other related species. The sequence data of adult *E. revolutum* were analyzed using the MEGA® software version 7 (MEGA®7). All nucleotide sequences were aligned using pairwise comparison and multiple alignments of nucleotide sequences by Clustal W 1.6 implemented in this program. The reference nucleotide sequences, which were imported from mitochondrial sequences in NCBI database, comprise of *E. caproni* (AP017706), *E. paraensei* (KT008005), two *Isthmiophora hortensis* (*Echinostoma hortense*) (KR0621821 and NC_0280101), and two *Echinochasmus japonicus* (KP844722 and NC_030518). The sequence of *Fasciola gigantica* (AP017707) was used as an outgroup. The maximum likelihood method (ML) implemented in MEGA®7 program was used to construct the phylogenetic tree using Kimura's two parameter model and the bootstrap method with 10,000 replica.

**The epidemiological mapping and calculation**

In the epidemiological study, two values were important for predicting the risk of parasite infections: prevalence and mean intensity. The prevalence is the percentage of infected hosts at the time of study. This value reflected the risk of infection in the population within the infected area. The mean intensity is the average number of metacercariae infection in each infected snail. Both prevalence and mean intensity were used for constructing the epidemiological mapping of *E. revolutum* infection in the infected area using GIS program. The formula for calculation was shown in Figure 13

$$\text{Prevalence (\%)} = \frac{\text{The number of infected snails (individuals)}}{\text{Total number of examined snails (individuals)}} \text{ X } 100$$

$$\text{Mean intensity} = \frac{\text{Summary of all metacercariae in all infected snails (individuals)}}{\text{Total number of infected snails (individuals)}} \text{ X } 100$$

FIGURE 13 The formula for calculating prevalence and mean intensity value

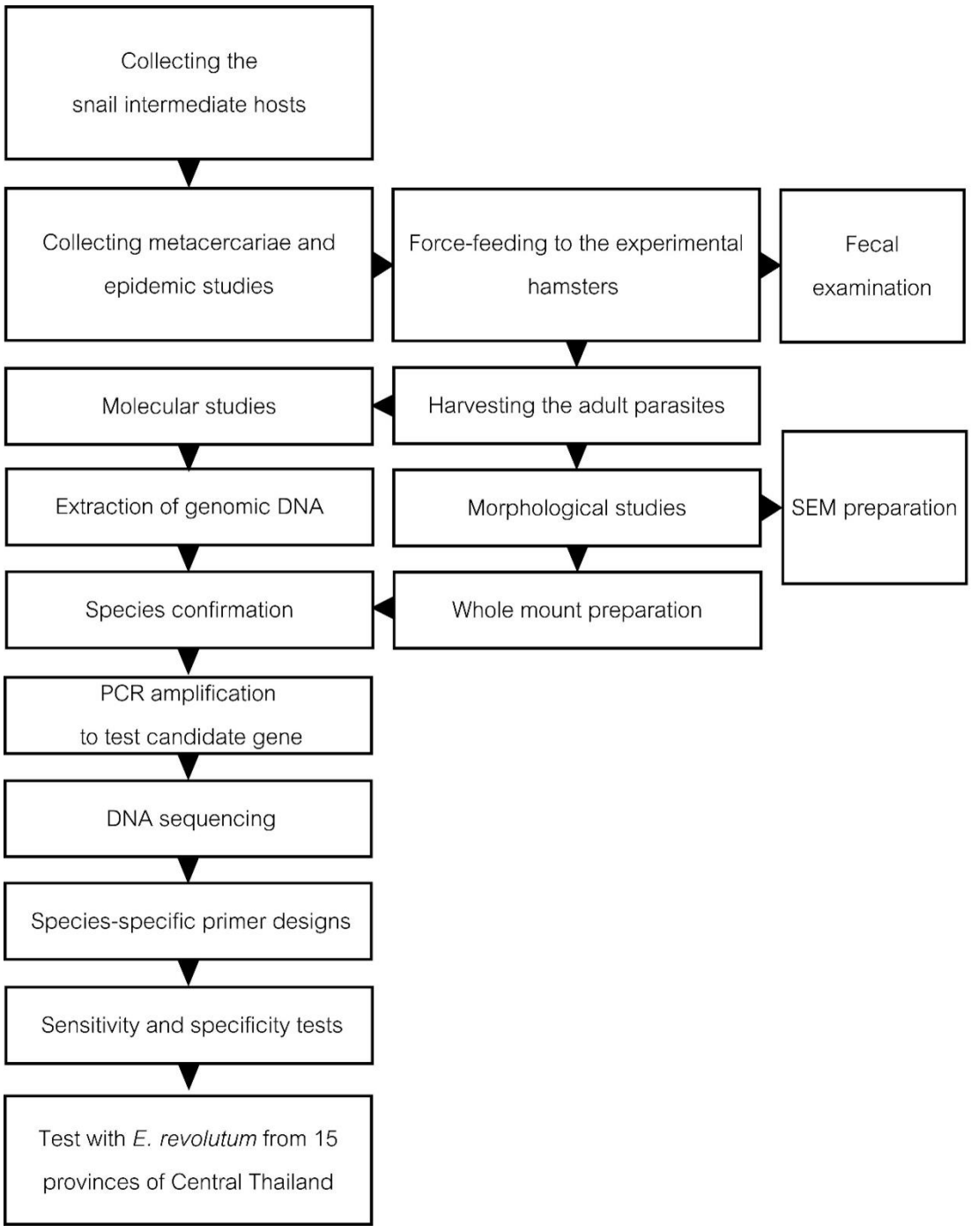



FIGURE 14 Summary of the methods used in this study

# CHAPTER FOUR
# RESULTS

**Collection of snail intermediate hosts and larvae of trematodes**

The total number of 20,302 snail specimens were collected from 254 collecting sites across 15 provinces of Central Thailand. They were classified into 12 species, comprising of 228 *Adamietta housei*, 5,910 *Bithynia siamensis*, 335 *Clea helena*, 1,430 *Filopaludina martensi*, 3,311 *F. sumatrensis polygramma*, 1,964 *Indoplanorbis exustus*, 3,568 *Lymnaea auricularia*, 1,605 *Melanoides tuberculata*, 256 *Pomacea canaliculata*, 384 *Segmentina* sp., 1,204 *Tarebia granifera* and 107 *Thiara scabra* (Table 4). Several species were found in each province. The highest number of snail species was found in Nakhon Sawan province with total numbers 12 snail species. The lowest number of snail species was found in Samut Songkhram province (2 species). The highest proportion of snail species found within the study area was *B. siamensis* (29.11%) followed by *L. auricularia* (17.57%), *F. sumatrensis polygramma* (16.31%), *I. exustus* (9.67%), *M. tuberculata* (7.91%), *F. martensi* (7.04%), *T. granifera* (5.93%), *Segmentina* sp.(1.89%), *C. helena* (1.65%), *P. canaliculata* (1.26%), *A. housei* (1.12%) and *T. scabra* (0.53%), respectively (Figure 15).

Echinostome cercaria were found only in six species of snails, *B. siamensis*, *I. exustus*, *L. auricularia*, *M. tuberculata*, *Segmentina* sp., and *T. granifera.* The metacercarial stage was found in four snail species, *B. siamensis*, *F. martensi, F. sumatrensis polygramma* and *I. exustus* (Figure 16).

TABLE 4 The number of snail species found in Central Thailand.

| Provinces | Number of snail specimens in each species | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AH | BS | CH | FM | FS | IE | LA | MT | PC | SE | TG | TS | Totals |
| Ang Thong | 0 | 500 | 53 | 50 | 72 | 50 | 252 | 59 | 0 | 0 | 0 | 0 | 1,036 |
| Ayutthaya | 80 | 1,118 | 56 | 165 | 172 | 266 | 457 | 217 | 0 | 0 | 250 | 50 | 2,831 |
| Chainat | 4 | 500 | 50 | 39 | 78 | 231 | 235 | 35 | 0 | 59 | 0 | 0 | 1,231 |
| Lopburi | 0 | 637 | 0 | 50 | 543 | 152 | 209 | 27 | 0 | 50 | 50 | 0 | 1,718 |
| Nakhon Sawan | 50 | 596 | 21 | 69 | 257 | 253 | 321 | 286 | 151 | 150 | 350 | 57 | 2,561 |
| Sing Buri | 0 | 300 | 0 | 85 | 214 | 189 | 102 | 100 | 0 | 75 | 0 | 0 | 1,065 |
| Suphanburi | 0 | 573 | 0 | 36 | 346 | 357 | 491 | 169 | 105 | 0 | 50 | 0 | 2,127 |
| Uthai Thani | 0 | 350 | 10 | 200 | 148 | 100 | 311 | 68 | 0 | 50 | 0 | 0 | 1,237 |
| Nakhon Pathom | 0 | 321 | 50 | 171 | 150 | 0 | 221 | 31 | 0 | 0 | 154 | 0 | 1,098 |
| Nonthaburi | 0 | 200 | 0 | 50 | 93 | 100 | 200 | 0 | 0 | 0 | 100 | 0 | 743 |
| Pathum Thani | 0 | 253 | 0 | 167 | 138 | 116 | 250 | 0 | 0 | 0 | 0 | 0 | 924 |
| Samut Songkhram | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 200 | 0 | 0 | 0 | 0 | 300 |
| Samut Sakhon | 0 | 0 | 0 | 3 | 100 | 50 | 50 | 100 | 0 | 0 | 0 | 0 | 303 |
| Nakhon Nayok | 0 | 50 | 50 | 171 | 200 | 50 | 50 | 100 | 0 | 0 | 0 | 0 | 671 |
| Saraburi | 94 | 412 | 45 | 174 | 800 | 50 | 419 | 213 | 0 | 0 | 250 | 0 | 2,457 |
| Totals | 228 | 5,910 | 335 | 1,430 | 3,311 | 1,964 | 3,568 | 1,605 | 256 | 384 | 1,204 | 107 | 20,302 |

Abbreviation represented the snail species, *A. housei* (AH), *B. siamensis* (BS), *C. helena* (CH), *F. martensi* (FM), *F. sumatrensis polygramma* (FS), *I. exustus* (IE), *L. auricularia* (LA), *M. tuberculata* (MT), *P. canaliculata* (PC), *Segmentina* sp. (SE), *T. granifera* (TG) and *T. scabra* (TS).

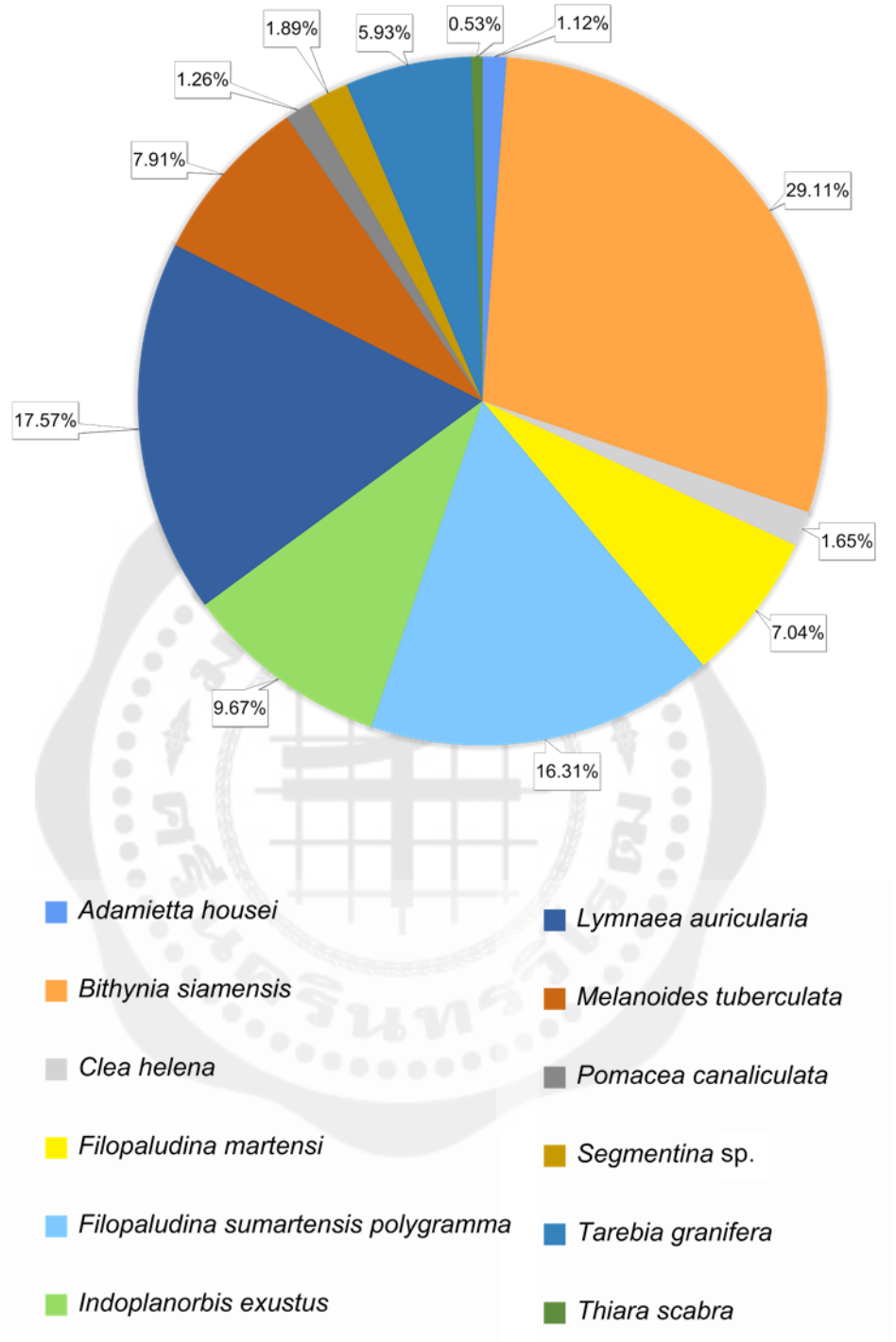



FIGURE 15 The proportion of each snail species which collected from 15 provinces in central Thailand.

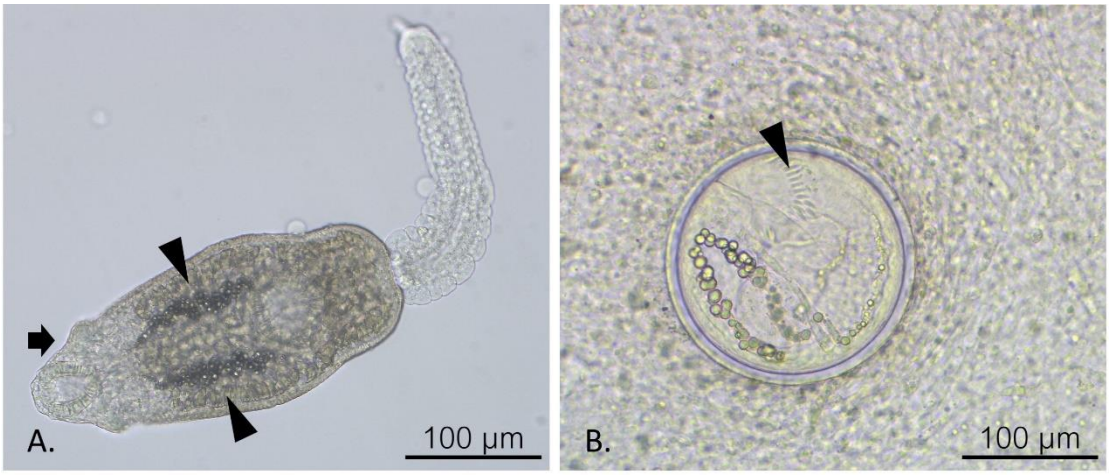



FIGURE 16 The larvae stage of echinostome found in the snails; A: echinostome cercaria with the distinct collar head (black arrow) and two descending excretory granules (black arrowhead); B: Metacercaria of *E. revolutum* with clearly visible collar spines (black arrowhead).

**Morphological study of *Echinostoma revolutum***

The living *E. revolutum* metacercariae were collected from the pericardial sac of *Filopaludina* snails. The morphological appearances were identified according to other previous report[15, 77, 78]. The main characteristics used to identify the parasites were number and arrangement of collar spines, size of metacercaria cyst, the presence of excretory granules. The description of *E. revolutum* metacercaria based on 100 metacercarial cysts. The bilayer transparent spherical cyst diameter was 193.20 – 220.00 (200.70 ± 7.46) µm. The thickness of the outer layer wall was 8.40 – 9.00 (8.89 ± 0.10) µm, while the inner layer wall was 1.40 – 1.80 (1.76 ± 0.05) µm. Thirty-seven collar spines on the collar head and clearly visible excretory granules were observed from all specimens (Figure 17). The metacercariae were fed to the experimental hamsters at the ratio of metacercariae and hamsters described in Chai 2011[15]. Adult worms were collected from the small intestine of hamsters at 11 – 17 days post-infection.

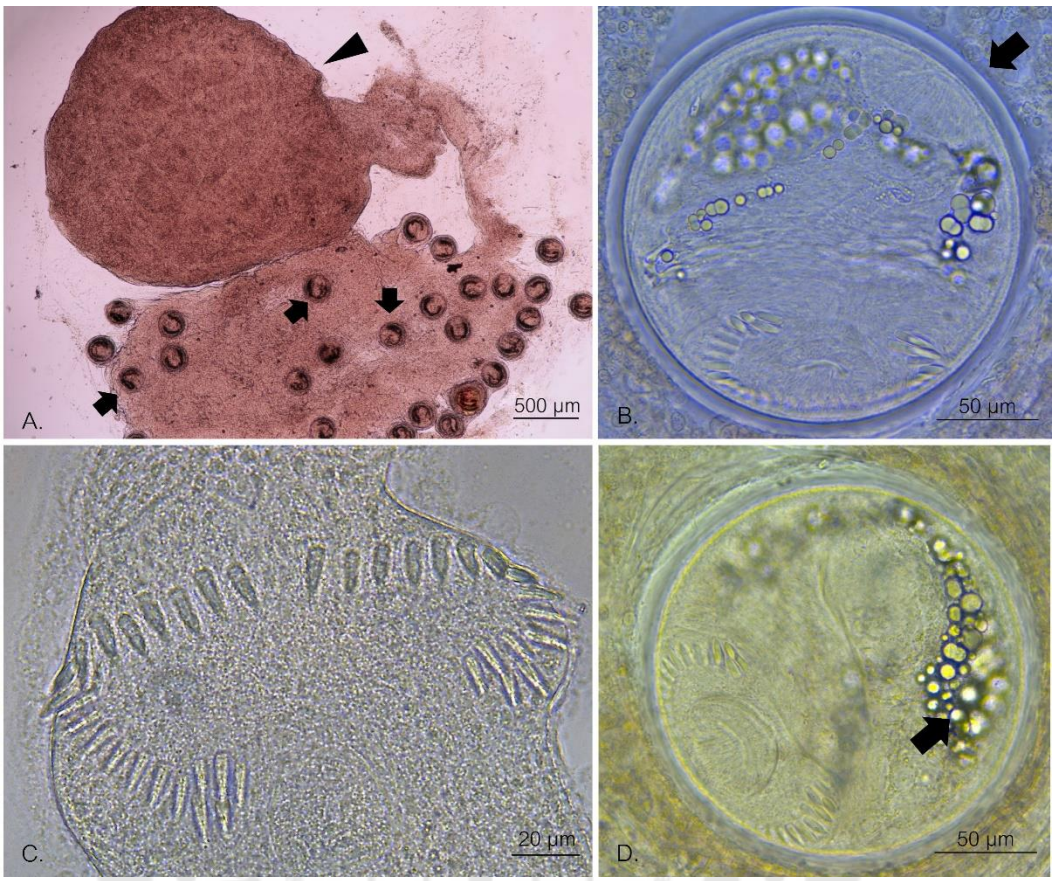



FIGURE 17 *E. revolutum* metacercariae; A: The metacercaria attached to the pericardial sac of *Filopaludina* snail. The arrowhead indicated the cardiac of snail and the black arrow indicated the metacercariae. B: The black arrow indicated the bilayer transparent wall of metacercaria. C: The hatched metacercaria revealed thirty-seven collar spine. D: The black arrow indicated the excretory granules.

**Morphological appearances of *E. revolutum* from permanent slide**

Adult worms were recoverd from the experimental hamster from 14 to 17 days post feeding of metacercariae isolated from *F. sumatrensis polygramma* and *F. martensi.* The morphological characters of adult stage were described from 11 specimens as follows; body flattened and elongated form. Body size was 4.79 – 5.86 (5,323.54 ± 305.92) mm in length and 0.94 – 1.14 (1,045.07 ± 56.62) mm in width. The well-developed collar head surrounded with the thirty-seven collar spines arranged by 5 – 6 – 15 – 6 – 5 (5 corner spines, 6 lateral spines on each side and 15 spines in two-alternating row) same as a metacercarial stage (Figure 18). Subterminal oral sucker was round and 121.46 – 140.07 (132.21 ± 5.33) µm in size. The extremely short prepharynx was approximately 5 µm. The muscular pharynx was clearly visible. The huge acetabulum was 445.03 – 472.56 (456.56 ± 8.12) µm in length and 385.07 – 478.43 (436.86 ± 27.16) µm in width. The esophagus was 137.11 – 149.76 (145 ± 3.74) µm in length, connected to a bifurcated intestine that elongated throughout their body and ended near the posterior terminal. The slightly spherical ovary was located posterior to acetabulum and connected to the moderate loop of the uterus. The uterus was filled with numerous eggs. Two slightly lobe testes tandemly located posterior to the ovary. The cirrus was well-developed and located in the cirrus sac near the acetabulum (Figure 19). All morphological data of *E. revolutum* and related species from various reports were sumumarized in Table 5. Our results were concordance with the characters of *E. revolutum*.


FIGURE 18 The collar spine arrangement of *E. revolutum*; A: ventral view show 5 corner spines (white arrow) and six lateral spines (black arrow); B: dorsal view with 15 spines in two-alternating row (black arrow).

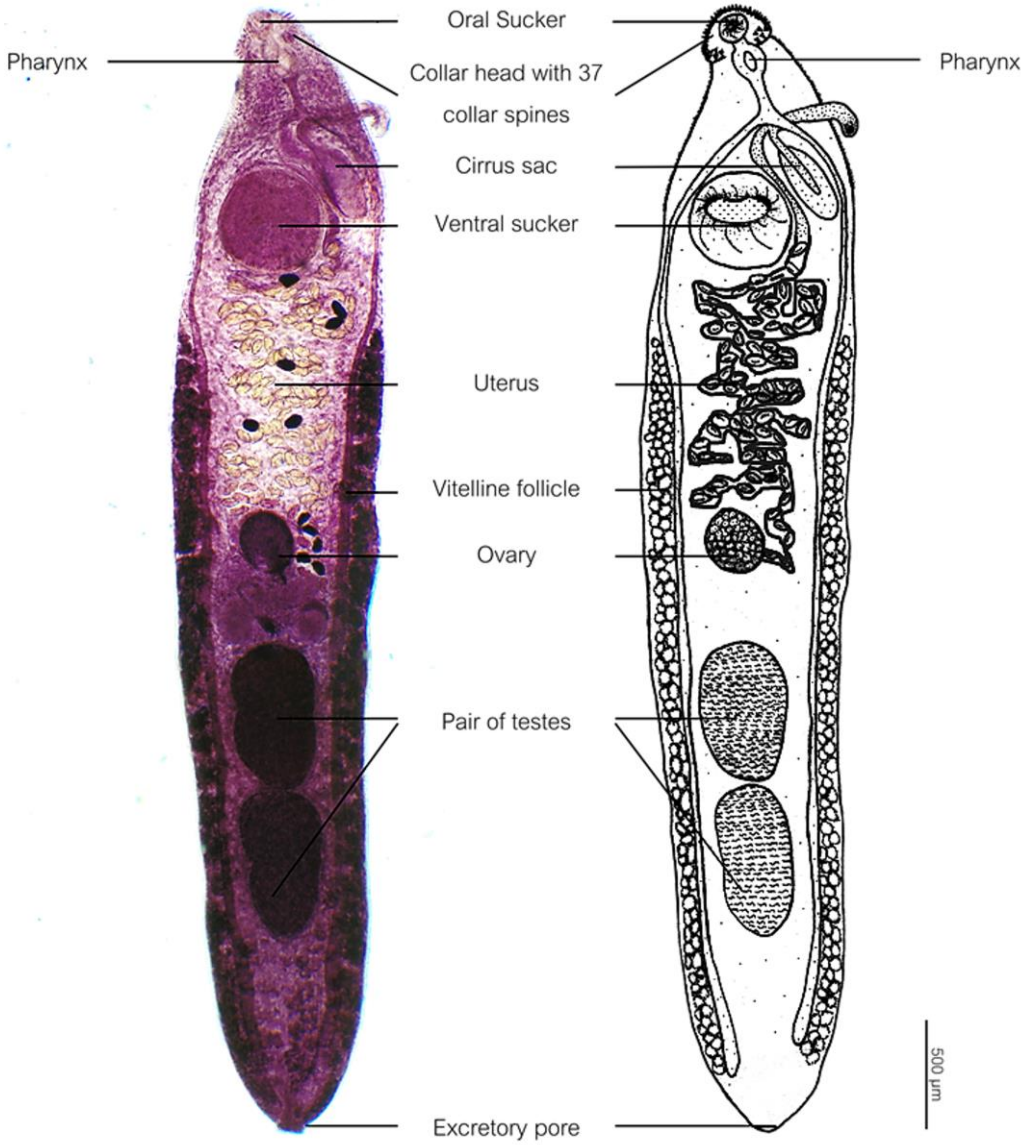



FIGURE 19 Adult stage of 17 days *E. revolutum* collected from experimental hamster

TABLE 5 The morphological characteristics of *E. revolutum* compare to related species found in Thailand.

| Morphological characters | Present specimens (11 specimens) | *E. revolutum*[15, 77-79] | *E. miyagawai*[80] | *E. ilocanum*[81-83] | *Echinoparyphium recurvatum*[78, 84] | *Hypoderaeum conoideum*[85] |
|---|---|---|---|---|---|---|
| Body size (μm.) | L: 5,323.54 (4,794.32 – 5,860.32) W: 1,045.07 (939.17 – 1139.68) | L: 4,000 – 6,349 W: 850 – 1,300 | L: 9,163 – 11,010 W: 1,028 – 1,272 | L: 5,200 – 9,204 W: 620 – 1,560 | L: 2,010 – 4,700 W: 460 – 650 | L: 7,618 – 11,427 W: 1,057 – 1,482 |
| Oral sucker (μm.) | 132.21 (121.46 – 140.07) | 100 – 208 | 262–337 | 180 – 240 | 92 – 150 | L: 242 – 296 W: 168 – 242 |
| Collar spine | 37 | 37 | 37 | 49 – 51 | 45 | 47-54 |
| Spine length (μm.) | 55.09 (52.28 – 57.05) | 28 – 90 | 52 – 75 | 18-40 | 53-70 | 26-34 |
| Prepharynx | Very shot | Very shot or absent | Absent or very short | Shot | Shot | Very short |
| Pharynx (μm.) | L: 150.08 (139.43 – 160.89) W: 122.51 (112.95 – 129.25) | L: 120 – 184 W: 80 – 149 | L: 262 – 337 W: 187 – 355 | L: 180 – 228 W: 135 – 210 | L: 92 – 130 W: 61 – 110 | L: 162 – 231 W: 151 – 202 |
| Esophagus (μm.) | 145 (137.11 – 149.76) | 100 – 200 | 542 – 655 | 172 – 228 | 160 – 337 | Shot |
| Ventral sucker (μm.) | L: 456.56 (445.03 – 472.56)) W: 436.86 (385.07 – 478.43) | L: 400 – 736 W: 380 – 763 | L: 655 – 748 W: 655 – 785 | L: 469-643 W: 469-737 | L: 296 – 400 W: 306 – 390 | L: 804 – 954 W: 701 – 942 |
| Genital opening | Post-bifurcate | Post-bifurcate | level of esophageal bifurcation | Post-bifurcate | Post bifurcate | Post bifurcate |
| Cirrus sac length (μm.) | 444.76 (432.24 – 459.67) | 300 – 550 | 468 – 598 | No data record | 300 – 400 | 735-1,206 |
| Vitelline follicle | Commence from below the ventral sucker to near the end of body | Commence from below the ventral sucker to near the end of body | Extending from short distance posterior to ventral sucker to the posterior body end | Visualized laterally on posterior three fourth of the body | Follicular and extend from posterior 1/3 level of uterus to the end of body | Extending from short distance posterior to ventral sucker to the posterior body end |
| Ovary (μm.) | Spherical, L: 263.70 (245.34 – 276.85) W: 226.16 (200.56 – 244.55) | Spherical, L: 200 – 350 W: 300 – 400 | Slightly transversely-oval L: 243–355 W: 337–449 | Spherical or subspherical L: 180-442 W: 165-402 | Spherical L: 130 – 220 W: 130 – 200 | Spherical or Slightly spherical L: 310 – 494 W: 241 – 414 |
| Testes (μm.) | Tandem, Slightly lobe Ant L: 638.89 (583.42 – 702.71) Ant W: 412.55 (384.32 – 452.62) Post L: 735.63 (656.81 – 824.45) Post W: 405.05 (371.30 – 441.54) | Tandem, Slightly lobe Ant L: 368 – 780 Ant W: 343 – 620 Post L: 368 – 960 Post W: 315 – 580 | Tandem, subglobular Ant L: 430 – 692 Ant W: 337 – 505 Post L: 449–655 Post W: 337–524 | Tandem, Deep or Slightly lobe Ant L: 469-1,179 Ant W: 241-804 Post L: 523-1,206 Post W: 228-697 | Tandem, Smooth and oval Ant L: 235 – 420 Ant W: 153 – 280 Post L: 265 – 480 Post W: 163 – 290 | Tandem Smooth and oval Ant L: 620 – 1183 Ant W: 322 – 643 Post L:701 – 1183 Post W: 287 – 655 |

**The tegumental surface of *E. revolutum***

The anterior part of tegumental surface coverd with numerous tiny spines, directed toward the posterior part of body. The tiny papillae located between rows of tegumental spines and acetabulum. The papillae were also found at the oral sucker and collar head. Thirty-seven collar spines arranged in specific pattern. The corner spines were clearly visible. The huge ventral ridge was clearly visible at anterior to a acetabulum. The unarmed cirrus situated on the upper area of acetabulum (Figure 20-24).

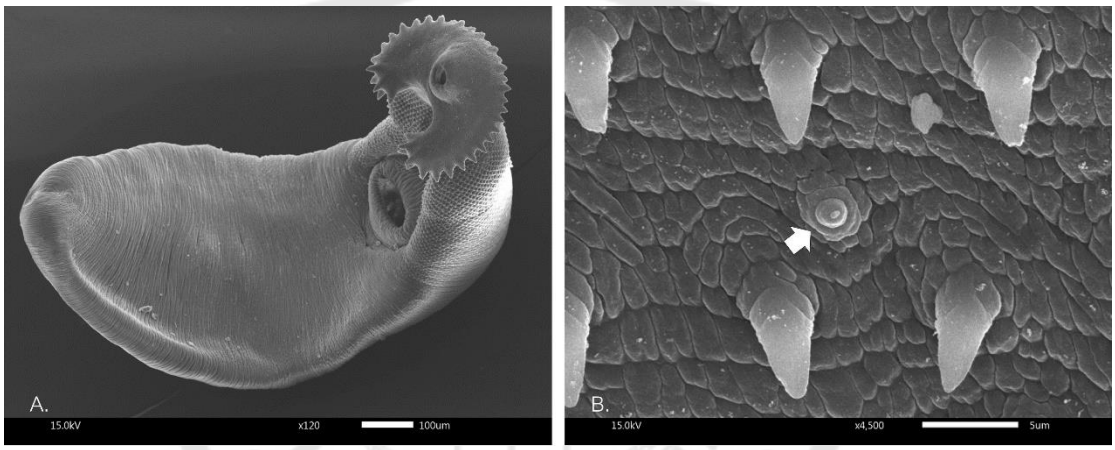



FIGURE 20 *E. revolutum* at 15 days post infection. A: The elongated body covered with numerous tiny spines. B: The tiny papillae (white arrow) found between rows of tegumental spines.

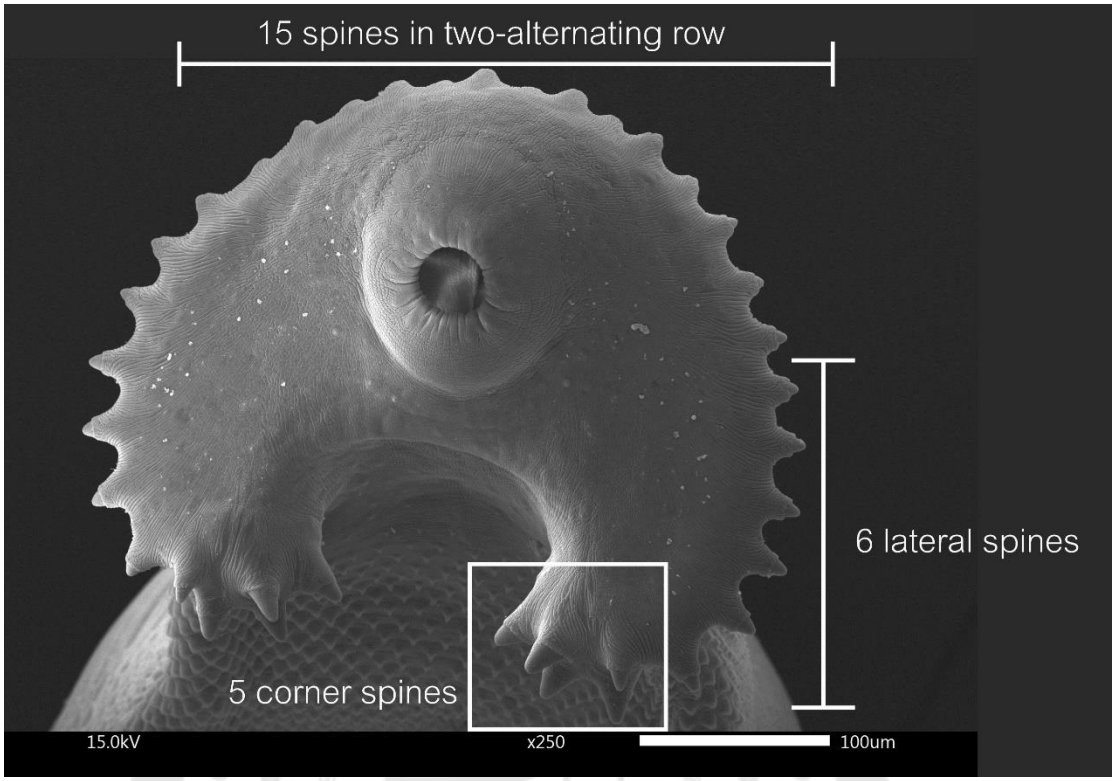



FIGURE 21 The collar head of *E. revolutum* showed thirty-seven collar spines.

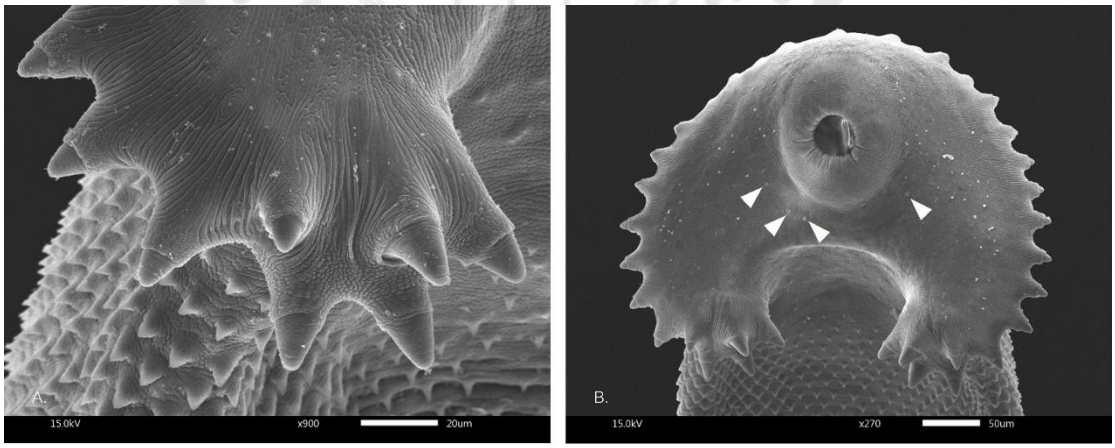



FIGURE 22 The collar head of 15 day *E. revolutum*; A: Five collar spines at a corner of the collar head; B: The papillae at the oral sucker (white arrow)

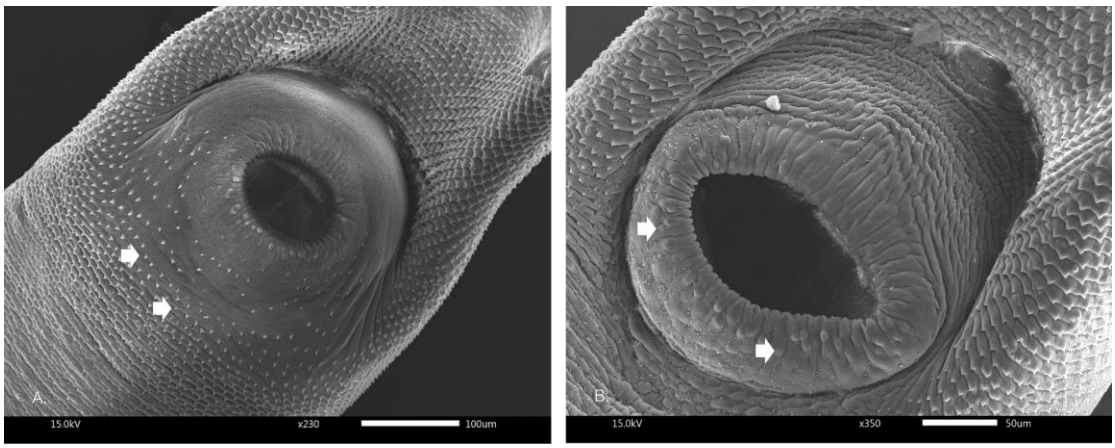



FIGURE 23 The acetabulum of *E. revolutum* A: The scale-like tegumental spines (arrow), B: The papillae on the acetabulum (arrow)

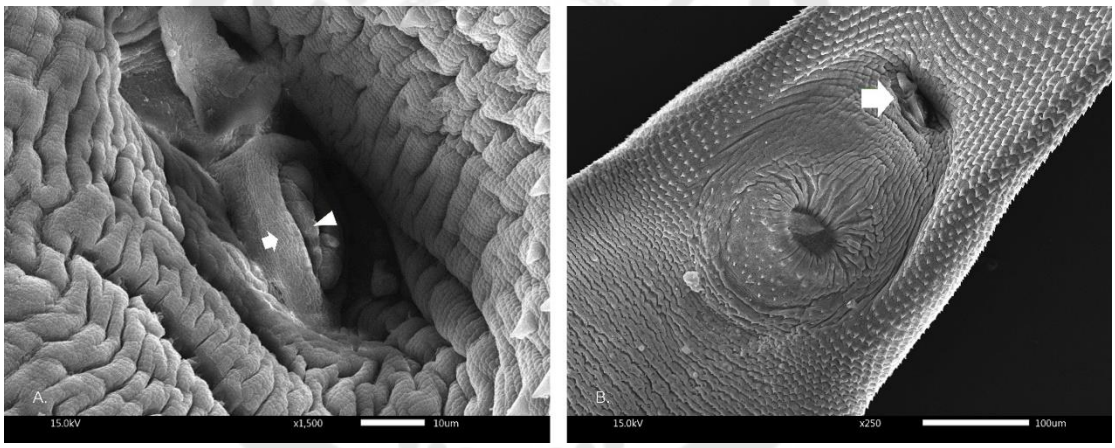



FIGURE 24 The unarmed cirrus (white arrowhead) at the cirrus sac (white arrow)

**Fecal examination**

The feces of infected experimental hamsters were investigated at first day, post-infection, by the direct smear and sedimentation method using formalin-ether technique. Eggs were yellowish, elliptical, operculated with a small abopercular wrinkle. The size was 110 – 125 (116.52 ± 4.33) × 72 – 75 (73.82 ± 0.94) µm (n=50) (Figure 25). For the direct smear method, the unembryonated eggs first appeared at 13 days post-infection. The number of eggs was 40 EPG. For sedimentation method, the egg was initially found at 12 days post-infection. The number of eggs was 58 EPG. (Table 6).

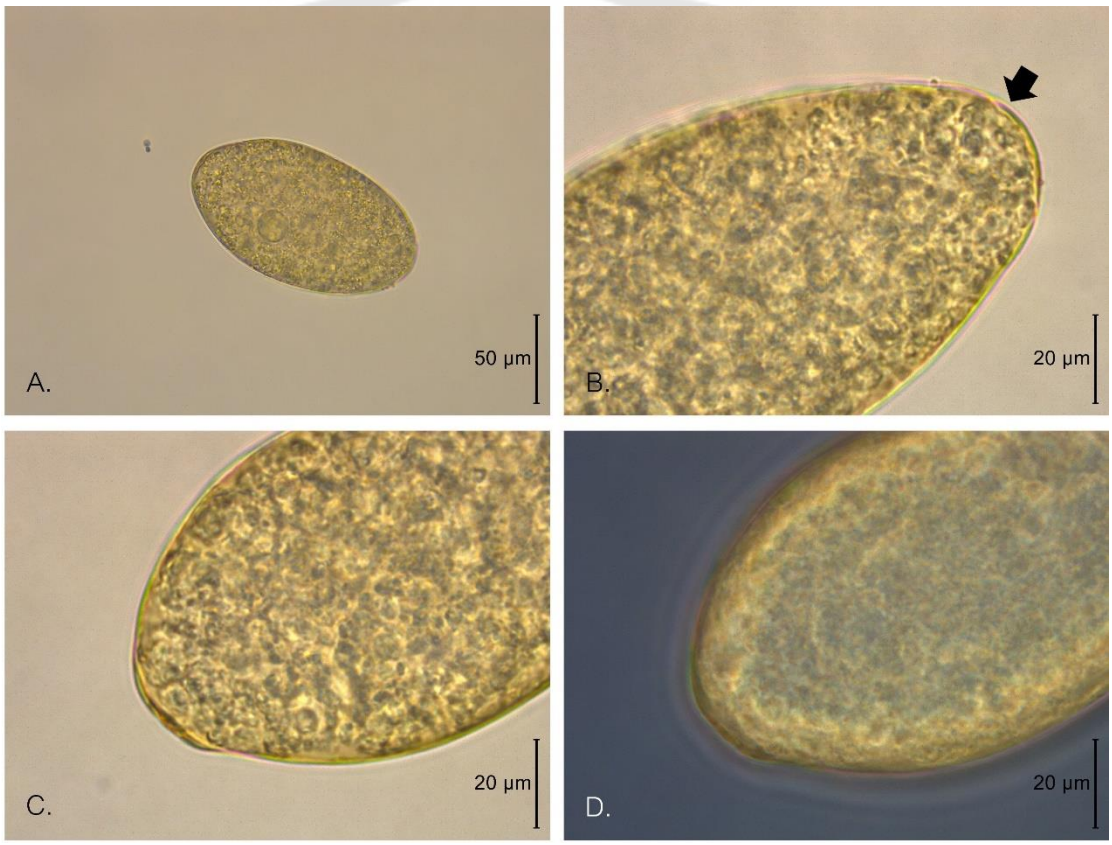



FIGURE 25 The egg of *E. revolutum*; A: An embryonated egg obtained from formalin-ether technique; B: A small abopercular wrinkle (arrow); C: An operculum observed under light microscope; D: An operculum observed under phase-contrast microscope

TABLE 6 The number of eggs found in fecal specimens (EPG)

| Methods / Days | EPG; Mean ± SD. (3 replicates) | |
|---|---|---|
| | Direct smear | Formalin-ether technique |
| 1 – 10 | 0 | 0 |
| 11 | 0 | 0 |
| 12 | 0 | 50 ± 20 |
| 13 | 30 ± 10 | 60 ± 10 |
| 14 | 34 ± 12 | 57 ± 23 |
| 15 | 54 ± 6 | 60 ± 0 |
| 16 | 40 ± 0 | 57 ± 15 |
| 17 | 44 ± 6 | 64 ± 15 |
| Mean | 40 ± 18 | 58 ± 14 |

*Echinostoma revolutum* recovery rate

Immature worms were first observed in the intestinal of the dead experimental hamster at 10 days post-infection. Twenty-three worms were recovered from the hamster, gave rise to the highest recovery rate (46.00%, 23/50). The high number of worms might have caused intestinal damage led to death of this hamster. Morphological characters of the worms were investigated under light microscope. All immature worms are soft body. Despite the body of worms were disintegrated, the muscular organs, ventral sucker, pharynx and collar head, were still intact (Figure 26). The unique 5 corner spines at the collar head and the DNA result indicated that they were *E. revolutum*.

The average recovery rate of worms from both naturally dead and euthanized hamsters was 6.00% (3/50). The detail of number of recovered worms per day was shown in Figure 27. Two adult *E. revolutum* were recovered from a euthanized hamster after 11 days of infection. Adult worms were 3.48 and 3.85 mm in length, which was shorter than the expected length of *E. revolutum*. The uterus contained a few eggs (Figure 28).

At day 12$^{th}$, the size of worm was more elongated, approximately 4.60 ± 172.78 (4.54 – 4.99 mm; n=4). The length of collar spines when observed by SEM were slightly short as shown in Figure 29. From day 13$^{th}$, adult stage was fully mature. The uterus was fulfilled with eggs (Figure 30). The length of collar spines was increased to approximate 11 µm (Figure 31).

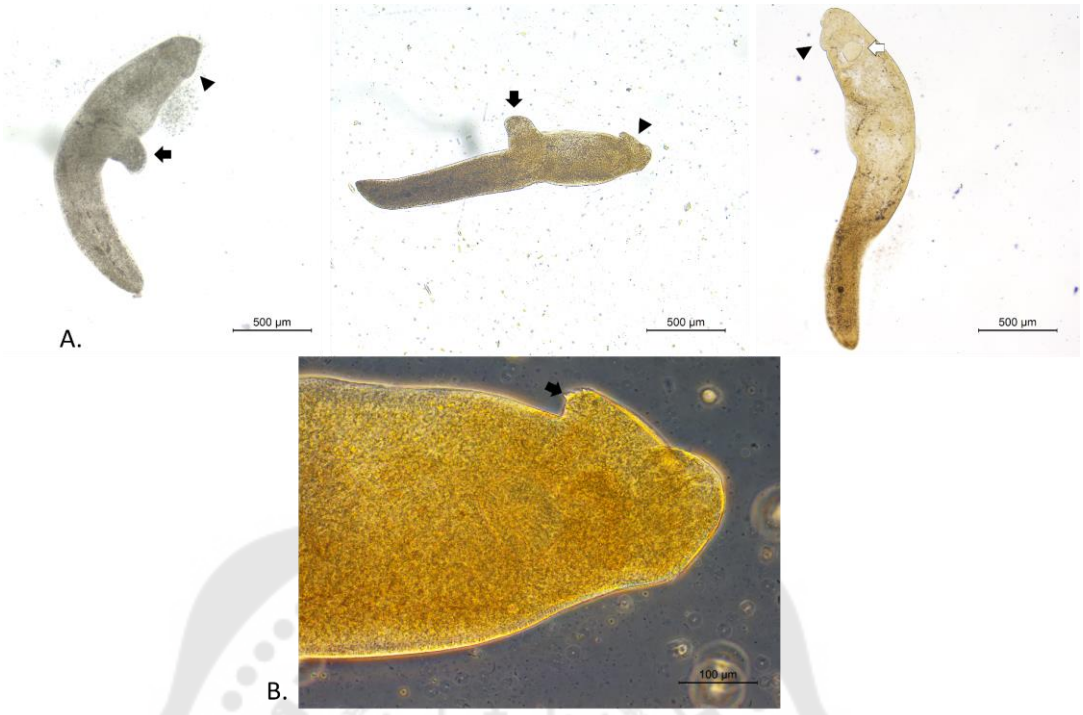
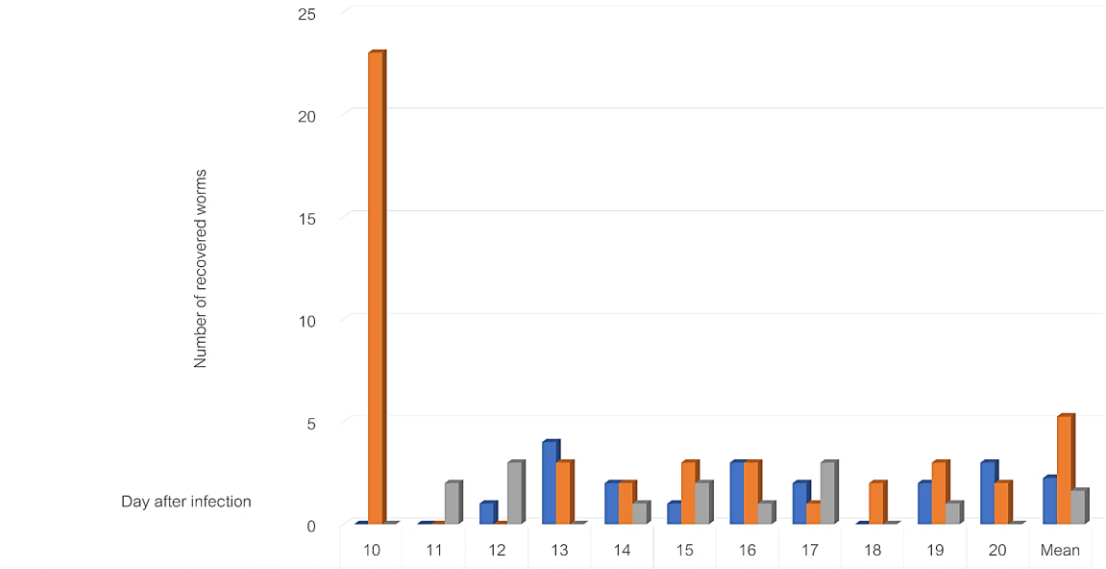



FIGURE 26 The immature worm collected from 10 days of post-infection; A: The decomposed body of immature worm, the black arrow heads indicated the collar head, the black and white arrows were ventral sucker and pharynx, respectively. B: The black arrow indicated the 5 corner spines observed under phase contrast microscope.




| Day after infection | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of recovered worms 1st hamster | 0 | 0 | 1 | 4 | 2 | 1 | 3 | 2 | 0 | 2 | 3 | 2 |
| Number of recovered worms 2nd hamster | 23 | 0 | 0 | 3 | 2 | 3 | 3 | 1 | 2 | 3 | 2 | 5 |
| Number of recovered worms 3rd hamster | 0 | 2 | 3 | 0 | 1 | 2 | 1 | 3 | 0 | 1 | 0 | 2 |

FIGURE 27 The bar chart displayed the recovery rates of worms in each day of infection.


FIGURE 28 Eleven-day *E. revolutum* showed a few of eggs in the uterus (white arrow).

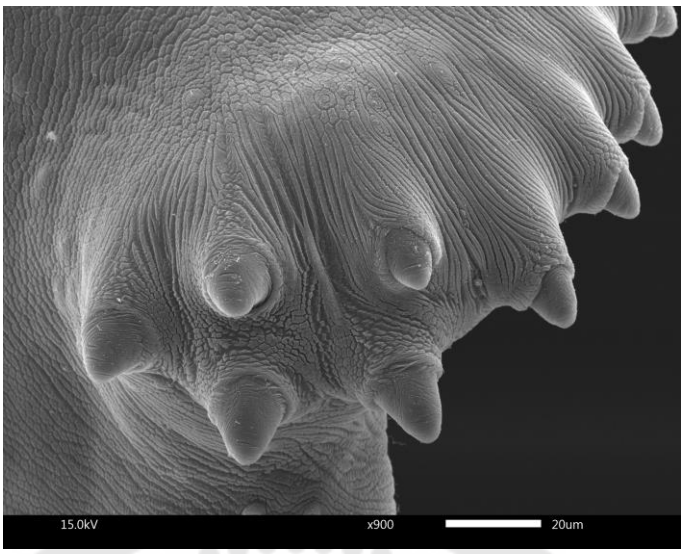



FIGURE 29 The collar spines of twelve-day *E. revolutum*

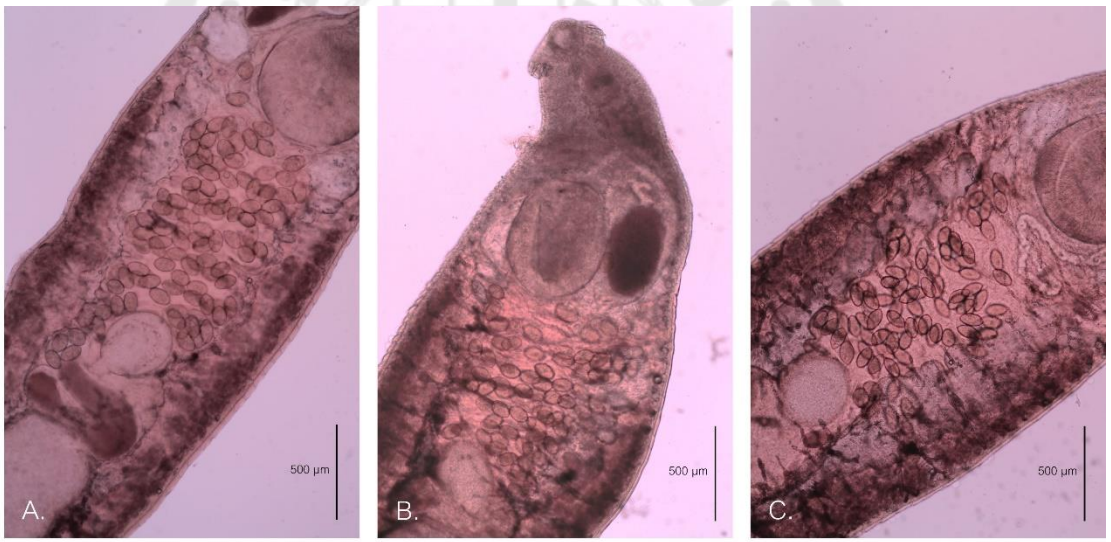



FIGURE 30 The living adult stage of *E. revolutum* revealed the full fill eggs in their uterus. A: 13 day post-infection; B. 15 day post-infection; C: 17 day post-infection

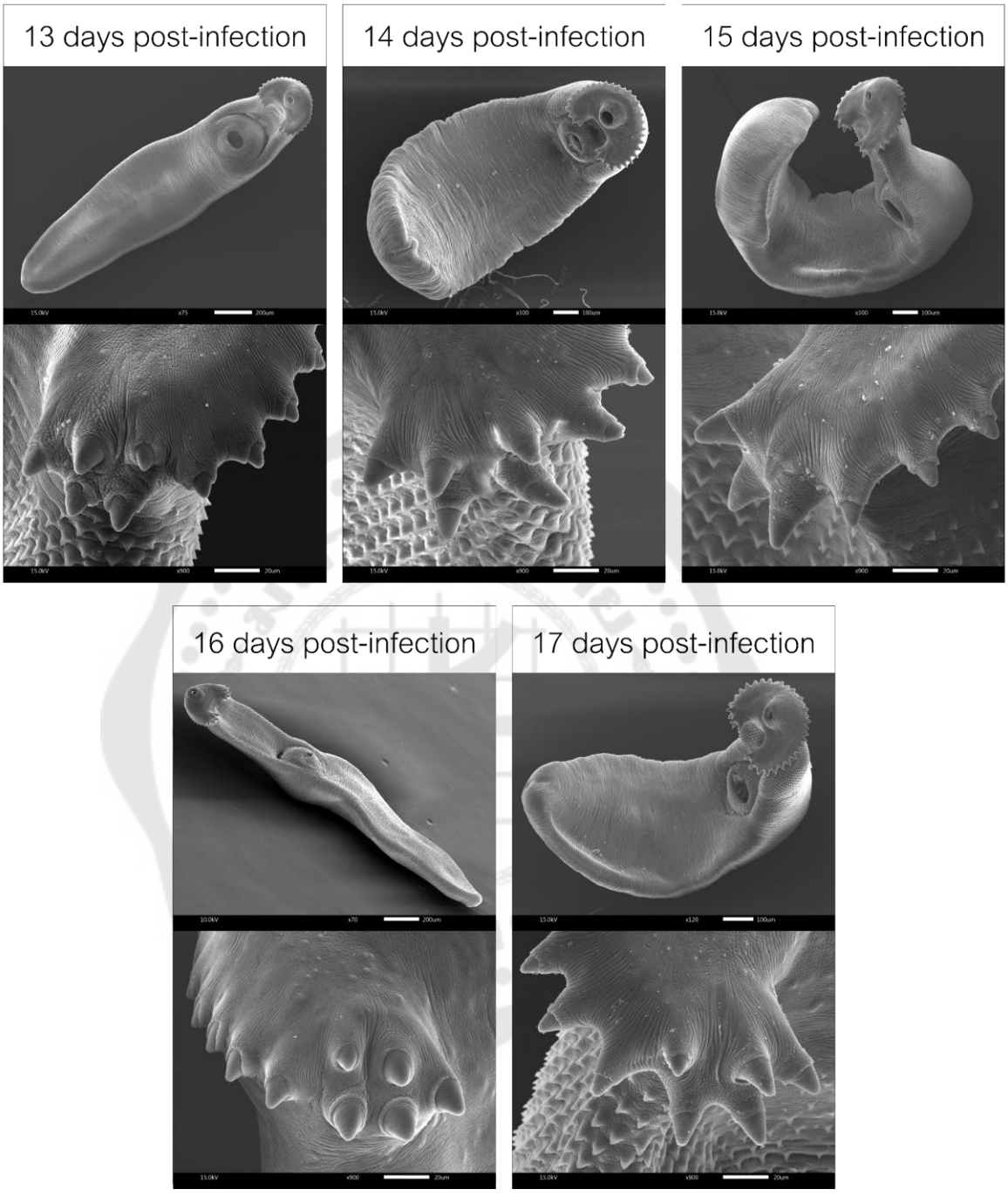



FIGURE 31 Adult worm recovered from hamsters from 13 to 17 days.

**The epidemiological studies**

The total number of 20,302 snail specimens were investigated. Eight of twelve species were infected with the echinostome larvae (echinostome cercaria and the *E. revolutum* metacercaria) based on the morphological characteristics. The highest prevalence of metacercarial infection was observed in *Filopaludina* snails (13.35% in *F. sumatrensis polygramma*). The highest prevalence of echinostome cercaria infection was from *L. auricularia* (1.54%). Five snail species also infected by echinostome cercaria and metacercaria, comprising *B. siamensis* (0.02% for metacercaria and 0.10% for cercaria), *I. exustus* (0.05% for metacercaria and 0.66% for cercaria), *M. tuberculata* (0.06% for cercaria), *Segmentina* sp. (0.26% for cercaria), and *T. granifera* (0.33% for cercaria), respectively. Figure 32 showed the proportion of echinostome infection in all snail species.

The prevalence data based on morphological appearances revealed that the snails collected from 13 of 15 provinces were infected with the echinostome larvae, comprising Ang Thong (1.54%, 16/1,036), Ayutthaya (0.49%, 14/2,831), Chainat (1.62%, 20/1,231), Lopburi (13.74%, 236/1,718), Nakhon Nayok (2.83%, 19/671), Nakhon Pathom (4.74%, 52/1,098), Nakhon Sawan (0.27%, 7/2,561), Pathum Thani (0.32%, 3/924), Samut Sakhon (2.64%, 8/303), Saraburi (5.94%, 146/2,457), Sing Buri (1.60%, 17/1,065), Suphanburi (1.69%, 36/2,127) and Uthai thani (1.21%, 15/1,237), respectively. The overall prevalence of echinostome infection was 2.90% (589/20,302). The highest prevalence of *E. revolutum* infection was found in *F. sumatrensis polygramma* from Lopburi province (42.91%). The detail of prevalences was shown in Figure 33.

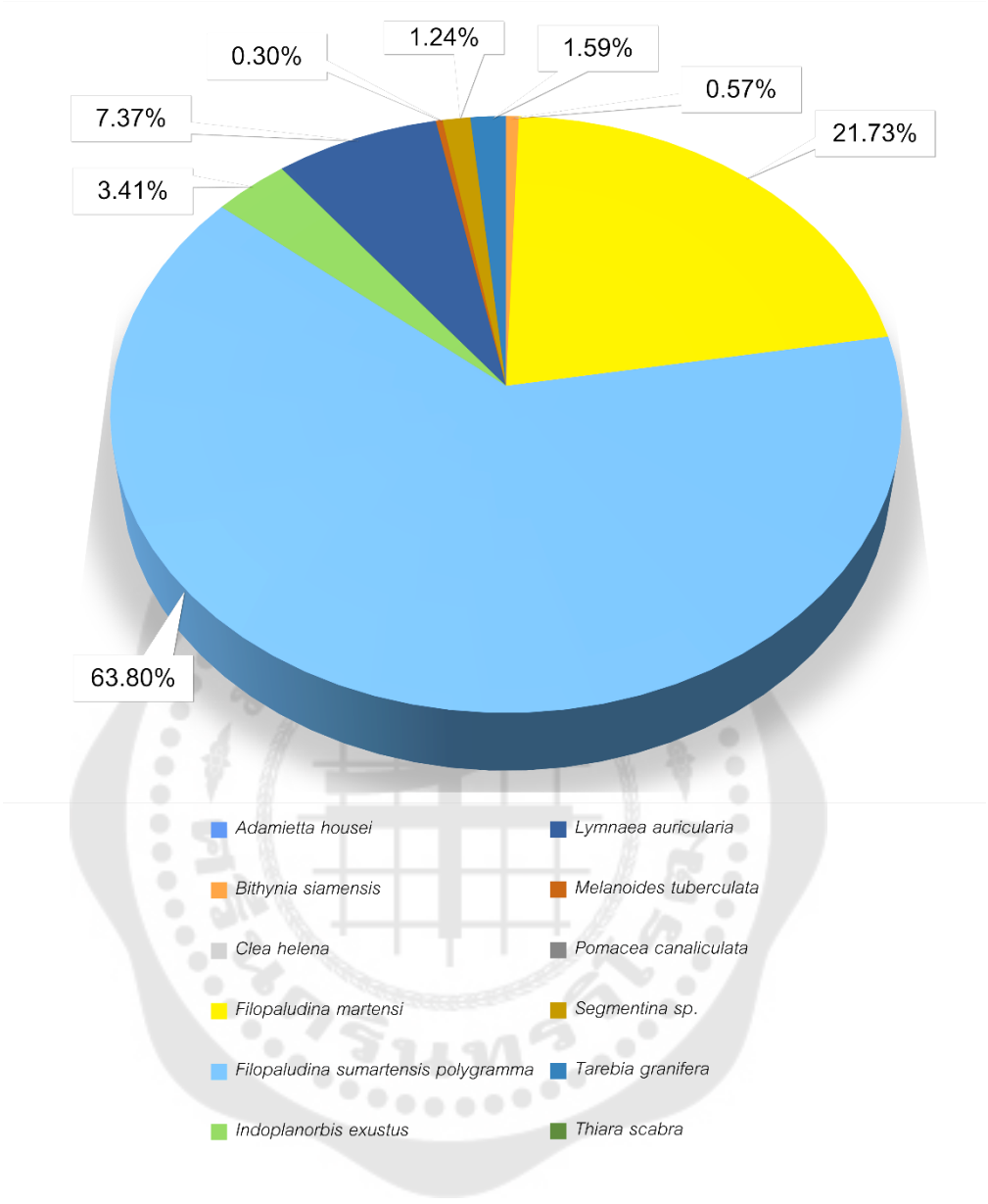



FIGURE 32 The proportion of snail species infected by larval stages of echinostome

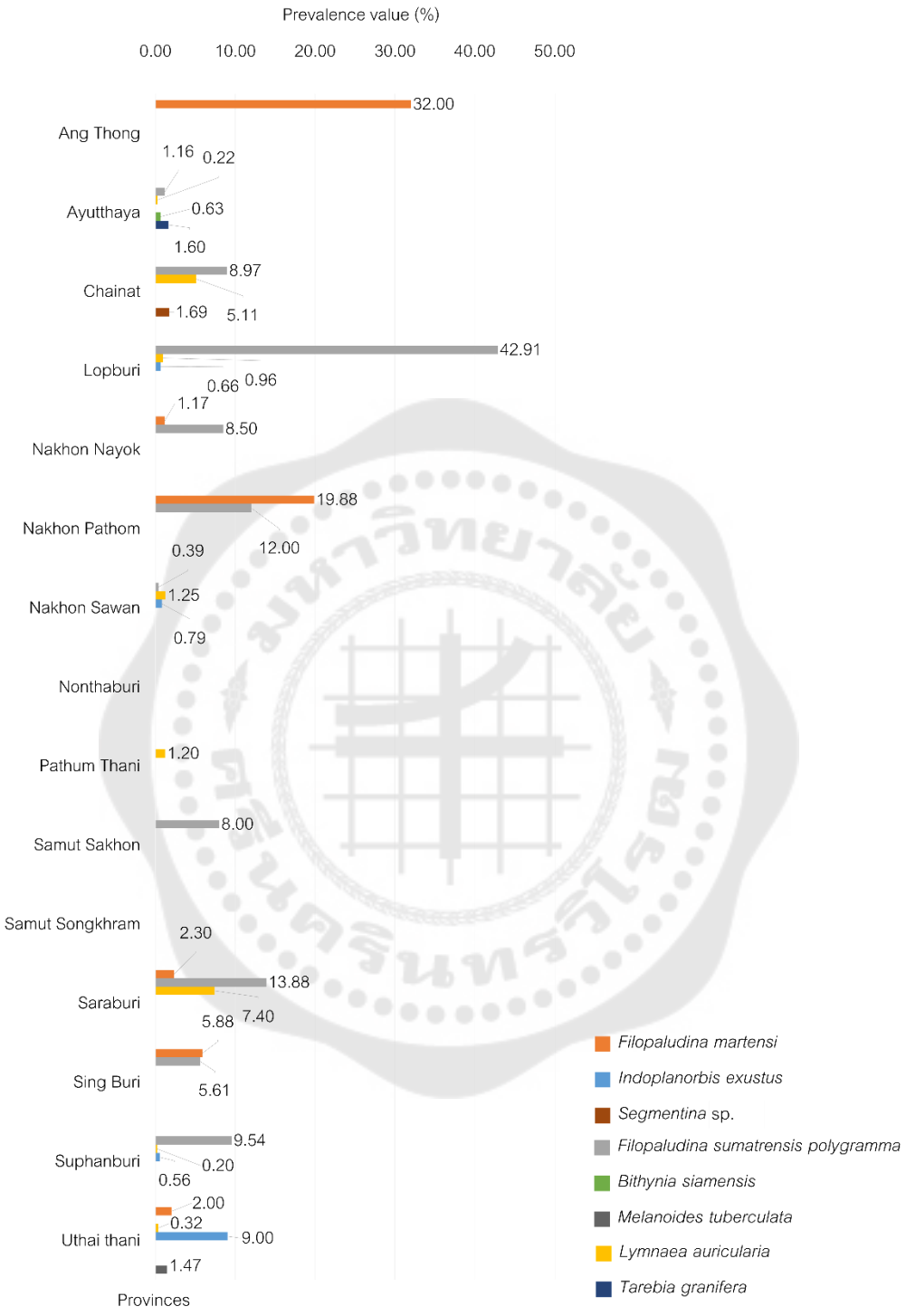



FIGURE 33 The prevalence of echinostome larvae (cercaria and metacercaria) in each snail species of each province.

**Molecular study of *Echinostoma revolutum***

**Species confirmation based on molecular method**

For *CO*1 gene, the result obtained from the Basic Local Alignment Search Tools (BLAST) with BLASTn algorithms revealed that the specimens were highly identical to *E. revolutum* and *E. miyagawai* (100 and 100% identities). The retrieved nucleotide data of the revolutum complex from NCBI database were used to construct a phylogenetic tree with the specimen sequences data. All specimens were clustered in clade of *E. revolutum / E. miyagawai*, as were the sequences of *E. miyagawai* and *E. revolutum* from the database (Figure 34A). However, the morphological appearances of all adult specimens were contrasted to the morphological characteristics of *E. miyagawai*, which showed extremely larger than average size of *E. revolutum*[80]. Average size of *E. miyagawai* is 9,163 – 11,010 µm, while average size of adult specimens is only 5,323.54 ± 305.92 µm (4,794.32 – 5,860.32 µm). Moreover, the location of genital pore of the specimen is at the posterior of bifurcal point, while *E. miyagawai* genital pore is at the same level of esophageal bifurcation[80]. Therefore, both morphological characters and phylogenetic tree based on *COI* revealed that all adult specimens obtained in this study were *E. revolutum*.

For *ND*1 gene, the specimens were clustered to the same clade of other *E. revolutum* sequences. Moreover, the *E. revolutum* clade was separated from other species in revolutum complex compose of *E. miyagawai*, *E. cinetorchis, E. robustum*, and *E. trivovis*, with high boostrap support (Figure 34B).

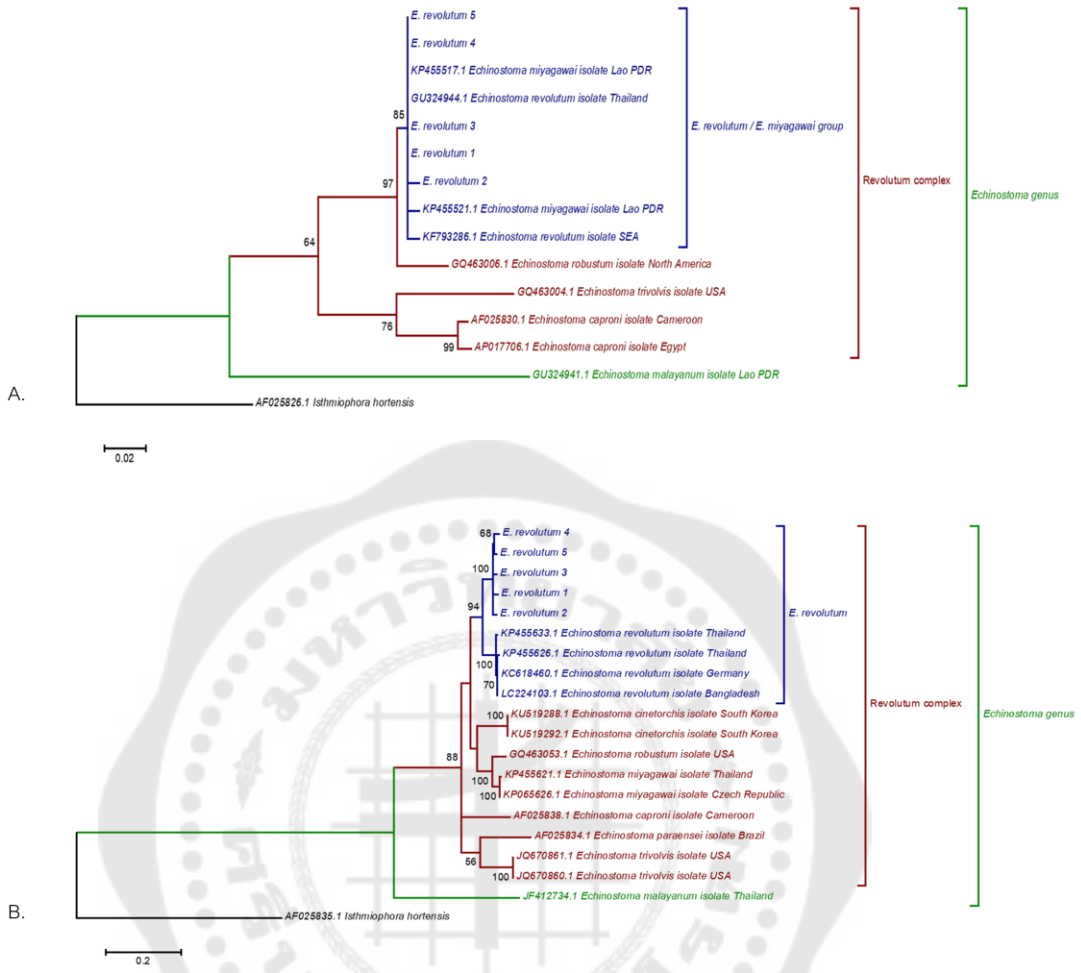



FIGURE 34 The maximum likelihood (ML) tree of *Echinostoma*. Nodal supports are bootstrap values. A sequence of *Isthmiophora hortensis* (*Echinostoma hortense*) was used as outgroup; A: The ML tree based on 181 nucleotides of *CO*1. B: The ML tree based on 443 nucleotides of *ND*1.

Regarding to *18s* rDNA gene used[75] to confirm species of specimens, all of them were positively amplified. A specific band (71 base pairs) were shown after amplification by PCR method (Figure 35). *E. revolutum* specimens were used to screen for gene candidate in the next experiment.

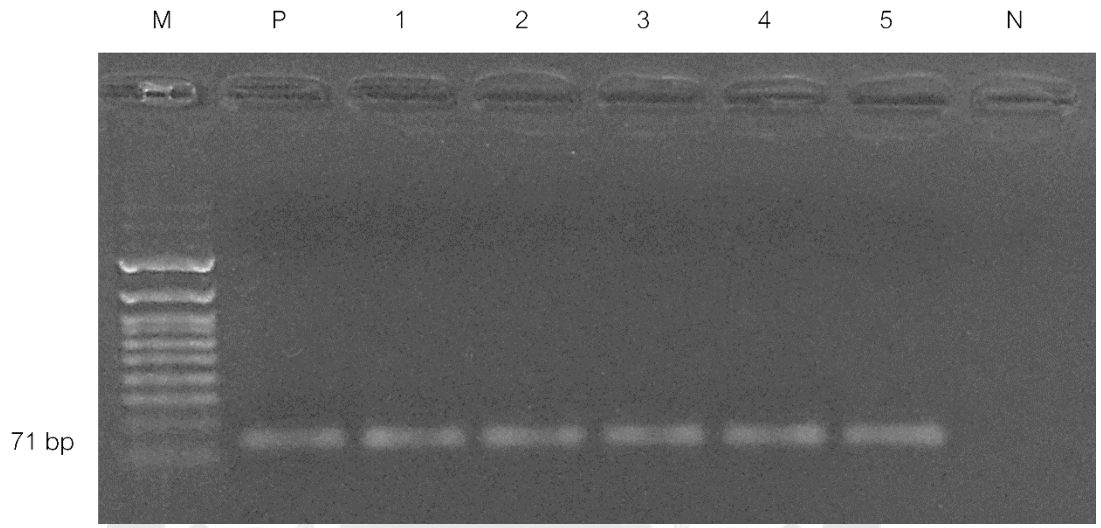



FIGURE 35 The specific amplification tests of five adult worm specimens collected from the experimental hamster. M was 50 bp Ladder, P was adult stage of *E. revolutum* that are positive control, the number 1 to 5 refer to each adult worm and N was negative control.

**Gene candidate for developing the species-specific primer**

Two nuclear genes (*TYR* and *CTSB*) and one mitochondrial gene (*CYTB*) were selected as candidate genes. The result of each gene were explained below.

**Tyrosinase gene**

The TYR degenerated primers namely, Tyr-deg-F and Tyr-deg-R, reported in Bae, et al. were used for amplification of this gene[49]. After several attempts to adjust the optimize condition for PCR amplification, these primers were unsuccessful to amplify DNA of *E. revolutum*. Therefore, the newly primers were generated in this study for amplifying the tyrosinase gene of *E. revolutum*.

Nucleotide sequences of *TYR* from the NCBI database accession number KC195914 was analyzed in BLASTX program. Four amino acid sequences of common central domain of trematodes, accession number, OON23210, OON23212, OON20329, PIS88377, were selected. They were used as templates for TBLASTN to find the nucleotide sequences data. Eleven sequences of *TYR* were found (Table 7). Two pairs of primer specific to TYR gene were designed based on the conserved region of this gene, TYRuni1 (F: 5'- AAAGACAGAGATGGTATCCG -3'; R: 5'- TTTGTTGTTCGT CCAAAGAC -3') and TYRuni2 (F: 5'- AATCAAGAATTTCGCACTCC -3'; R: 5'- TGTACTTTGTTGTTC GTCCA -3'). The expected PCR product were 309 and 505 base pairs, respectively. The PCR condition of both sets of primer were tested and optimized for amplifying the DNA of *E. revolutum*. The optimized conditions were described in Table 8.

TABLE 7 The accession number of *TYR* sequences from related family of trematodes.

| Nucleotide accession number | Descriptions | Location site of common central domain in each sequence (base pairs) |
|---|---|---|
| KC195914 | *Clonorchis sinensis* tyrosinase (TYR2) mRNA complete cds | 633 - 1,233 |
| AY812904 | *Schistosoma japonicum* SJCHGC06782 protein mRNA complete cds | 633 - 1,236 |
| KC195913 | *Clonorchis sinensis* tyrosinase (TYR1) mRNA complete cds | 615 - 1,215 |
| KC195914 | *Clonorchis sinensis tyrosinase* (TYR2) mRNA complete cds | 633 - 1,233 |
| KC195915 | *Clonorchis sinensis* tyrosinase (TYR3) mRNA complete cds | 633 - 1,233 |
| KC195916 | *Clonorchis sinensis* tyrosinase (TYR4) mRNA complete cds | 618 - 1221 |
| KM658170 | *Paragonimus westermani* tyrosinase (TYR) mRNA complete cds | 627 - 1,227 |
| XM_009172646 | *Opisthorchis viverrini* hypothetical protein partial mRNA | 615 - 1,215 and 2,049 - 2,649 |
| XM_009172647 | *Opisthorchis viverrini* hypothetical protein partial mRNA | 633 - 1,233 |
| XM_012937294 | *Schistosoma haematobium* Tyrosinase mRNA | 633 - 1,236 |
| XM_018799099 | *Schistosoma mansoni* tyrosinase precursor partial mRNA | No indicated domain site |

TABLE 8 The optimized condition of primers for amplifying the DNA of *E. revolutum* based on *TYR* gene.

| Primer sets | Steps | Temperatures (°C) | Times | Cycles |
|---|---|---|---|---|
| TYRuni2 | Pre-denature | 95 | 4 minutes | - |
| | Denature | 95 | 60 seconds | 35 |
| | Annealing | 53.2 | 50 seconds | 35 |
| | Extension | 72 | 1.5 minutes | 35 |
| | Final extension | 72 | 10 minutes | - |

Only TYRuni2 primer set was successfully amplified DNA of *E. revolutum* at the optimized annealing temperature of 53.2ºC (Figure 36). And the optimized concentration of $MgCl_2$ was 1.0 mM (Figure 37). All targeted PCR products were sequenced, and the sequences were compared with amino acid sequences via BLASTX program. The results showed that all sequences are similarity to tyrosinase protein reported from various trematode species (Table 9).

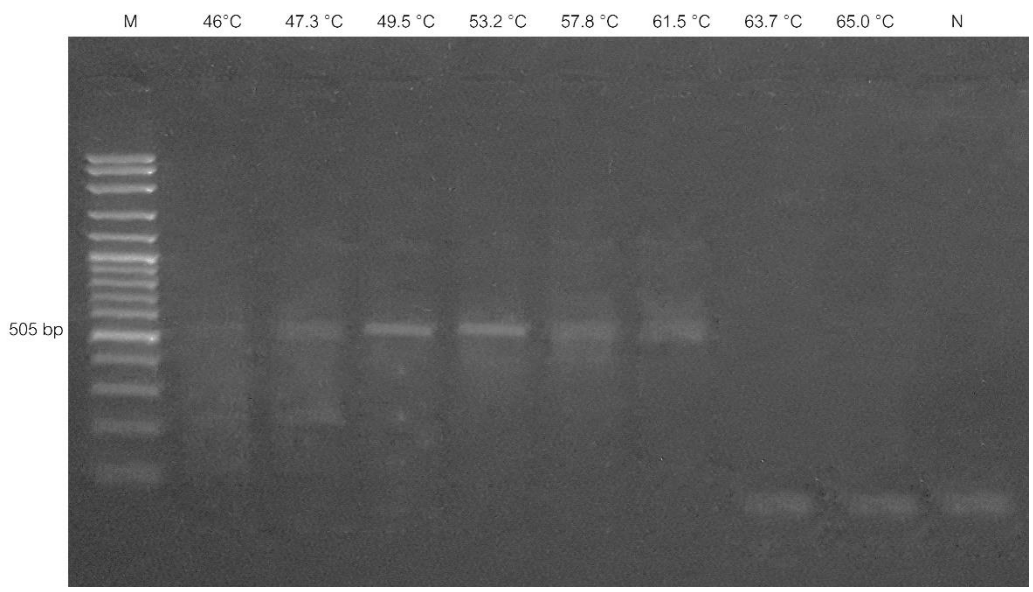



FIGURE 36 The annealing temperature optimization of TYRuni2 primer set. M referred to 100 bp marker, N referred to negative control. The annealing temperature of each PCR reaction was shown at the top of each lane.

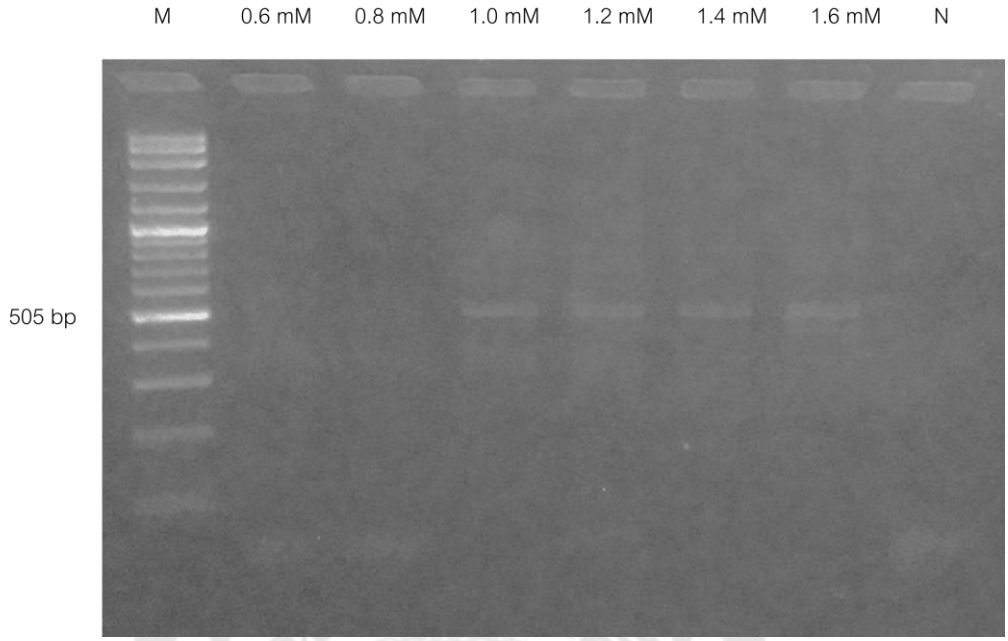



FIGURE 37 The concentration of $MgCl_2$ ranging from 0.6 mM to 1.6 mM, respectively. M referred to 100 bp marker and N referred to negative control

TABLE 9 The result from BLASTX showed similarity of the PCR products to tyrosinase protein in various species of trematodes.

| Accession number | Descriptions | E value | Percent identity (identity base pairs) |
|---|---|---|---|
| OON23210 | Common central domain of tyrosinase *Opisthorchis viverrini* | 9e-26 | 92.73 (51/55) |
| AGG11797 | Tyrosinase (*Clonorchis sinensis*) | 1e-21 | 84.91 (49/53) |
| AJE29953 | Tyrosinase (*Paragonimus westermani*) | 3e-19 | 81.13 (43/53) |
| XP_012801733 | Tyrosinase (*Schistosoma haematobium*) | 2e-14 | 60 (36/60) |
| AAW26996 | Tyrosinase 1 (*Schistosoma japonicum*) | 2e-13 | 66.04 (35/53) |
| RTG83478 | Tyrosinase (*Schistosoma bovis*) | 4e-13 | 66.04 (35/53) |
| AAP93838 | tyrosinase 1 precursor (*Schistosoma mansoni*) | 1e-12 | 62.26 (35/53) |

**Cathepsin B gene**

*CTSB* gene amplified with the protocol obtained from the previously report[57] was unsuccessful. Therefore, the new primers were generated in this study for amplify the sequences of this gene based on the conserved regions. Four sequences of *CTSB* were retrieved from NCBI database and analyzed by Prosite program (https://prosite.expasy.org/) to find the conserved area that can be used to generate the primers and the result was shown in Table 10.

TABLE 10 The site of conserved region in *CTSB* gene computed by Prosite program.

| Accession number | Descriptions | Location of active site (base pairs) |
|---|---|---|
| FN314853 | *Schistosoma japonicum* isolate Anhui full length mRNA | 336 - 369, 858 - 888, 909 - 966 |
| KP317840 | *Schistosoma_mekongi*_cathepsin_B_mRNA_complete_cds | 337 - 369, 858 - 888, 909 - 966 |
| KT149795 | *Fasciola_gigantica*_cathepsin_B5 (CB5) _mRNA_complete_cds | 324 - 357, 900 - 957 |
| KT781072 | *Fasciola_gigantica*_cathepsin_B7(CB7) _mRNA_complete_cds | Not found any domain protein |

The primers CTSB2_Uni4 F (5'- GAGTCTTTTGATGCTCGTTCTCAATGG -3') and R 5'- CGTGCAACAACTGAGTGGATCTGC -3') were manually designed based on these conserved sequences and the expected product size was 450 base pairs. These primers were capable of amplifying five adult specimens by PCR method at various annealing temperature as described in Table 11.

TABLE 11 The optimized condition of primers for amplifying the DNA of *E. revolutum* based on *CTSB gene.*

| Primer sets | Steps | Temperatures (°C) | Times | Cycles |
|---|---|---|---|---|
| CTSB2_Uni4 | Pre-denature | 95 | 4 minutes | - |
| | Denature | 95 | 60 seconds | 35 |
| | Annealing | 54.4 | 50 seconds | 35 |
| | Extension | 72 | 1.5 minutes | 35 |
| | Final extension | 72 | 10 minutes | - |

The best annealing temperature was 54.4ºC, which yielded the non-specific bands (Figure 38) Adjusting the concentration of $MgCl_2$ could not solve the problem (Figure 39). After gel electrophoresis, PCR products from five DNA of adult *E. revolutum*, appeared at the position equivalent to the expected PCR product, were cut, purified and diluted at 10 folds. These PCR products were used as the template for re-amplification.

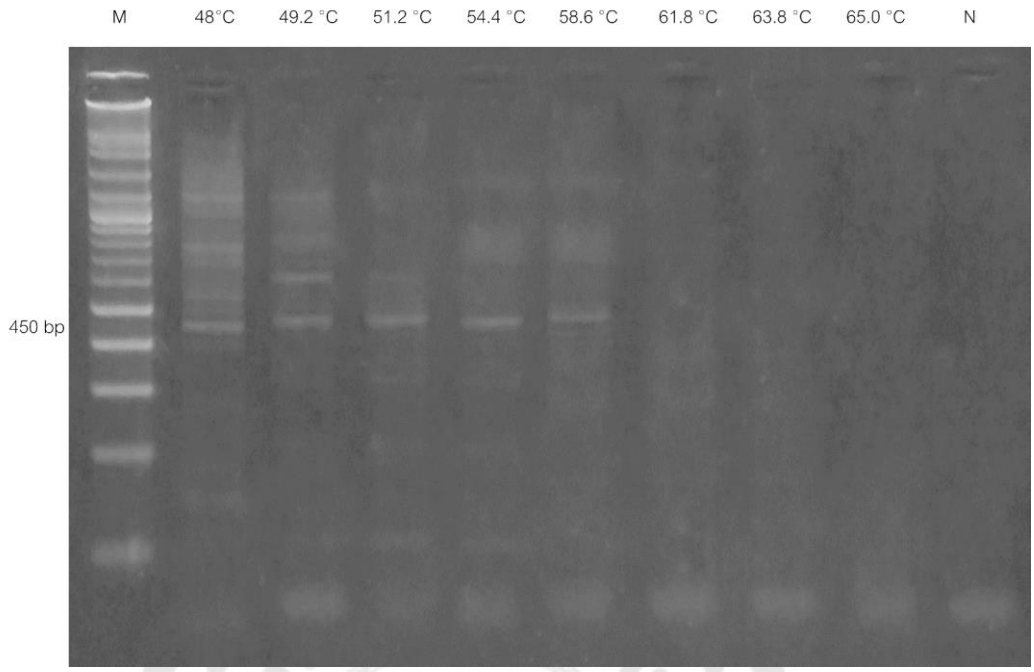



FIGURE 38 The annealing temperature optimization of CTSB2_uni4 primer set. M referred to 100 bp marker, N referred to negative control.

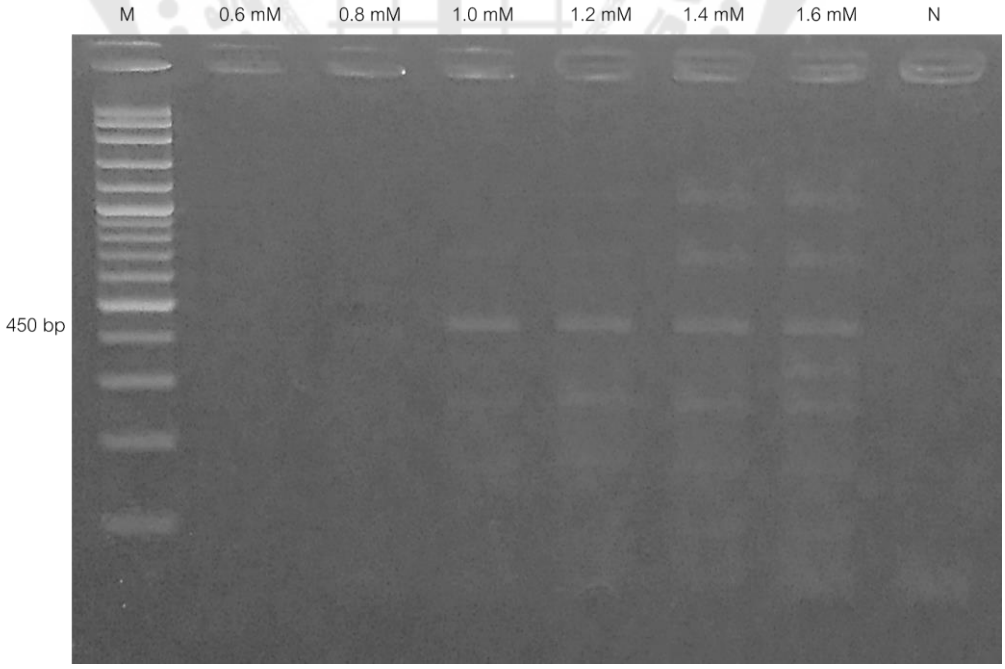



FIGURE 39 The concentration of $MgCl_2$ ranging from 1.6 mM to 0.6 mM, respectively. M referred to 100 bp marker and N referred to negative control.

The result of re-amplification was shown in Figure 40. A single specific-sized band (450 base pairs) was observed and subjected to DNA sequencing. All sequencing result were analyzed with BLASTn program. The result found that all specimens were similar to the partial CDS of cathepsin B2 mRNA of *F. hepatica* (DQ534444) and complete CDS of cathepsin B2 mRNA of *F. gigantica* (AY227674) with 100% identity.

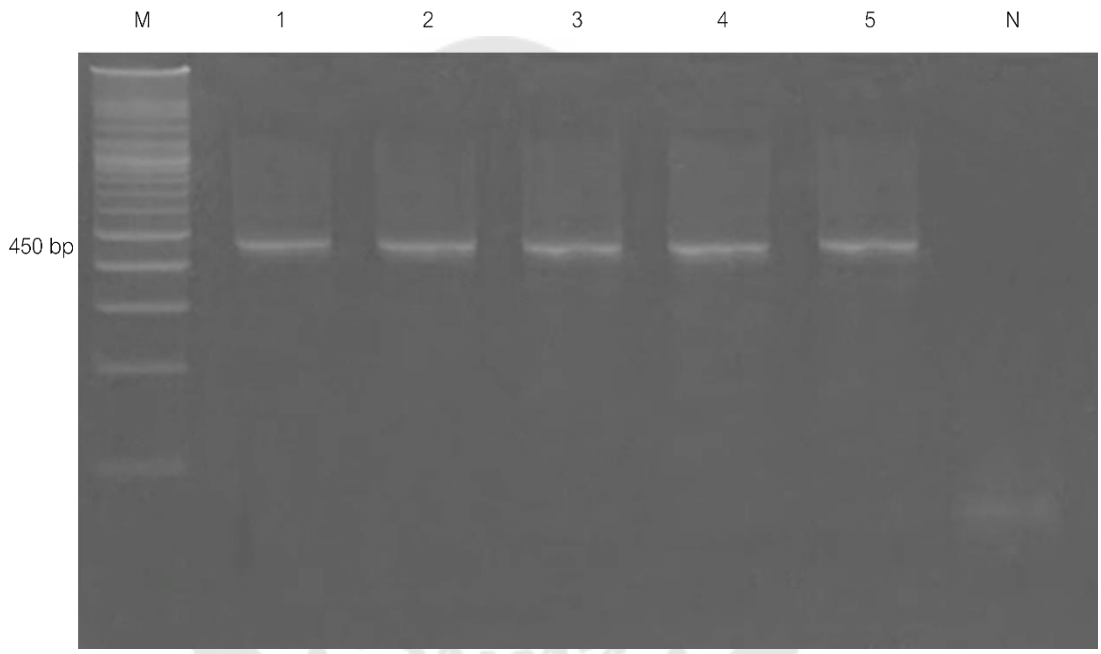



FIGURE 40 The CTSB PCR product re-amplified by using the purified amplicon as a template. The number 1 to 5 referred to amplicon of five adult specimens. M referred to 100 bp marker and N referred to negative control.

**Cytochrome B gene**

The novel primer of *Echinostoma CYTB*; namely, EchCBF (5'- TGC TGA TTC ATG TTT GAG GTT TGG - 3') and EchCBR (5'- AAG GGT ACT CTG GAT GAC AAG C -3') were designed to amplify this gene. The optimum annealing temperature ($T_a$) of EchCYTB primer set was 59.4 ºC and the PCR product was 837 base pairs. (Figure 41). After optimization, five adult specimens were used in the PCR reaction with the optimized protocol and the result showed a sharp single band of each adult specimen (Figure 42). The sequencing results were subjected to further analysis with BLASTX program. The result found that all PCR products were similar to sequence of CYTB protein of various species (Table 12).

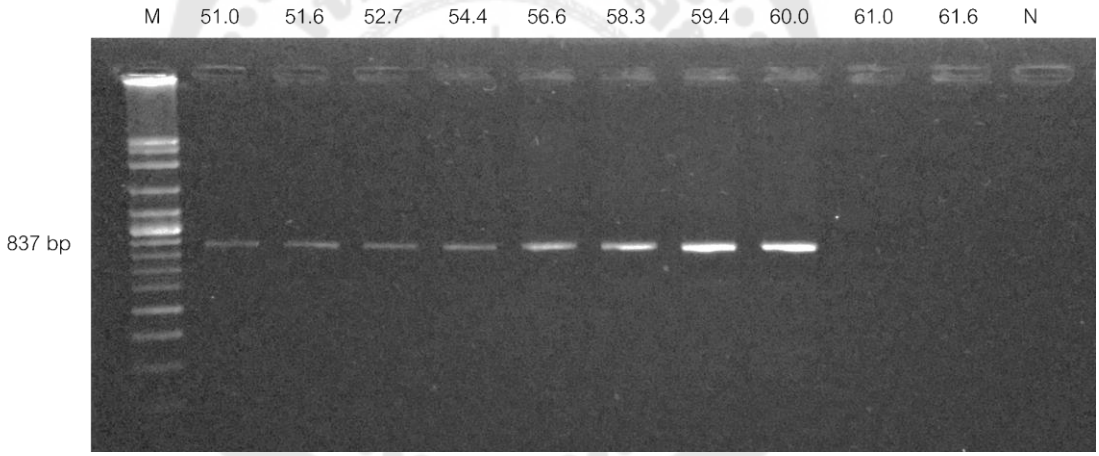



FIGURE 41 The annealing temperature optimization of EchCB primer set. M referred to 100 bp marker, N referred to negative control. The annealing temperature was shown at the top of each lane.

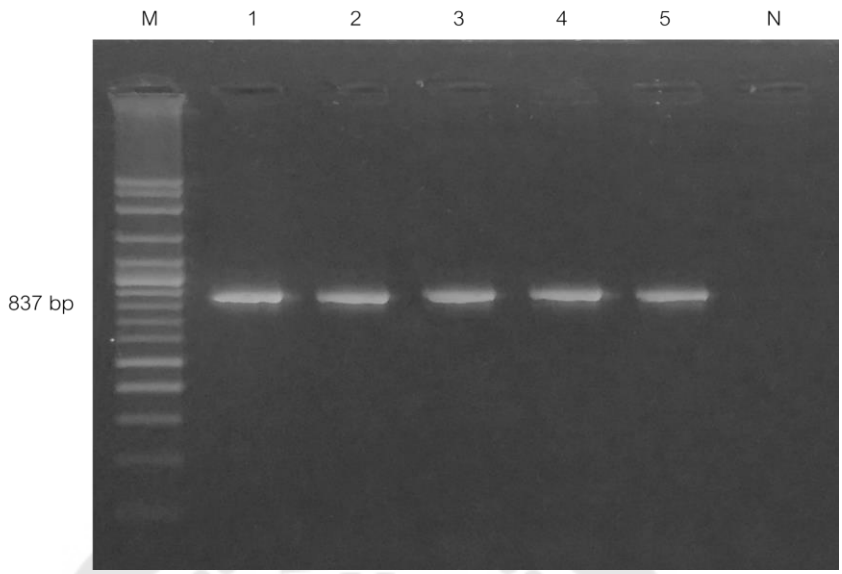



FIGURE 42 A specific band amplified by using the optimized protocol. Lane 1 - 5 were five adult specimens, M referred to 100 bp marker and N referred to negative control.

TABLE 12 The BLASTX comparing *E. revolutum* sequences with CYTB protein of various trematode species.

| Accession number | Descriptions | E value | Percent identity (identity base pairs) |
|---|---|---|---|
| YP_009519769 | Cytochrome B (*Echinostoma miyagawai*) | 1.00E-159 | 92.94 (250/269) |
| BAV82927 | Cytochrome B *(Echinostoma caproni)* | 2.00E-157 | 91.08 (245/269) |
| AMB20936 | Cytochrome B (*Echinostoma paraensei*) | 3.00E-158 | 90.33 (243/269) |
| YP_009467178 | Cytochrome B *(Artyfechinostomum sufrartyfex)* | 2.00E-148 | 89.14 (238/267) |
| AIN37096 | Cytochrome B *(Hypoderaeum* sp. Hubei-2014*)* | 1.00E-139 | 86.62 (233/269) |
| ASN77657 | Cytochrome B *(Fasciola gigantica)* | 2.00E-137 | 79.93 (214/269) |
| YP_009236158 | Cytochrome B *(Fascioloides magna)* | 2.00E-137 | 79.78 (217/272) |
| YP_009166778 | Cytochrome B *(Explanatum explanatum)* | 4.00E-134 | 79.62 (211/265) |
| YP_009164286 | Cytochrome B *(Gastrothylax crumenifer)* | 3.00E-133 | 79.25 (210/265) |
| YP_009533198 | Cytochrome B *(Cyathocotyle prussica)* | 3.00E-134 | 78.87 (209/265) |
| AJR32859 | Cytochrome B *(Paramphistomum leydeni)* | 5.00E-134 | 78.87 (209/265) |
| YP_009169423 | Cytochrome B *(Fischoederius elongatus)* | 1.00E-132 | 78.49 (208/265) |
| ATD85496 | Cytochrome B *(Paragonimus heterotremus)* | 2.00E-131 | 78.11 (207/265) |

**Comparative of each candidate gene**

A suitable gene for developing the species-specific primers must produce a single band when observed on agarose gel electrophoresis. The PCR fragment must be adequate for design and creating the species-specific primers. *Cytochrome B* gene demomstrated the highest potential as the target for species-specific primers. On the other hand, both nuclear genes showed a low intensity band of PCR product when amplified with the same concentration of adult worm DNA. Moreover, the primers cannot amplified DNA from the eggs (Figure 43). *CTSB* was unsuccessful as a candidate for PCR primer. It needs reamplification with the amplicon to generate a single band. In addition, both of nuclear gene primers amplified only some part of gene regions. Therefore, it is necessary to studies more detail about the amino acid and DNA sequences of those genes for the propose of gene identification to generate larger length of known nucleotide sequences that might be useful for designing the new species-specific primers in the future. As a result, *cytochrome B* gene was selected and used as the template for species-specific primer designs in the next experiment.

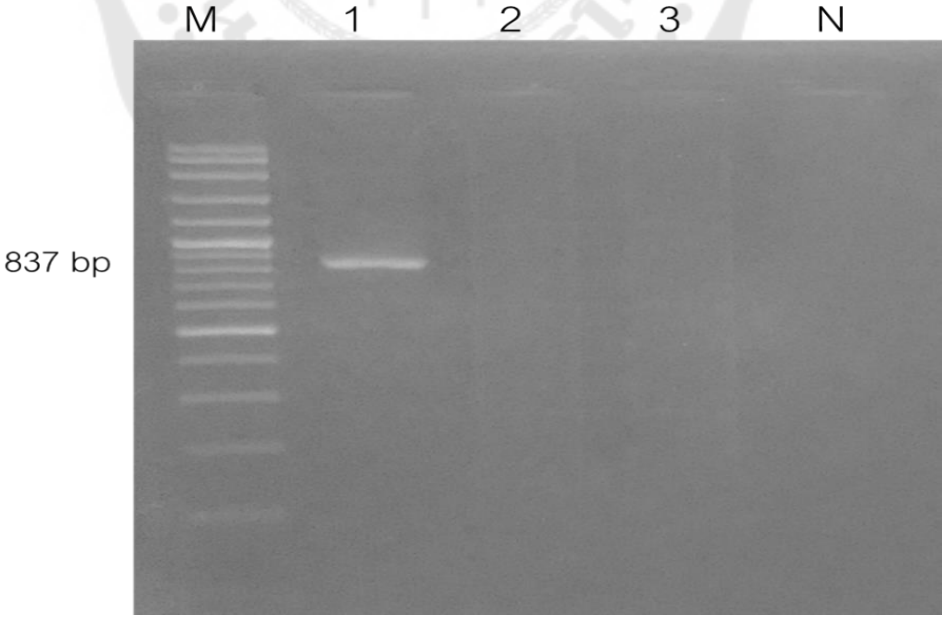



FIGURE 43 The *E. revolutum* eggs DNA amplification of *CYTB* gene (lane 1) *TYR (*(lane 2*)* and *CTSB* (lane 3). N referred to negative control.

### Primer design for specific PCR amplification

#### PCR primer design and optimization

The species-specific primer namely, ER1 (5'- ACCACATCACCATAT CCCGC-3') and ER2 (5'-GGGCAGCCACAGTTCTTACT-3') were designed based on *CYTB* sequences that obtain from previous experiments. The expected length of a PCR product was 235 base pairs. The target site of species-specific primers was shown in Figure 44. The primers were optimized for the annealing temperature using gradient PCR with temperature ranged from 51 to 61.6°C. The optimized annealing temperature was 60ºC for 50 s. PCR was carried out with 1.6 mM of $MgCl_2$. The specific band of PCR product was seen at 235 base pairs when verified by agarose gel electrophoresis. (Figure 45).


FIGURE 44 The target site of species-specific primers, the yellow label shown the area of forward and reverse primer.

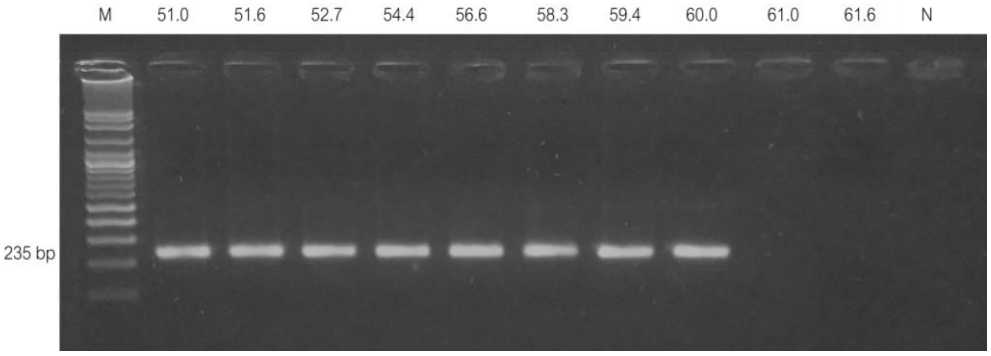



FIGURE 45 The PCR amplification of *E. revolutum* using species-specific primer (ER1 and ER2). M referred to 100 bp marker, N referred to negative control. The annealing temperature at the top of each lane

**PCR optimization and primer validations**

After validation of the optimal annealing temperature for ER primer set, the specificity test was performed with DNA of *E. revolutum,* related species and hosts. The results found that the primers can amplify *CYTB* gene in *E. revolutum*, comprised of an egg, cercaria, metacercaria and adult stage. There was no cross-amplification with other trematodes, including *E. cinetorchis, E. ilocanum*, *I. hortensis* (*E. hortense*), *Echinochasmus japonicus*, *T. anastomusa*, *H. taichui* and *S. falcatus*, respectively (Figure 46A). Moreover, these primers did not cross-amplify with DNA of intermediate and definitive hosts, *L. auricularia, F. sumatrensis, F. martensi, I. exustus, B. siamensis, C. helena, Phodopus roborovskii* (hamsters), *Gallus gallus* (domestic chicken) and *Anas platyrhynchos* (domestic duck) (Figure 46B). The minimum DNA concentration which can be amplified was 0.85 pg in *E. revolutum* DNA (Figure 47A) and 8.5 pg in mixed of *E. revolutum* and hosts DNA (Figure 47B).

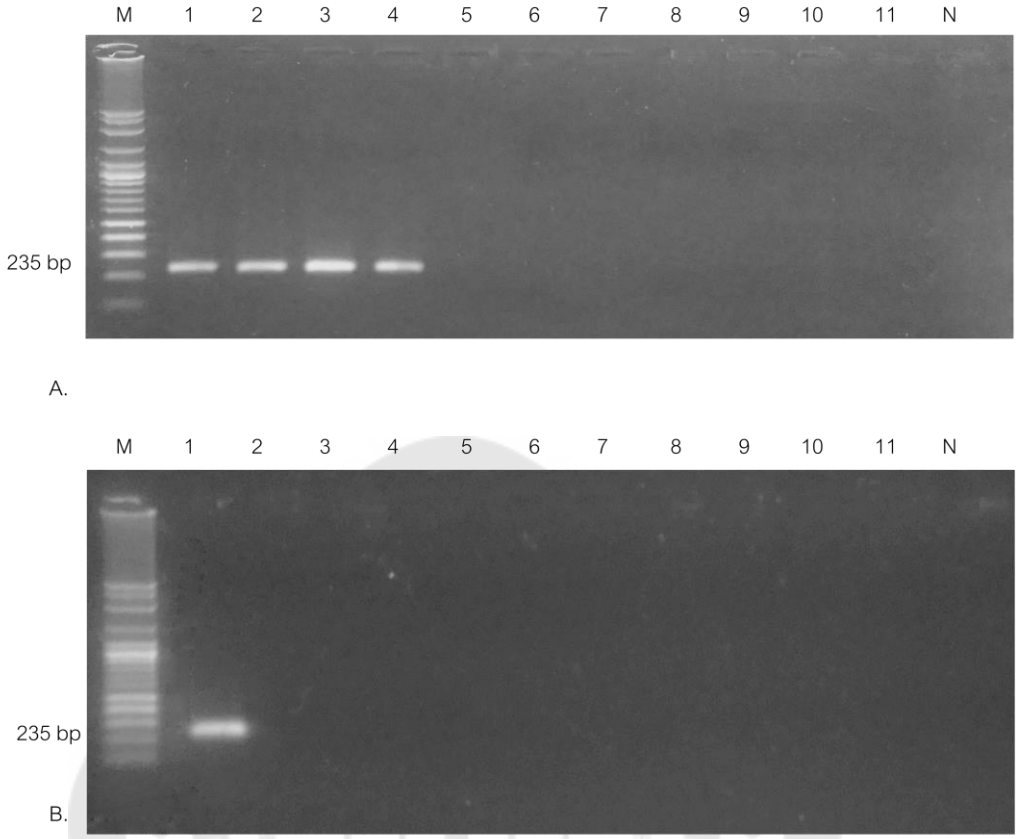



FIGURE 46 The specificity tests for the ER primer set. The letter M referred to 100 bp marker, N referred to negative control. A: (1) *E. revolutum* egg; (2) *E. revolutum* cercaria; (3) *E. revolutum* metacercaria; (4) adult of *E. revolutum* (5) *E. cinetorchis*; (6) *E. ilocanum*; (7) *I. hortensis* (*E. hortense*); (8) *Ec. japonicus*; (9) *T. anastomusa*; (10) *H. taichui*; and (11) *S. falcatus*, respectively. B: The specificity test for *E. revolutum* hosts include the following: (1) DNA of *E. revolutum*; (2) *L. auricularia*; (3) *F. sumatrensis polygramma*; (4) *F. martensi*; (5) *I. exustus*; (6) *B. siamensis*; (7) *C. helena*; (8) *P. roborovskii*; (9) *G. gallus*; and (10) *A. platyrhynchos*.

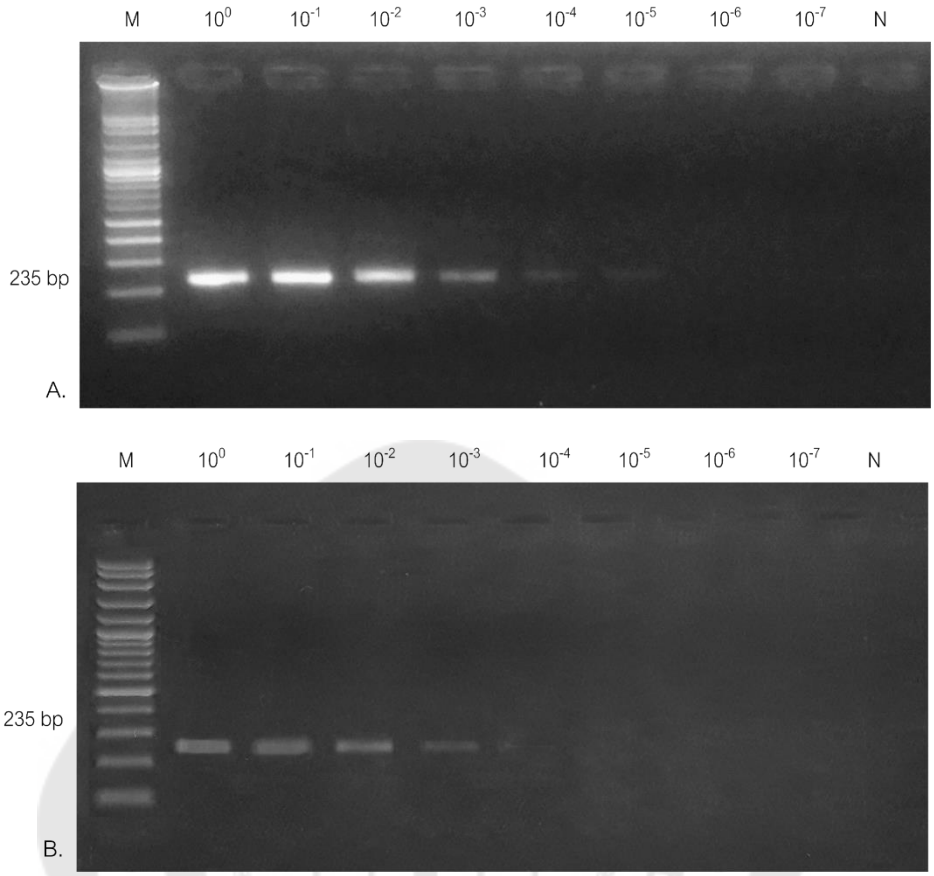



FIGURE 47 PCR results of the sensitivity testing of the ER primer set. A: *E. revolutum* DNA, B: *E. revolutum* and the intermediate host DNA mixture.

**Phylogenetic relationship analyses**

The nucleotide sequences of *CYTB* were trimmed to 793 base pairs for the purposes of accuracy in constructing a representation of the phylogenetic relationships. Base composition of the sequences were computed by Genomics %G~C Content Calculator program (https://www.sciencebuddies.org/science-fair-projects/ref erences /genomics-g-c-content-calculator) and the result shown A-T biased (63.4%) with A is the most common (46.26%) and G as the least (11.03%). The sequences were aligned to examine the nucleotide differences by Multalin program (version 5.4.1; http://multalin.toulouse.inra.fr/multalin/multalin.html) and the results showed that nucleotide sequences from all specimens are mostly identical with some differences, depending on the location sites that the specimens were collected from (Figure 48). The maximum-likelihood (ML) tree of *CYTB* sequences that amplified by EchCB primer set revealed three separating clades of *Echinostoma, Isthmiophora* and *Echinochasmus* species. Five adult specimens of *E. revolutum* from five provinces in Central Thailand were clustered together in *E. revolutum* clade which was distinct from other genera. Moreover, *E. revolutum* lineage was divided into two sister groups according to their localities consisting of the Eastern and Western provinces in Central Thailand (Figure 49).

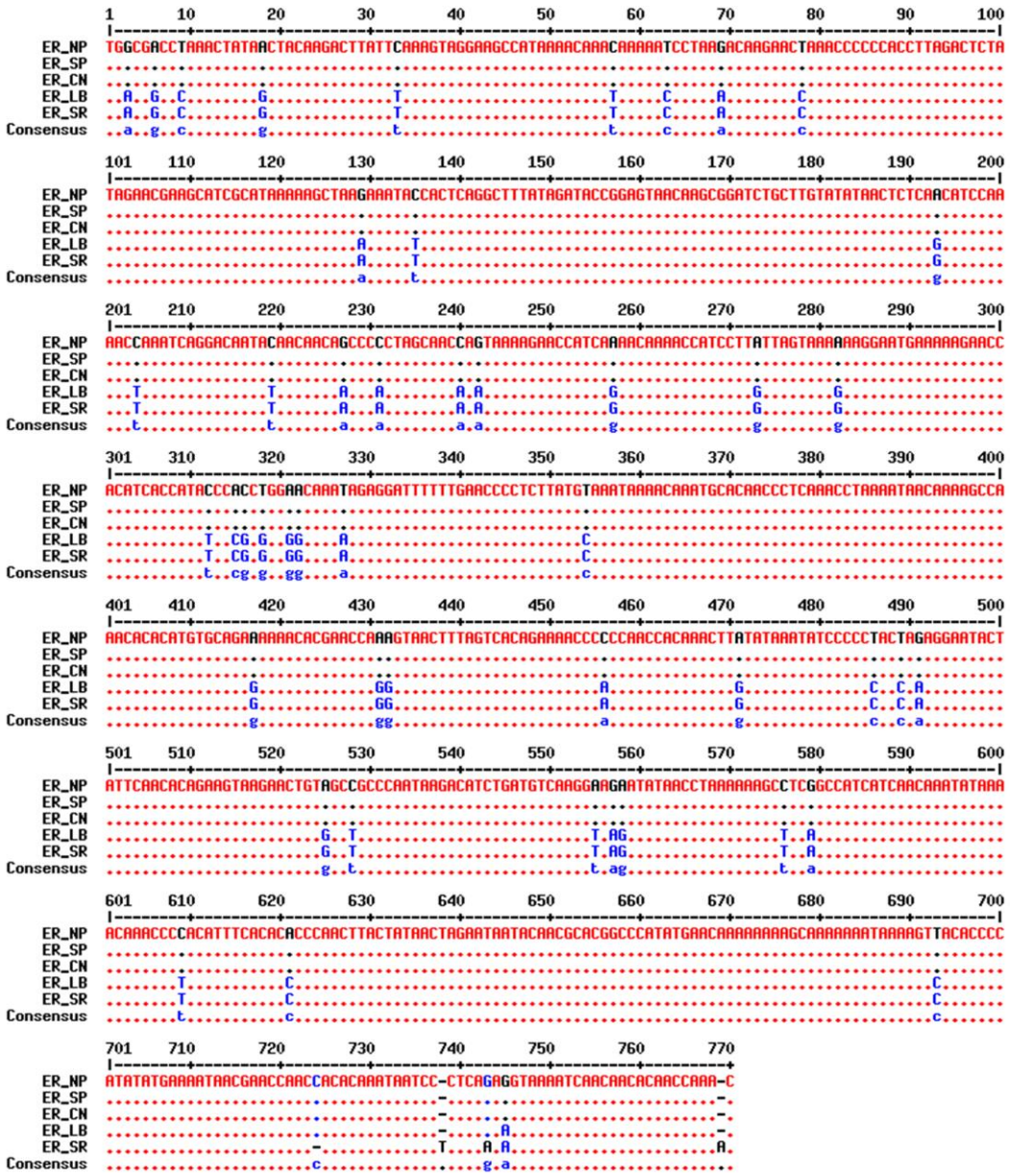


FIGURE 48 The partial *CYTB* sequences of 5 adult specimens from each province in the central, Thailand. A dot (.) indicates homology. The abbreviation ER_NP, SP, CN, LB, SR are referred to *E. revolutum* specimens from Nakhon Pathom, Suphanburi, Chainat, Lopburi and Saraburi provinces, respectively.

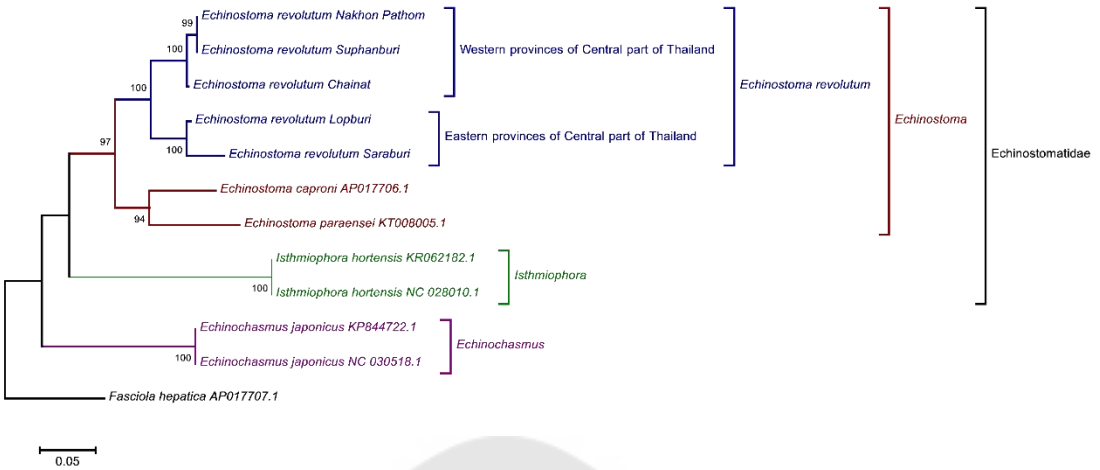



FIGURE 49 The ML tree of *E. revolutum* and other related species based on CYTB gene. The nodal support was estimated using 10,000 bootstrap re-sampling.

**Species-specific detection of *Echinostoma revolutum***

The specie-specific detection based on ER primer set were used to discriminate the larva of *E. revolutum* from related species with similar morphological appearances. The result revealed the echinostome cercariae in ten collecting sites could not be amplified with the ER primer set (Table 13). For the *E. revolutum* metacercaria, morphological characteristics of *E. revolutum* can be discriminated from other metacercariae. However, the metacercariae collected from *B. siamensis* in one collecting site of Tha Ruea district, Ayutthaya province could not be amplified with these primers. The result of species-specific-amplification that verified the species of echinostome cercariae and *E. revolutum* metacercariae were shown in Figure 50.

TABLE 13 The overall echinostome infected sites in the study area. Each infection site was verified by species-specific primer to discriminate *E. revolutum* from other species.

| Provinces | Districts (Site No.) | Snail species | Larval stage of worms | *E. revolutum* |
|---|---|---|---|---|
| Ang Thong (AT) | Mueang Ang Thong (AT3) | *Filopaludina martensi* | Metacercaria | Positive |
| Ayutthaya (AY) | Tha Ruea (AY1) | *Bithynia siamensis* | Metacercaria | Negative |
| | Uthai (AY8) | *B. siamensis* | Cercaria | Negative |
| | Bang Pahan (AY15) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Sena (AY31) | *Lymnaea auricularia* | Cercaria | Negative |
| | Sena (AY31) | *B. siamensis* | Cercaria | Negative |
| | Phra Nakhon Si Ayutthaya (AY34) | *Tarebia granifera* | Cercaria | Negative |
| Chainat (CN) | Manorom (CN1) | *Segmentina sp.* | Cercaria | Negative |
| | Manorom (CN2) | *L. auricularia* | Cercaria | Positive |
| | Mueang Chainat (CN4) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Sapphaya (CN5) | *L. auricularia* | Cercaria | Positive |
| | Sapphaya (CN6) | *L. auricularia* | Cercaria | Positive |
| Lopburi (LB) | Nong Muang (LB1) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Sa Bot (LB5) | *Indoplanorbis exustus* | Metacercaria | Positive |
| | Khok Samrong (LB8) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Chai Badan (LB14) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Tha Luang (LB18) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Ban Mi (LB19) | *L. auricularia* | Cercaria | Positive |
| | Tha Wung (LB21) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Nakhon Nayok (NY) | Ban Na (NY4) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Ongkharak (NY5) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Ongkharak (NY6) | *F. martensi* | Metacercaria | Positive |
| | Pak Phli (NY8) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Nakhon Pathom (NP) | Bang Lan (NP4) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Mueang Nakhon Pathom (NP10) | *F. martensi* | Metacercaria | Positive |
| | Nakhon Chai Si (NP13) | *F. martensi* | Metacercaria | Positive |
| | Nakhon Chai Si (NP13) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Nakhon Sawan (NS) | Tha Tako (NS5) | *I. exustus* | Cercaria | Negative |
| | Banphot Phisai (NS15) | *L. auricularia* | Cercaria | Positive |
| | Phayuha Khiri (NS27) | *L. auricularia* | Cercaria | Positive |
| | Takhli (NS29) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Pathum Thani (PT) | Sam Khok (PT12) | *L. auricularia* | Cercaria | Positive |
| | Lat Lum Kaeo (PT13) | *L. auricularia* | Cercaria | Positive |
| Samut Sakhon (SS) | Ban Phaeo (SS4) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Ban Phaeo (SS5) | *F. sumatrensis polygramma* | Metacercaria | Positive |

TABLE 13 (continued)

| Provinces | Districts (Site No.) | Snail species | Larval stage of worms | *E. revolutum* |
|---|---|---|---|---|
| Saraburi (SR) | Nong Saeng (SR3) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Nong Saeng (SR4) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Mueang Saraburi (SR5) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Mueang Saraburi (SR5) | *F. martensi* | Metacercaria | Positive |
| | Sao Hai (SR7) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Mueang Saraburi (SR12) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Chaloem Phra Kiat (SR14) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Phra Phutthabat (SR16) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Kaeng Khoi (SR17) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Wihan Daeng (SR19) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Wihan Daeng (SR20) | *L. auricularia* | Cercaria | Positive |
| | Nong Don (SR21) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Nong Don (SR22) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Muak Lek (SR24) | *L. auricularia* | Cercaria | Positive |
| Sing Buri (SB) | Khai Bang Rachan (SB3) | *F. martensi* | Metacercaria | Positive |
| | Phrom Buri (SB9) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Phrom Buri (SB10) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Suphanburi (SP) | Song Phi Nong (SP3) | *L. auricularia* | Cercaria | Positive |
| | U Thong (SP6) | *I. exustus* | Cercaria | Negative |
| | Bang Pla Ma (SP9) | *I. exustus* | Cercaria | Negative |
| | Dan Chang (SP14) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Nong Ya Sai (SP15) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| | Sam Chuk (SP17) | *F. sumatrensis polygramma* | Metacercaria | Positive |
| Uthai Thani | Huai Khot (UT6) | *Melanoides tuberculata* | Cercaria | Negative |
| | Nong Chang (UT10) | *F. martensi* | Metacercaria | Positive |
| | Nong Chang (UT10) | *L. auricularia* | Cercaria | Positive |
| | Nong Khayang (UT13) | *I. exustus* | Cercaria | Negative |

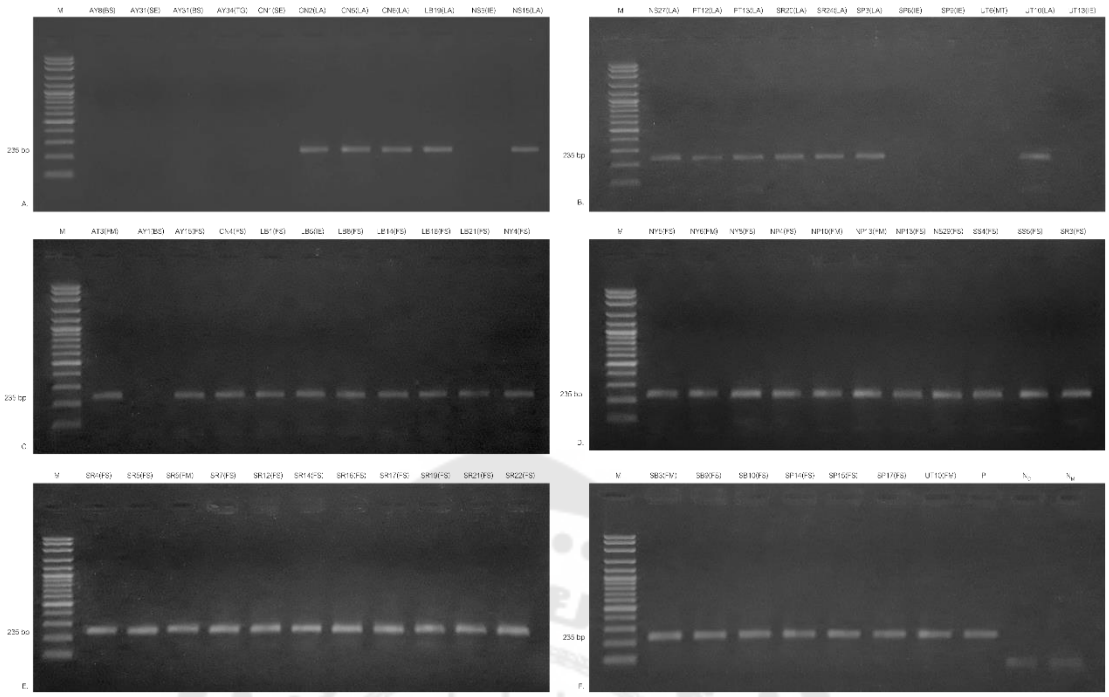


FIGURE 50 Species-specific amplification of echinostome larvae in each infected site, A: – B the PCR product of echinostome cercaria, C – F: the PCR product of metacercaria. DNA of adult *E. revolutum* was used as positive control (P) $N_C$ and $N_M$ are negative control for cercaria and metacercaria (distilled water).

**The epidemiological map of each province in the study area**

This study used both morphological and molecular methods for discriminating *E. revolutum* larvae from other echinostome larvae. The species-specific primer based on *CYTB* used to construct the epidemiological map of *E. revolutum* in each province as below.

**Ang Thong province**

The morphology showed that only Mueang Ang Thong districts was found the metacercaria infection. The prevalence of infection in *Filopaludina martensi* was 32.00% (16/50) and mean intensity was 11. These metacercariae were confirmed by the molecular study as *E. revolutum*. The comparative of epidemiological maps of this province were shown in Figure 51.

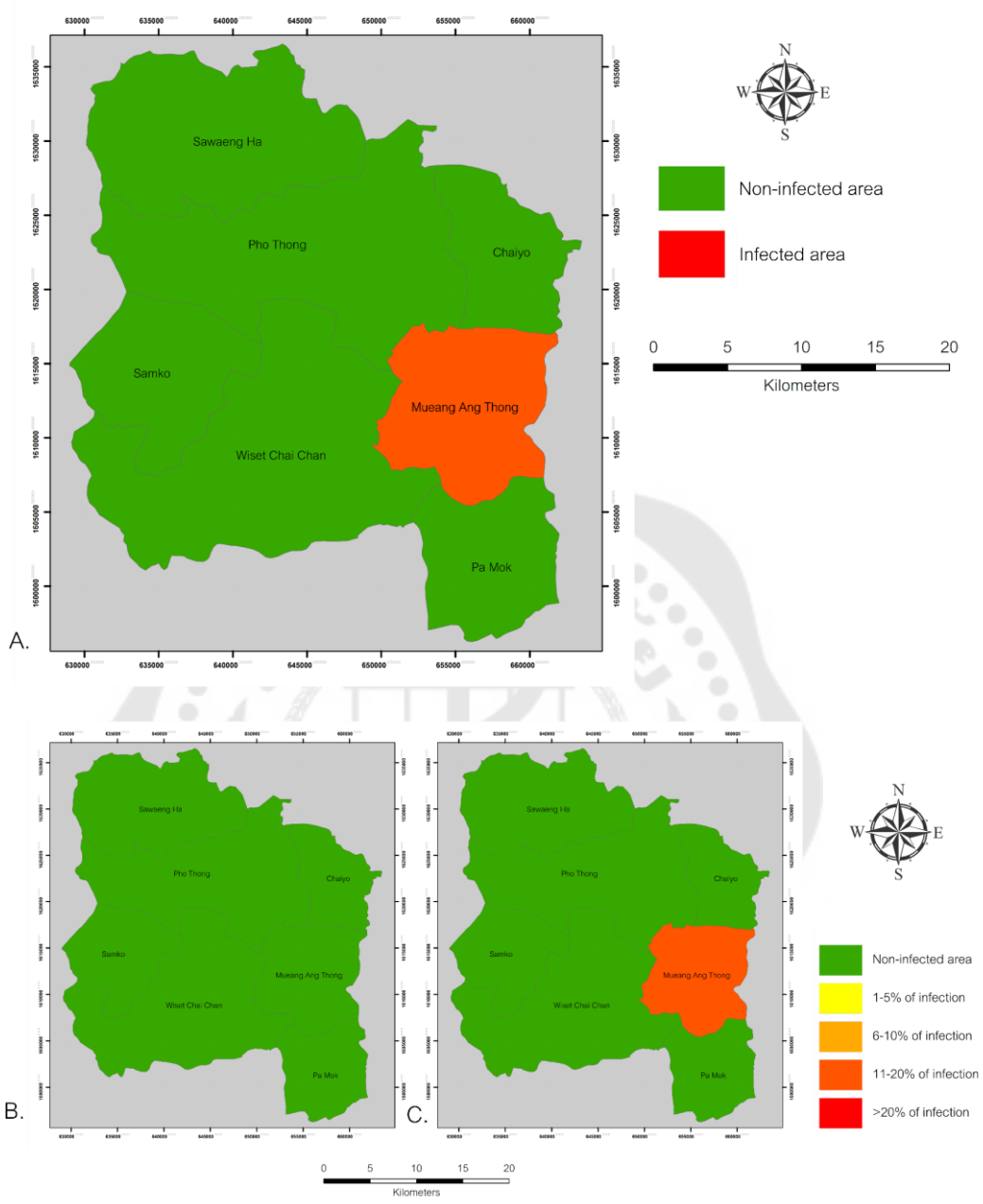



FIGURE 51 The epidemiological map of *E. revolutum* in Ang Thong province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Ayutthaya province**

Five out of sixteen districts were positive for echinostome larval stage based on morphological study, comprising Phra Nakhon Si Ayutthaya, Sena and Uthai (three for echinostome cercaria and two for metacercaria). For the echinostome cercariae, Phra Nakhon Si Ayutthaya, it was found only in *T. granifera* with a prevalence of 8.00% (4/50). For Sena district, *B. siamensis* and *L. auricularia* were infected with prevalence of 8.00% (4/50) and 2.00% (1/50), respecitively. Uthai district, *B. siamensis* were infected with prevalence of 4.00% (2/50).

The metacercariae were found in Bang Pahan (*F. sumatrensis polygramma*; prevalence was 10.00% (2/20) and mean intensity was 6) and Tha Ruea (*B. siamensis* prevalence was 2.00% (1/50) and mean intensity was 3). However, the molecular results suggested that echinostome cercaria was not *E. revolutum.* Only metacercariae from one *F. sumatrensis polygramma* collected from Bang Pahan can be amplified with the species-specific primers. The comparative of epidemiological maps were shown in Figure 52

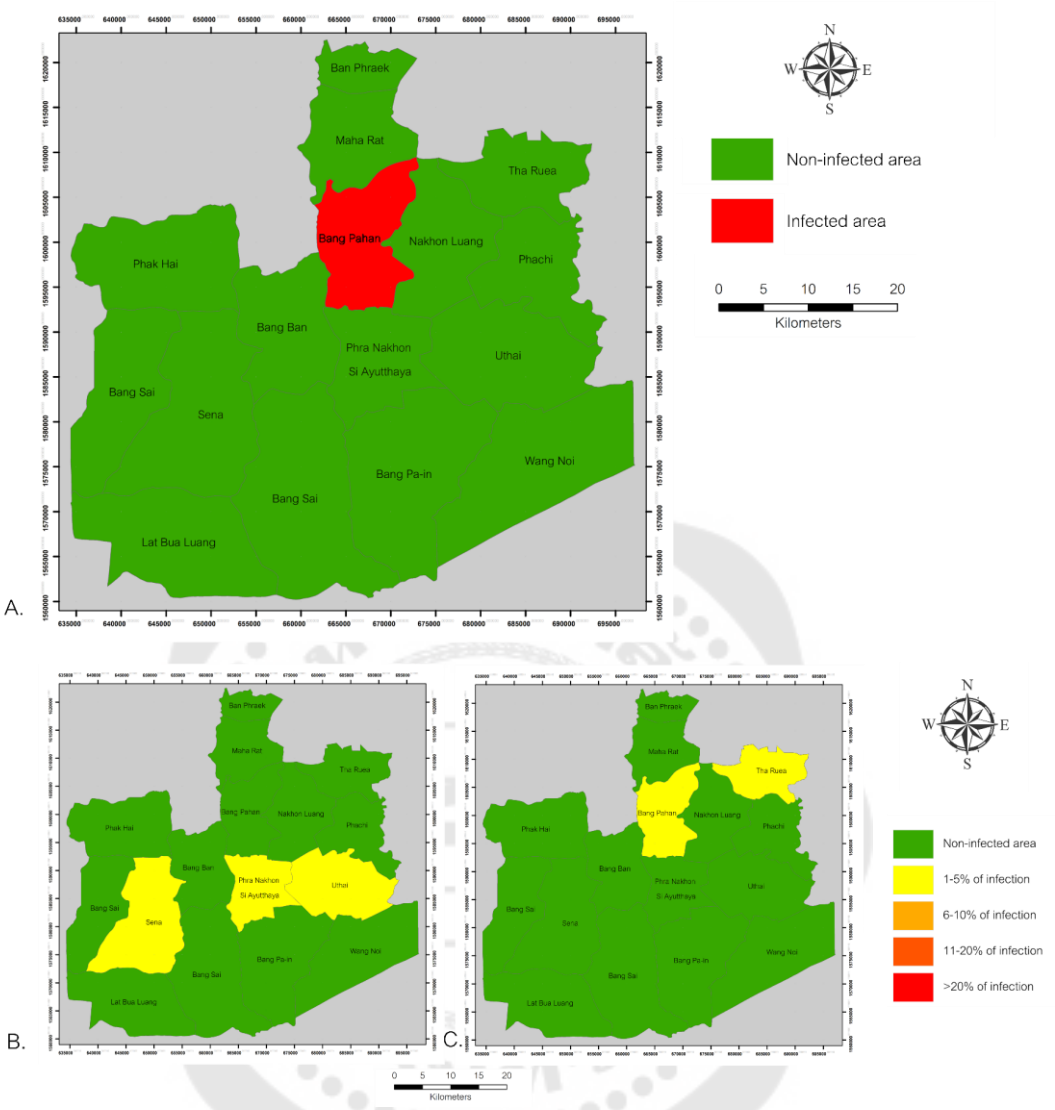



FIGURE 52 The epidemiological map of *E. revolutum* in Ayutthaya province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Chainat province**

Three districts were positive for the echinostome infection. Four snail species; *B. siamensis*, *I. exustus*, *L. auricularia*, and *Segmentina* sp. were collected from Manorom district. Two of them were infected by echinostome cercaria (*L. auricularia*, and *Segmentina* sp.) with the prevalence value at 3.13% (1/32) and 2.00% (1/50), respectively. *F. sumatrensis polygramma* in Mueang Chainat district had the metacercariae with a prevalence of 14.00% (7/50) and mean intensity was 6. For Sapphaya district, *L. auricularia* had high infection rate of echinostome cercariae (11/65). The molecular results revealed that only echinostome cercariae collected from *Segmentina* sp. was not *E. revolutum.* The comparative of epidemiological maps were shown in Figure 53.

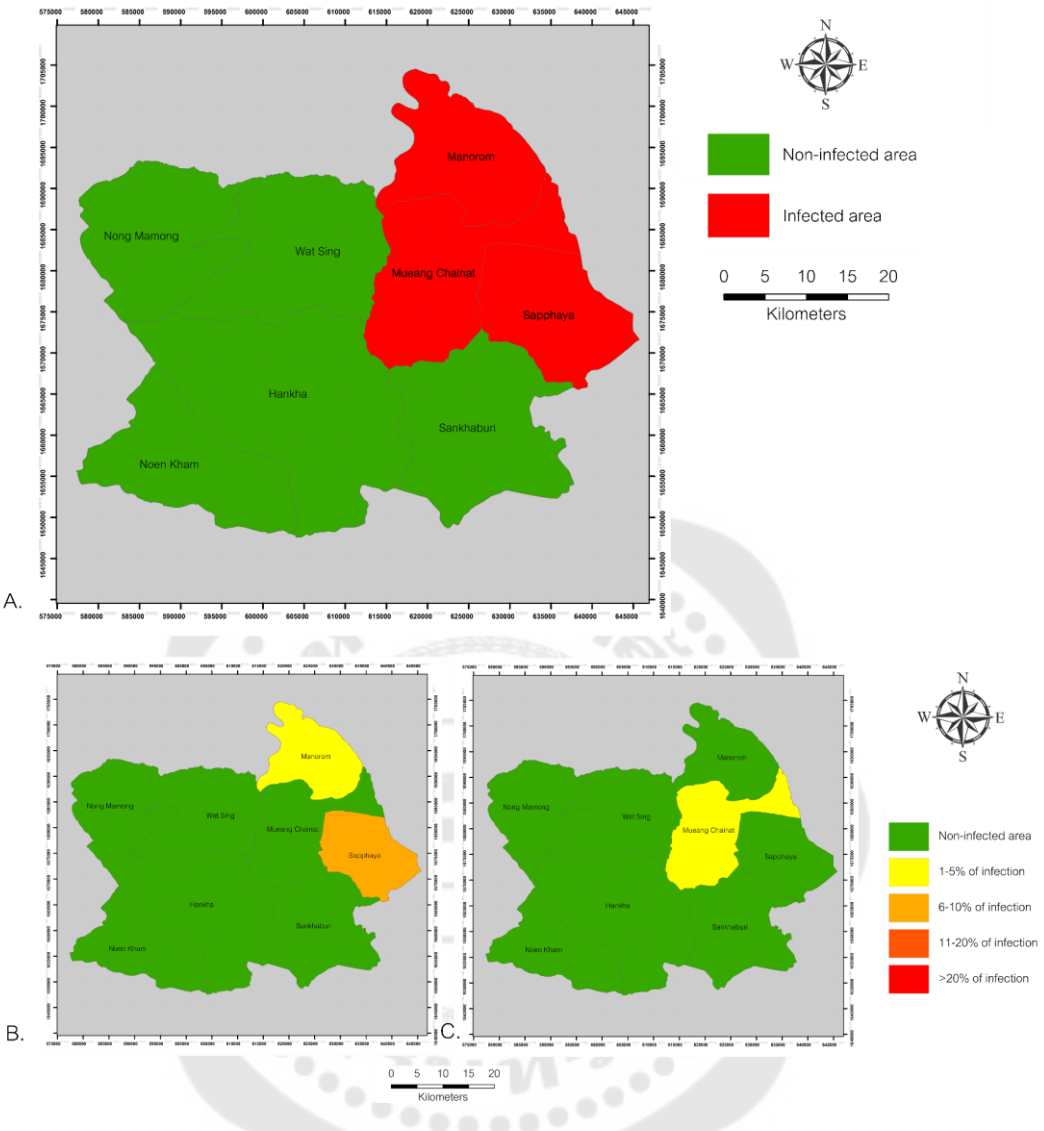



FIGURE 53 The epidemiological map of *E. revolutum* in Chainat province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Lopburi province**

The morphological results revealed that Lopburi province had the highest metacercaria prevalence, nearly 100.00% in some sites. Seven districts were found the infection of echinostome larvae. The metacercarial stage infection were found in Chai Badan, Khok Samrong, Nong Muang, Sa Bot, Tha Luang and Tha Wung. The prevalence of each district was 81.00%(81/100), 2.00% (1/50), 2.00% (1/50), 2.00% (1/50), 98.00% (147/150) and 9.38% (3/32), with the mean intensity 15, 23, 1, 7, 21 and 7, respectively. Most of metacercariae were found in *F. sumatrensis polygramma* and some of metacercariae were found in *I. exustus*. The infection of echinostome cercariae was found in *L. auricularia* from Ban Mi district with prevalence of 4.00% (2/50). The molecular data confirmed that all districts are infected with *E. revolutum.* The comparative of epidemiological maps were shown in Figure 54.

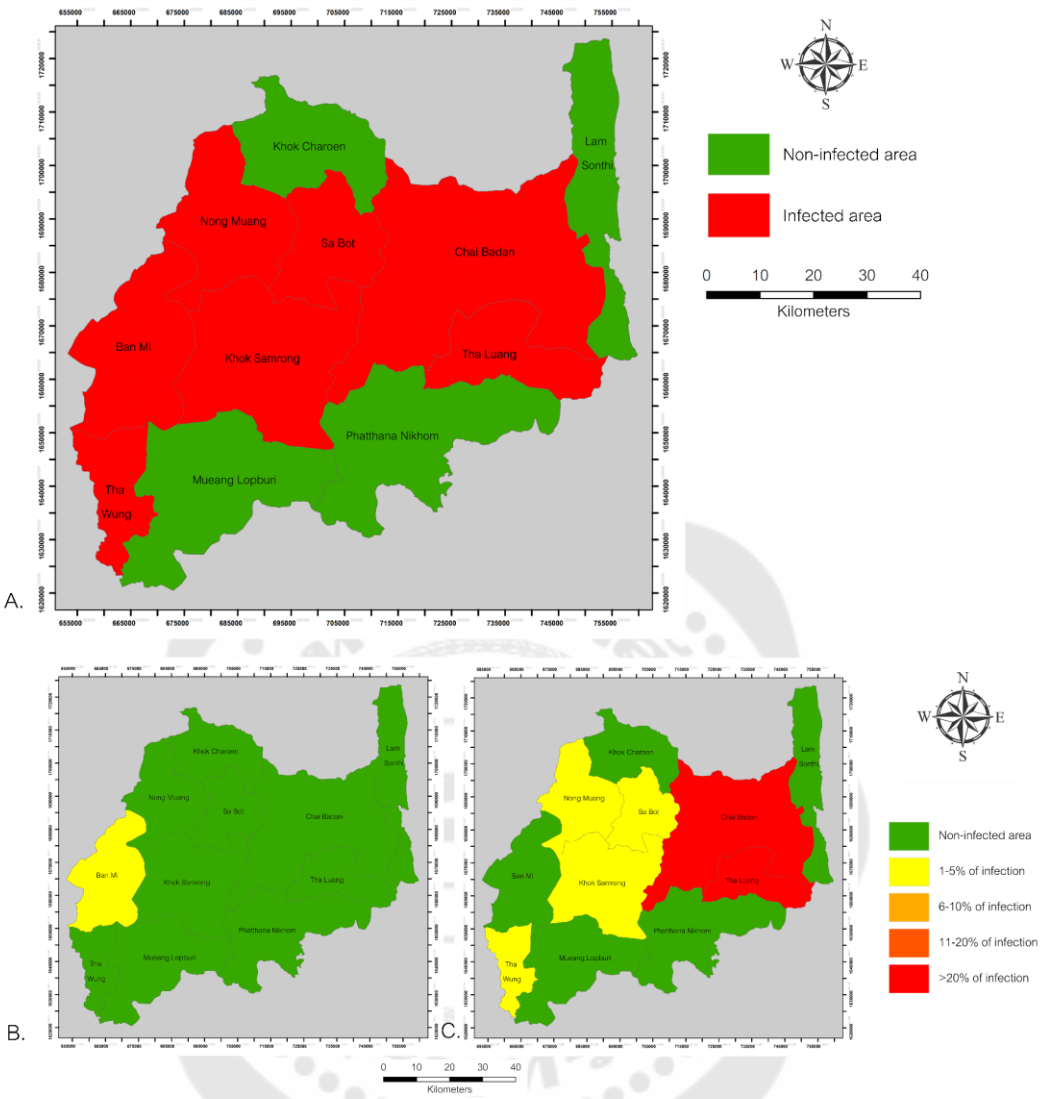



FIGURE 54 The epidemiological map of *E. revolutum* in Lopburi province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Nakhon Nayok province**

The metacercariae infected in *Filopaludina* snails were *E. revolutum* based on both of morphological and molecular methods. *Filopaludina* snails collected from three districts, namely Ban Na, Ongkharak, and Pak Phli were infected with *E. revolutum* metacercariae. The result found that *F. sumatrensis polygramma* in Ban Na district was infected with prevalence of 4.00% (2/50), mean intensity was 10. *F. sumatrensis polygramma* and *F. martensi* from Ongkharak district infected with metacercarial stage of *E. revolutum* with prevalence of 6.00% (3/50) and 4.00% (2/50), respectively (mean intensity was 2 and 16). The comparative of epidemiological maps were shown in Figure 55.

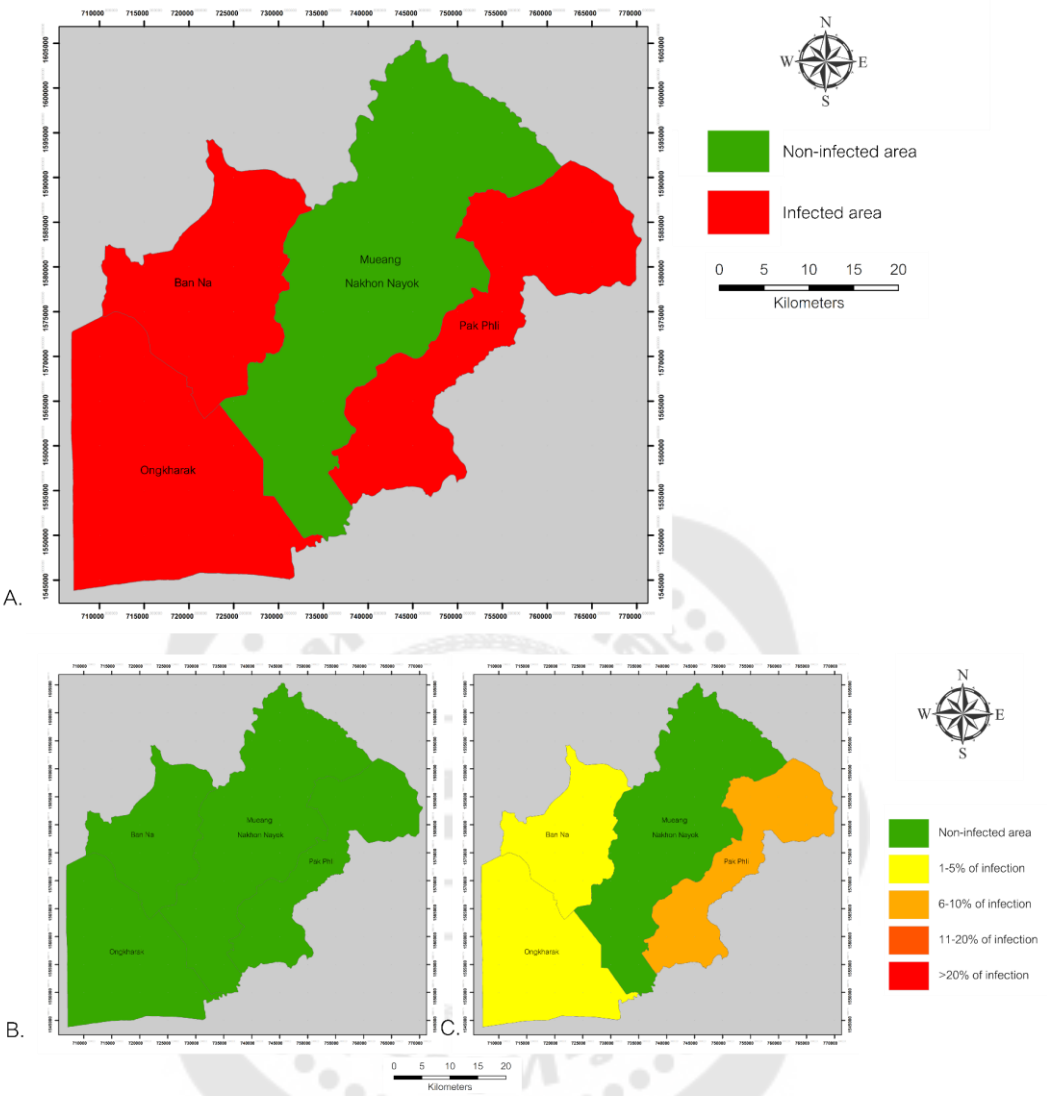



FIGURE 55 The epidemiological map of *E. revolutum* in Nakhon Nayok province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Nakhon Pathom province**

Most of infection site in this province had high prevalence value than 20.00%. Echinostome lavae infection was found in three districts comprising, Bang Len, Mueang Nakhon Pathom and Nakhon Chai Si. The prevalence of infection in *F. sumatrensis polygramma* collected from Bang Len district was 2.00% (1/50) and mean intensity was 9. The prevalence of infection in *F. sumatrensis polygramma* and *F. martensi* collected from Nakhon Chai Si district were 34.00% (17/50) and 21.31% (13/61) and mean intensity was 18 and 6, respectively. Moreover, *F. martensi* in Mueang Nakhon Pathom had a infection rate at 42.00% (21/50). The specimen from all infection districts were verified by PCR and the result showed all of them as *E. revolutum.* The comparative of epidemiological maps were shown in Figure 56.

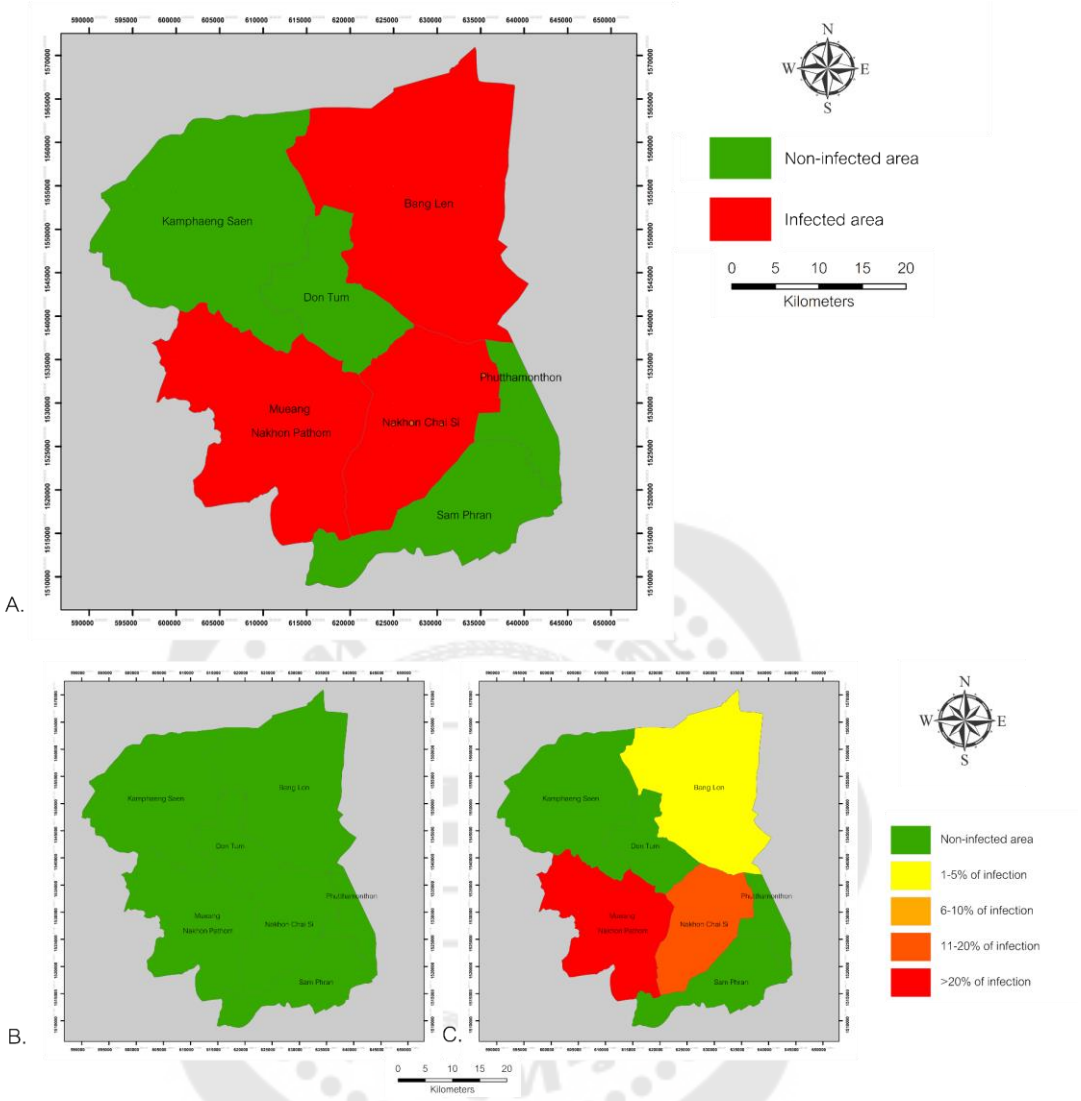



FIGURE 56 The epidemiological map of *E. revolutum* in Nakhon Pathom province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Nakhon Sawan province**

This province contained the highest number of snail species. Three districts were infected by echinostome cercaria comprising, Banphot Phisai (*L. auricularia* with prevalence of 1.65% (2/121)), Phayuha Khiri (*L. auricularia* with prevalence of 4.00% (2/50)) and Tha Tako (*I. exustus* with prevalence of 3.57% (2/56)). The molecular results showed that echinostome cercariae from Tha Tako district that collected from *I. exustus* was not *E. revolutum*. Only *F. sumatrensis polygramma* from Takhli district was infected by metacercariae of *E. revolutum* with the prevalence of 2.00% (1/50) and mean intensity was one. The comparative of epidemiological maps were shown in Figure 57.

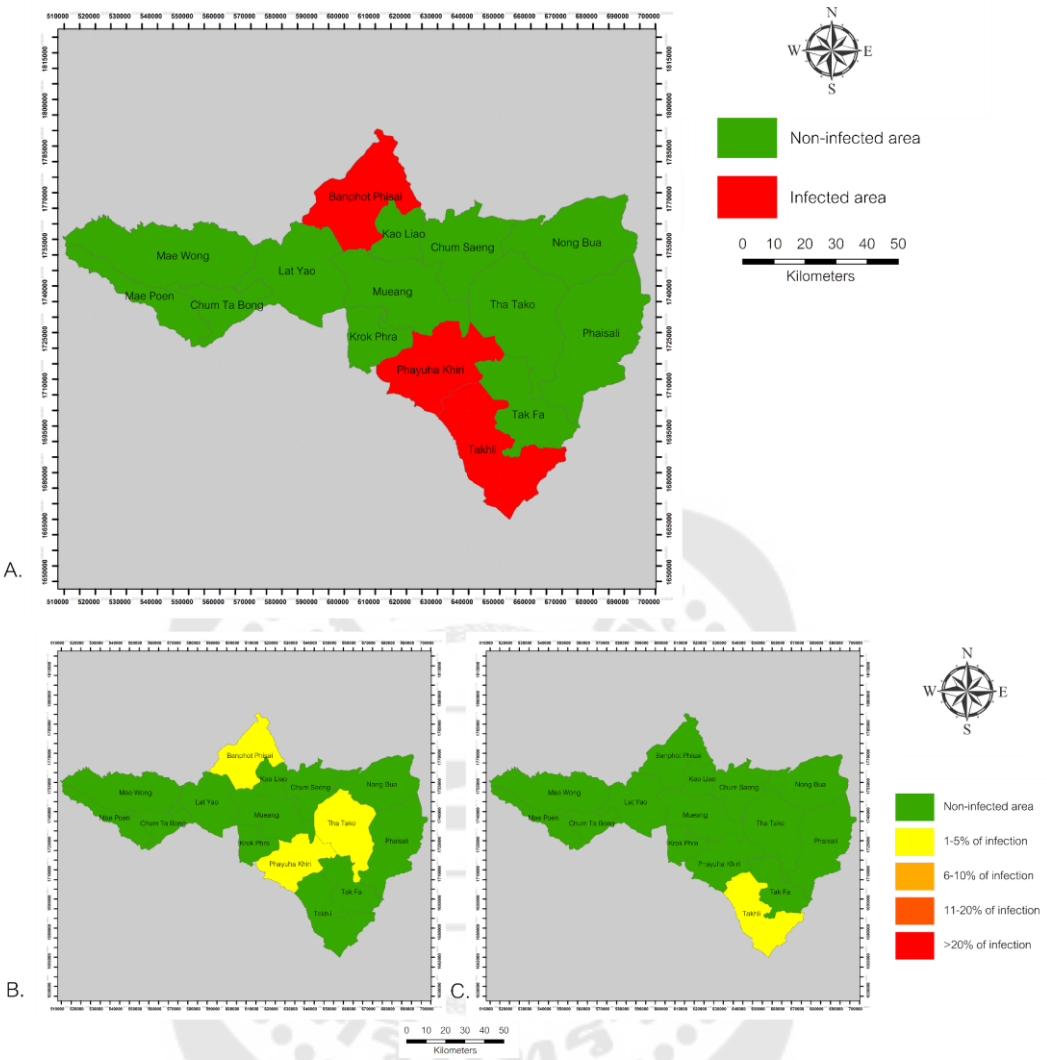



FIGURE 57 The epidemiological map of *E. revolutum* in Nakhon Sawan province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Pathum Thani province**

This province was found five number of snail species. Only *L. auricularia* in Lat Lum Kaeo and Sam Khok districts were infected by echinostome cercaria with prevalence of 2.00% (1/50) and 4.00% (2/50), respectively. For the molecular identification of echinostome cercaria, the result showed that the specimens from all sites were *E. revolutum*. The comparative of epidemiological maps were shown in Figure 58.

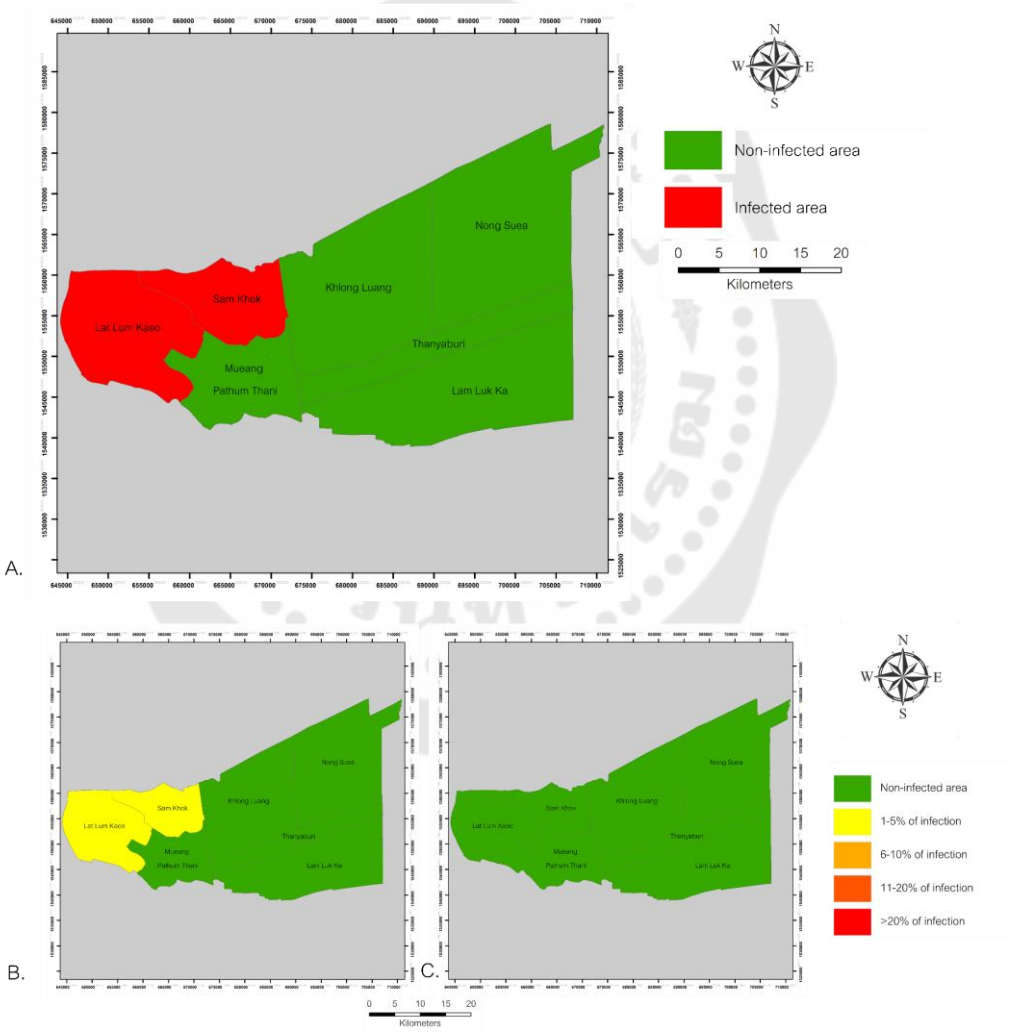



FIGURE 58 The epidemiological map of *E. revolutum* in Pathum Thani province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Samut Sakhon province**

The result shown two collecting sites in Ban Phaeo district was found the infection of metacercarial stage in *F. sumatrensis polygramma* with the prevalence of 10.00% (5/50) and 6.00% (3/50), respectively (mean intensity was 7 and 4). The PCR from all infection sites showed the positive result. The comparative of epidemiological maps were shown in Figure 59.

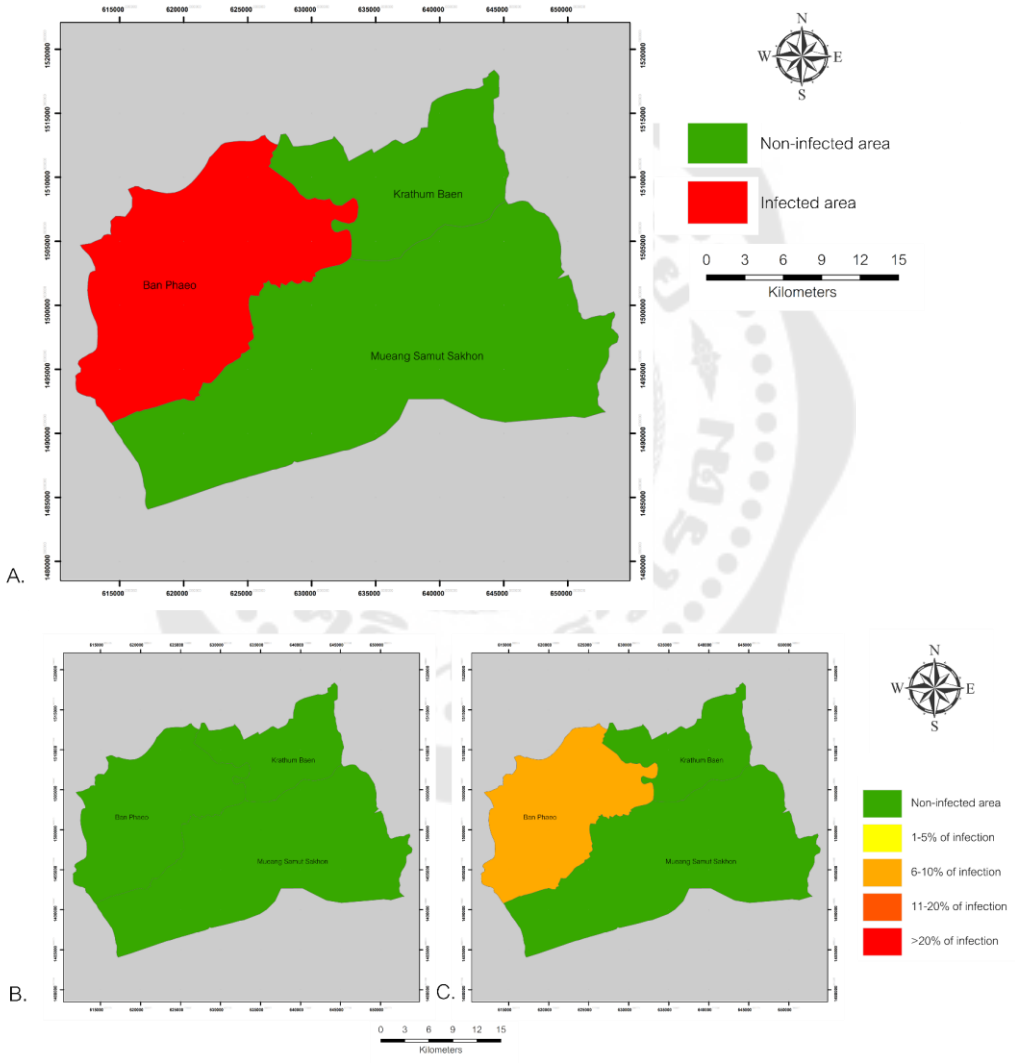



FIGURE 59 The epidemiological map of *E. revolutum* in Samut Sakhon province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Saraburi province**

Three snail species collected from nine out of thirteen districts were infected by echinostome cercaria and metacercariae, namely *L. auricularia*, *F. sumatrensis polygramma* and *F. martensi*. The prevalence of cercariae infection of Muak Lek and Wihan Daeng was 10.67% (16/150) and 10.00% (15/150). The highest infection rate of metacercarial stage was found in Chaloem Phra Kiat district, that had prevalence of infection at 22.33% (35/150) and mean intensity was 15. For other districts, Mueang Saraburi, Sao Hai, Nong Saeng, Nong Don, Phra Phutthabat, Wihan Daeng and Kaeng Khoi districts were infected by metacercaria with prevalence of 14.12% (25/177), 7.39% (17/230), 7.37% (14/190), 5.80% (13/224), 2.84% (6/211), 2.00% (3/150) and 1.04% (2/193), respectively. The mean intensity of these districts were 13, 9, 4, 5, 3, 5 and 11 respecitively. The PCR from all infection sites in this province showed the positive result. The comparative of epidemiological maps were shown in Figure 60.

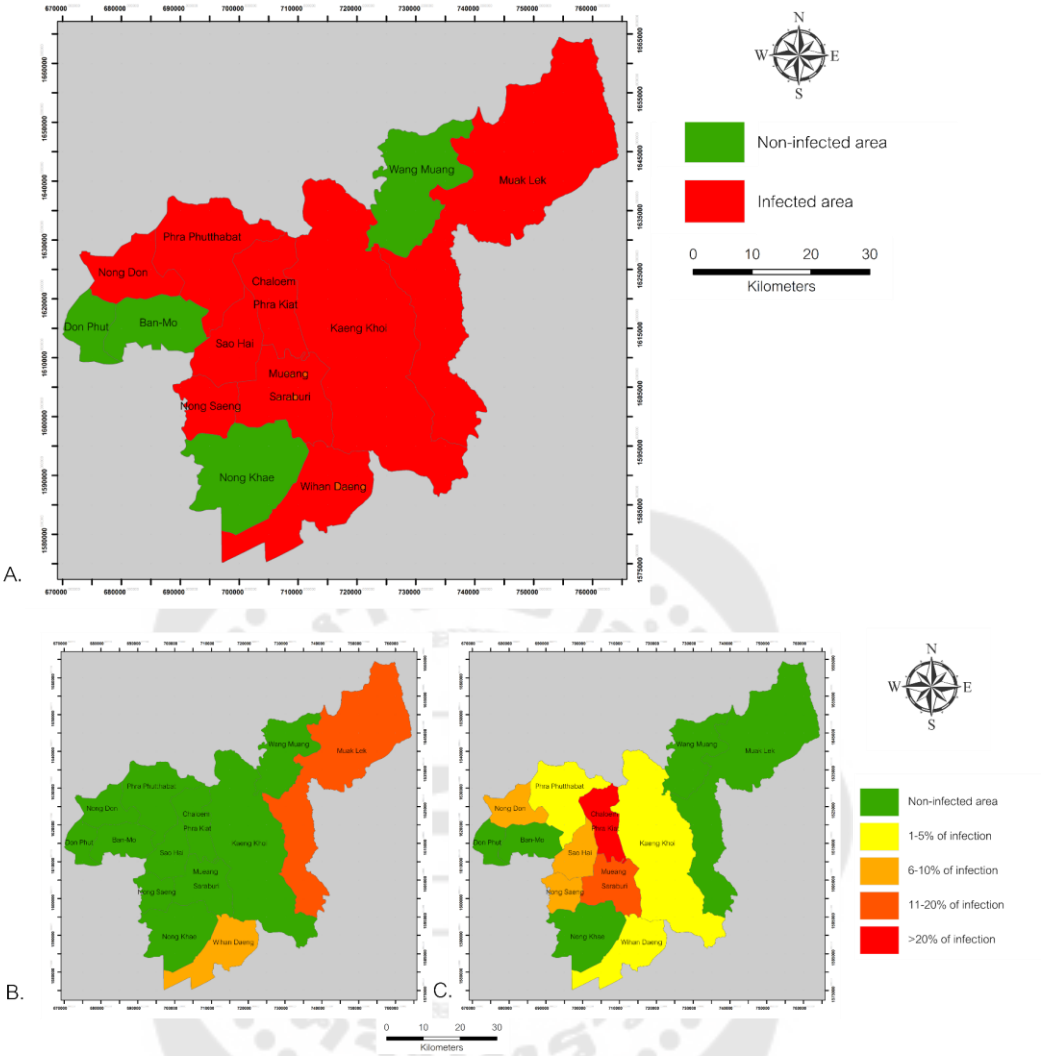



FIGURE 60 The epidemiological map of *E. revolutum* in Saraburi province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

Sing Buri province

Two species of *Filopaludina* snails from Khai Bang Rachan and Phrom Buri districts were infected by *E. revolutum* metacercariae. The prevalence of infection in Khai Bang Rachan and Phrom Buri districts was 2.59% (5/193), 5.74% (12/209) and mean intensity was 16 and 8, respecitively. The PCR from all infection sites showed the positive result. The comparative of epidemiological maps were shown in Figure 61

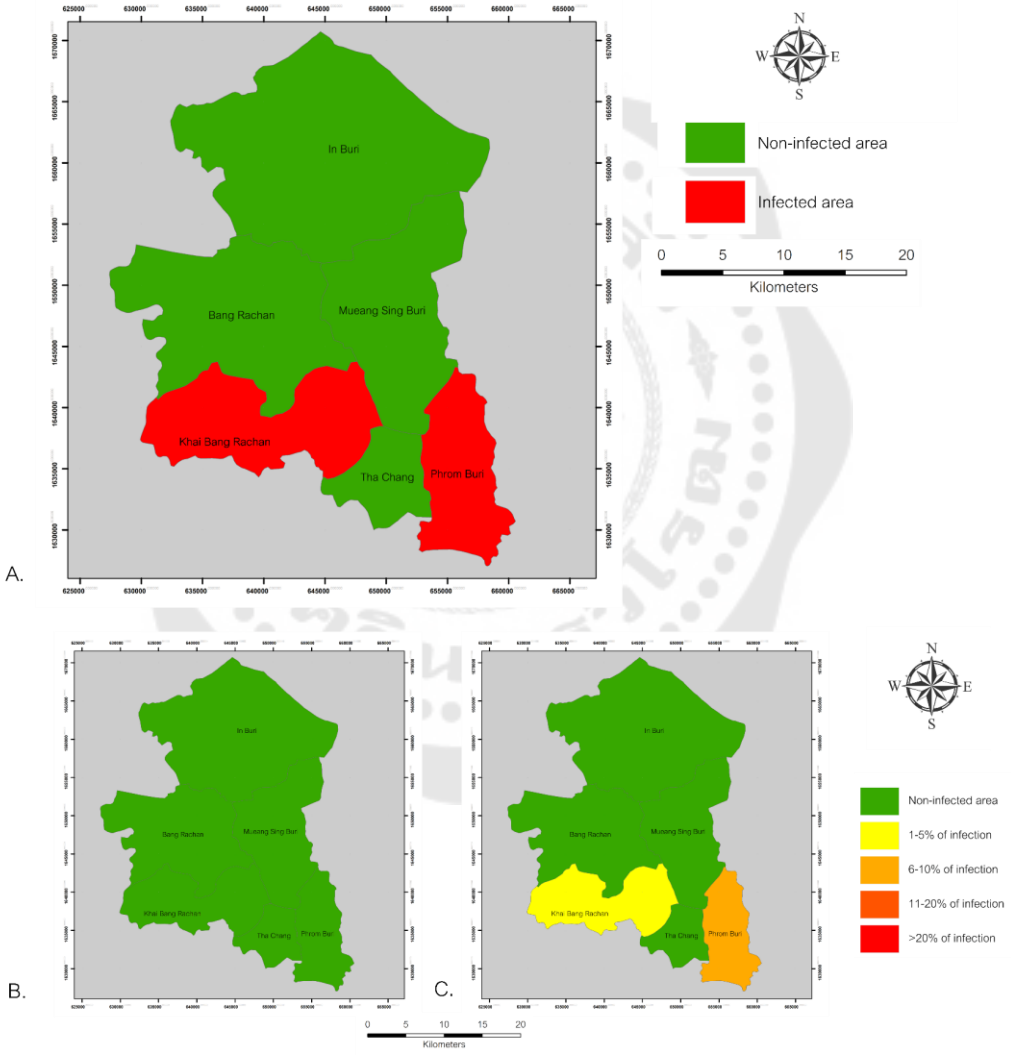



FIGURE 61 The epidemiological map of *E. revolutum* in Sing Buri province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Suphanburi province**

Six out of ten districts were found the infection of echinostome larvae. Three snail species were infected, *I. exustus* and *L. auricularia* were first intermediate host and *F. sumatrensis polygramma* was second intermediate host. The prevalence of cercariae infection of Bang Pla Ma, Song Phi Nong and U Thong was 2.07% (2/283), 1.38% (4/289) and 1.19% (5/419), respectively

Three districts were found the infection of metacercariae in *F. sumatrensis polygramma*. The infection were found in Dan Chang, Sam Chuk and Nong Ya Sai with the prevalence of 12.59% (18/143), 5.70% (9/158), 2.60% (6/231) and mean intensity was 12, 11 and 20, respectively. The molecular results showed echinostome cercaria from

*L. auricularia* and metacercaria from *F. sumatrensis polygramma* are *E. revolutum*. The comparative of epidemiological maps were shown in Figure 62

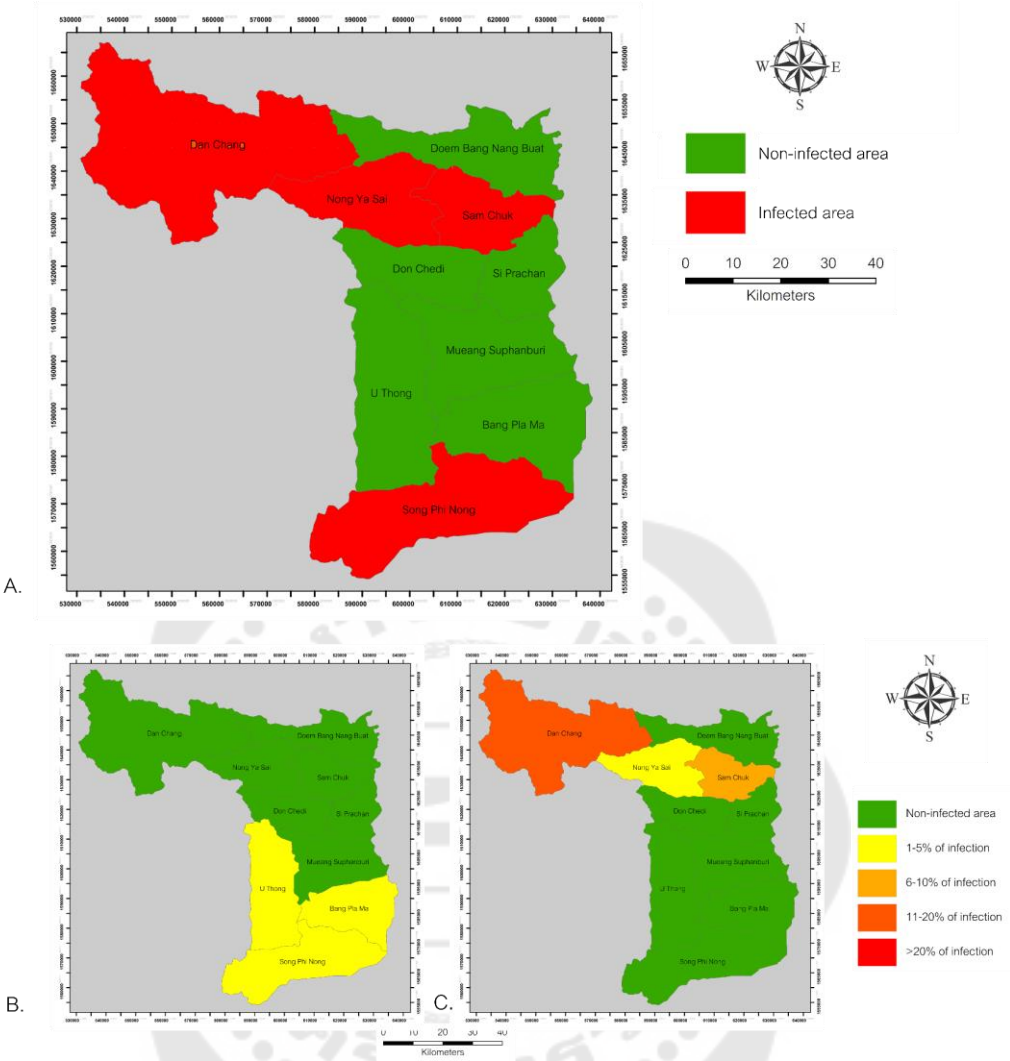



FIGURE 62 The epidemiological map of *E. revolutum* in Suphanburi province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

**Uthai Thani province**

Three districts were found the echinostome cercaria infection, comprising Nong Khayang, Huai Khot and Nong Chang with the prevalence of 5.29% (9/170),1.82% (2/110) and 1.30% (3/231). Only Nong Chang district was found the metacercaria infection in *F. martensi*. The prevalence of metacercaria was 1.73% (4/231) and mean intensity was 2. The PCR result showed both metacercaria from *F. martensi* and echinostome cercaria from *L. auricularia* in Nong Chang were *E. revolutum.* The comparative of epidemiological maps were shown in Figure 63 and the overall infection area of *E. revolutum* in Central Thailand were shown in Figure 64

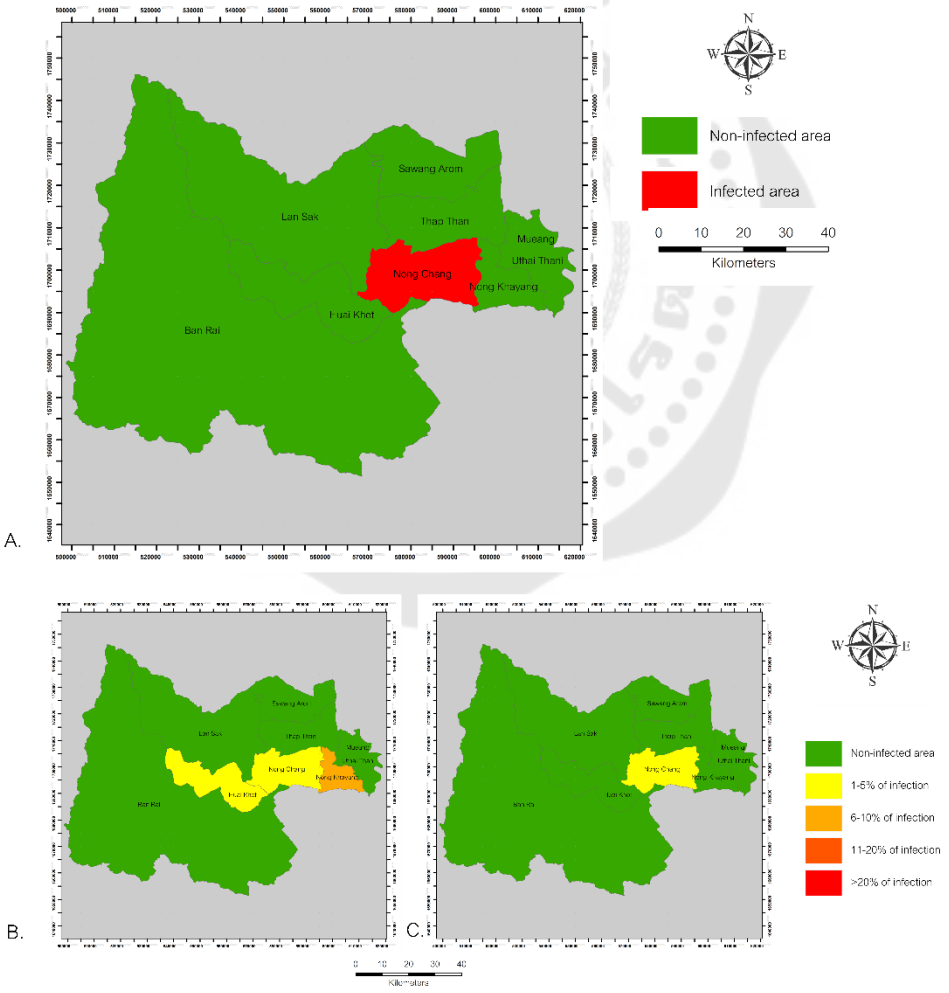



FIGURE 63 The epidemiological map of *E. revolutum* in Uthai Thani province. A: Positive areas of *E. revolutum* DNA, B: cercarial stage infection C: metacercarial stage infection.

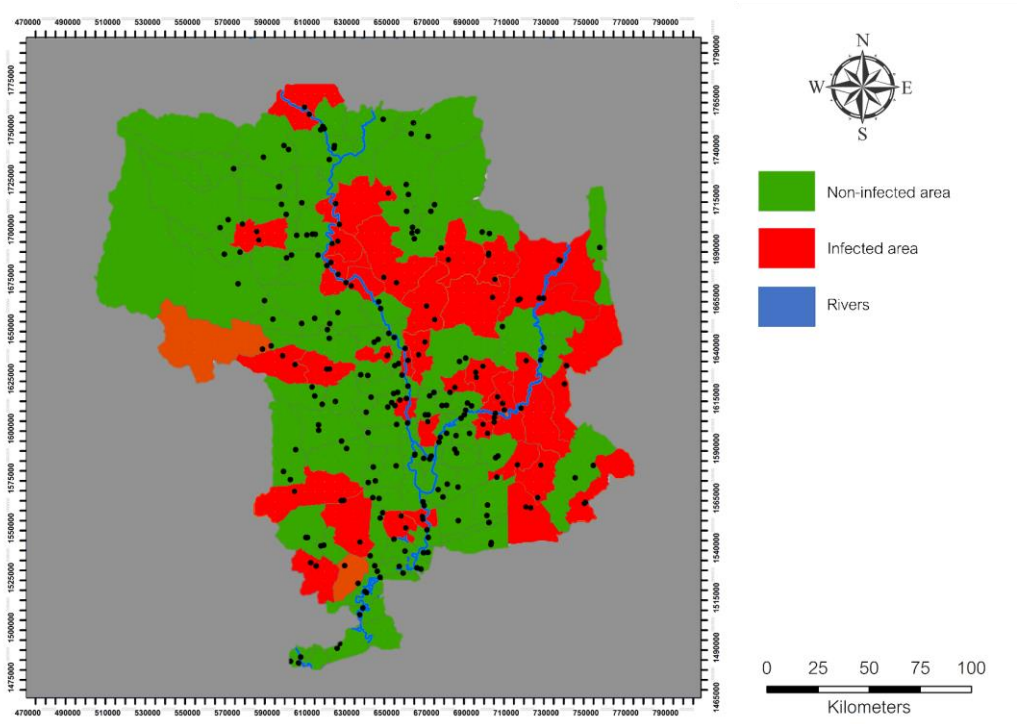



FIGURE 64 The overall infection area of *E. revolutum* in Central Thailand.

# Chapter 5
# Discussion

**Morphological study of *E. revolutum* in metacercaria and adult specimens**

In the initial of study, the differential features, the life cycle, morphology, and geographical distribution, were used to discriminate the *E. revolutum* metacercariae from other related species. Thirty-seven collar spines around their head is the first criteria to classify them as a species in revolutum group[13, 15, 86]. Approximate diameter of the metacercariae in this study is 193.2 – 220 (200.70 ± 7.46) μm, which are different from other reports. The metacercaria reported by Chai and colleagues is approximately 170 – 190 μm in diameter[15]. The metacercaria from other continents, such as South Australia, was reported as 117 – 125 μm[87]. In Thailand, a previous report of *E. revolutum* metacercariae in Chiang Mai revealed diameter of metacercariae as 136-195 μm[7]. However, all the metacercariae obtained from this study were within the usual size range of *E. revolutum*[13, 15, 26]. There are some species in revolutum group with a diameter overlapping within this range, comprising *E. friedi*, *E. luisreyi* and *E. trivolvis*[13]. Nevertheless, these species are confined to the different geographical areas. For examples, *E. friedi* can be found only in Europe, *E. luisreyi* can be found in North or South America and *E. trivolvis* can be found in South America[13]. Therefore, majority of metacercariae collected from Central Thailand are initially classified as *E. revolutum* metacercariae and used for experimental infection in the hamsters.

The metacercariae from *B. siamensis* in Tha Ruea (AY1) district are not *E. revolutum* when verified by the species-specific primers. This specimen was not be amplified with the ER primer set or *18s* species-specific primer from the previously report[75]. This result revealed that, to the precise identification of *E. revolutum* species, the researcher required to use morphological appearance together with species-specific primers or DNA sequencing data.

The metacercarial size may vary depending on the amount of pressure placed on the cover slip. The pressure may flatten a metacercaria to a size smaller than 170 μm

which was not within the size range of *E. revolutum* leading to misidentification. This presumption is supported by the previous report discussed the different size of the metacercariae as a result of the coverslip pressure[15]. On the other hand, if the researcher attempts to measure size of metacercaria before covered with coverslip, the measure size will be smaller than other reports leading to misidentification.

Regarding the fecal examination and worm recovery rate, EPG in this study cannot be done directly with 1 gram of mass feces. The hamster feces are very tiny and very lightweight that cannot collect to 1 gram. Therefore, 0.1 gram of feces was used to calculate EPG and done with three replicates each day. Eggs can be recovered from the feces by sedimentation method. It appeared, on average, 12 days post-infection with 58 EPG. The direct smear method found eggs within the next days. This result showed both methods can be used for investigating the eggs in the feces of hosts at 13 days post-infection. Although a formalin-ether technique is more effective and can detected an egg earlier than direct smear method, it is necessary to prepare the reagents and consume time[74]. On the other hand, the direct smear method is more convenient, but the sensitivity of this method is lower than formalin-ether technique for approximately one-third folds. Therefore, both methods have the different advantages depend on the situations.

The recovery rate of *E. revolutum* is only 6.00%, which is lower than *E. revolutum* from Vietnam stain (35%)[15]. The previously reports in Thailand were based on the naturally infection of *E. revolutum* in the free-grazing ducks in the various provinces[34], while the experimental infection of *E. revolutum* in Vietnam cannot be successful in any experimental ducks[15]. These results may revealed that there are some differences in term of hosts – parasites relationships in each strain of *E. revolutum* that necessaries to study in the future. Regarding to the worm developments, adult of *E. revolutum* could be collected from the small intestine of hamster at 11 days of infection. This worm has few numbers of egg in uterus. They cannot be found in the fecal examination. Although 11 days *E. revolutum* can produced the eggs in their uterus, the worm is not fully developed. The actual size of *E. revolutum* is 3.48 and 3.85

mm, while the fully developed *E. revolutum* is average 4.79 – 5.86 mm in length (5,323.54 ± 305.92). The result revealed that this trematode will be fully developed after 13 days of infection. Thus, the hamster model should be terminated after 13 days of infection to make sure that the worms are fully developed. When compare to other definitive hosts, such as a young pigeon, the *E. revolutum* can develop to produce eggs in ten days after infection and the massive egg production starts several days later[27]. On a contrary, E. revolutum becomes ovigerous on day 12 in the intestine of the domestic chicks[17, 88]. These results shown that *E. revolutum* development may be slightly different in the different definitive hosts. Although the maturation time in each definitive host is different, the unique morphological characteristic of *E. revolutum* are the same. *E. revolutum* specimens in this study have the morphological appearances and body sizes similar to other previous reports[13, 15, 86] when observed with both light and scanning electron microscope.

**The molecular studies and species-specific primer**

Although the microscopic examination has been the most effective method of investigating the cercariae and metacercariae in intermediate hosts, it is virtually impossible to identify them precisely at the species level, especially in the case of multiple infection in definitive hosts. Therefore, three candidate genes were validated to overcome this problem. Two nuclear genes were studied for the first time. The *tyrosinase* gene (*TYR*), the first gene attempted to amplify, encoded the protein that plays an important role during the egg production trematodes[46-48]. In this study, this gene cannot be amplified with the primers obtained from previously report[49] neither by vary the concentration of reagent nor annealing temperature. Therefore, the primers were newly designed from the conserved regions of this gene and validated the PCR condition. After optimizing protocol, *TYR* of *E. revolutum* was successfully amplified and the BLASTX result shown that it is similar to *tyrosinase* in various species such as *Opisthorchis viverrini* (OON23210), *Clonorchis sinensis* (AGG11797), *Paragonimus westermani* (AJE29953), *Schistosoma haematobium* (XP_012801733). Similarly, *CTSB*

required the newly developed primers for amplifying *E. revolutum* DNA. However, the sensitivity of *TYR* and *CTSB* primers are lower than *cytochrome B* (*CYTB*) primers. Furthermore, *CTSB* primers fail to generate a single band of product in one PCR reaction and require processing and re-amplification to generate a sharp band under gel electrophoresis.

Regarding to *CYTB*, the sensitivity of this gene is the highest when compared to other genes. *CYTB* primers can amplify even a small amount of DNA from the eggs. The length of *CYTB* is adequate for designing species-specific primers. All mitochondrial genes are well protected by mitochondrial membrane, maternally inherited and presence in multiple copies per cell[89]. These properties made *CYTB* more tolerance to DNA degradation than nuclear gene, leading to more sensitivity, especially in detecting the eggs that naturally contaminated with feces and DNA degradation may occur easier than other stages found in the cavity of hosts. The previous reports supported this reason. They revealed that *CYTB* is suitable for the identification of difficult or degraded specimens, such as species identification in processed food products[89, 90] and degraded DNA, such as forensic samples[91]. Moreover, it can amplify DNA from eggs that was collected from the feces by formalin-ether technique in this study. This technique is a concentration method, which typically used for trematode egg detection in feces[92]. In addition, a recently report in 2016 recommended *CYTB* as one of the best marker genes for discriminating *E. revolutum* larva from other related species.

The phylogenetic tree of *E. revolutum* based on *CYTB* confirmed that *E. revolutum* which remains in Central Thailand demonstrated genetic structures and can be divided into two sister groups depending on their specimen geographies. This result caused by maternal effect of mitochondrial gene. It made *E. revolutum* in each geography may have some different DNA sequences that inherited from the different ancestors. It can also be useful in the epidemiological studies. This gene can reveal the source of emerging infection in the infected area by cluster them to the group that has most similarity sequences. Thus, a comprehensive investigation of the genetic variations and the relationships between the populations in this complex group based on the *CYTB*

gene should be continued with larger sample sizes covering all species in the complex to increase the validity of the sequence data. This may enable the researcher to overcome systematic problems of revolutum complex species in the future.

Regarding to species-specific primer based on *CYTB* gene, this is the first study to apply the *E. revolutum CYTB* gene. There are only two sequence data of the *Echinostoma CYTB* gene in the database, comprising *E. caproni*, *E. paraensei* and two sequences of related species *Isthmiophora hortensis* (*E. hortense*) were retrieved from the GenBank database for comparing the sequences before designed primers. This is because these primers were designed based on conserved region found only in *E. revolutum* and *CYTB* is highly diverse among different species[59]. Therefore, the primers specific to *E. revolutum* should not be used to amplify other *Echinostoma* species.

The specificity test was done with an *in-silico* program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the result shown no cross-amplification with other Trematoda (taxid:6178), mammals (taxid:40674), gastropods (taxid:6448), chicken (taxid:9031) and ducks (taxid:8835). For the specificity test, various snail genera were reported as *E. revolutum* intermediate hosts such as *Clea*, *Bithynia*, *Filopaludina*[7]. Therefore, all of them were used for validating the specific of primer. The result found that the primers can amplify E. revolutum DNA with no cross-amplification with host DNA. Interestingly, the ER primers have a limit of detection at 0.85 pg, while the report of concentration obtained from a single egg in common trematodes is range from 3.57 to 17.53 ng[93]. Consequently, the level of sensitivity is enough to detection only one egg of *E. revolutum* in the specimen. This is the high-performance method for detecting the eggs in patient feces and is more convenient than the classical method that was usually used to determine trematode infection in humans. Finally, this species-specific primer can be useful in the future studies for detection and surveillance program for *E. revolutum* outbreaks in their hosts as well.

**The epidemic of *E. revolutum***

The present study is the first report that applied the species-specific primer based on *cytochrome B* to identify the larval stages infection of *E. revolutum*. Most provinces show a high prevalence value of the larval stage of echinostome in various snail species, but the species-specific primer revealed that only four snail species are infected with *E. revolutum,* comprising *L. auricularia, F. sumatrensis polygramma*, *F. martensi* and *I. exustus.* For the cercarial stage, *L. auricularia* is the only species that infected by cercarial stage of *E. revolutum.* This result is according with the previous reports that revealed the snail in this genus as a susceptible host for *E. revolutum*[27]. The previous studies support this result, they reported that first intermediate host of *E. revolutum* is only *Lymnaea* genus or relate species in Lymnaeidae[4, 13, 15, 27, 28, 31]. This result is cause by the life cycle of *E. revolutum*. When the miracidium hatched from the eggs, they swim and respond to the stimuli emanating (complex macromolecules) from snail mucus and directly swim to penetrate their hosts[28].

Regarding to metacercariae, most of infection is found in *Filopaludina* snails (*F. sumatrensis polygramma* and *F. martensi)*. These snail species are reported as susceptible host of *E. revolutum*[3, 11, 15]. From the results, nine provinces are the first recorded of *E. revolutum* in Thailand, comprising Ang Thong, Chainat, Nakhon Nayok, Nakhon Pathom, Samut Sakhon, Saraburi, Sing Buri, Suphanburi and Uthai Thani provinces. Four additional provinces reveal the infection rates similar the report of Saijuntha in 2013 that found *E. revolutum* infection in domestic ducks with high prevalence percent[34].

For the detail of each province, Ang Thong is found that infected snail only in *F. martensi* of Mueang Ang Thong district. This district is connected to Nong Saeng district in Saraburi provinces that has the prevalence of metacercaria at 24 % and *E. revolutum* is broadly distributed across this province. The irrigation canal and other water resources may support the snail intermediate hosts to transport and harbor the larva stage of *E. revolutum* between Saraburi and the nearby provinces[94]. First, Lopburi province, this province had the highest prevalence of *E. revolutum* in this study (98%).

This result is similarity to the previous study in 2013 that reported the highest prevalence of *E. revolutum* in Lopburi province (85.7%). The free-grazing ducks in this area usually found the infected with *E. revolutum* in their intestine[34]. Another province that connected to Saraburi is Nakhon Nayok. This province is found *E. revolutum* infection in all district that connected to Keang Khoi and Wihan Daeng district of Saraburi that has 30 and 4% of *E. revolutum* prevalence, respectively. In Chainat provinces, there are both cercaria and metacercaria infected in the freshwater snails in this study. All infected districts are located on the eastern part that connected to TaKhli and Phayuha Khiri district of Nakhon Sawan province which found *E. revolutum* infection with the prevalence at 2 and 4%, respectively. Moreover, Nakhon Sawan is one of the provinces that had report of *E. revolutum* infection in domestic ducks with the prevalence value at 50% in 2013[34]. Therefore, this study confirms that *E. revolutum* is still existence in this province until now. For the western part provinces, Uthai Thani has *E. revolutum* infection only in Nong Chang with both cercaria and metacercaria. Surprisingly, this infection area is not directly connected to another infection site. However, there are many water resources and canals such as Huai Khun Kaew canal, Huai Khun Kaew watershed and Huai Khot-Wang Man diversion canals that connect to the upstream river namely, Sakae Krang River. Therefore, the intermediate hosts and larval stage of *E. revolutum* may discharged from the northern provinces of Thailand, that known as the epidemic area of *E. revolutum*[7, 11], to Uthai Thani province. The southern province of Uthai Thani is Suphanburi province, the infection area in this province is separate into two distinct parts. The upper part is the area connected to Ayutthaya province that has the previous report of *E. revolutum* in domestic duck with very high prevalence value (76.7%)[34]. From this reason, the rotation of free-grazing ducks among several rice paddies and the connection of man-made irrigation canals in the agricultural area may support a spread of this zoonotic trematode in both intermediate and definitive hosts to the nearby provinces such as Suphanburi, Sing Buri and Pathum Thani. For another area of infection in Suphanburi province, this region is connected to Nakhon Pathom province that are moderate to high prevalence area of *E. revolutum* in this study. The

last province is Samut Sakhon, a small province with many ponds and other water resources of brackish water, also has *E. revolutum* infection in Ban Phaeo district that connected to Muang Nakhon Pathom district. From above mentioned, the ponds which connect to the irrigation canals in these provinces are suitable conditions for the spread of many freshwater snails[94], especially *Filopaludina* and *Lymnaea*, which are important intermediate hosts. The epidemiological map acquired from this study can be applied to create the prevention program to prevent the spread of *E. revolutum* from the infection area to neighboring area.

## Conclusion

The successfully development of a high-performance species-specific primers of *E. revolutum* can detect the parasite at various larval stages, especially the cercarial stage that is difficult to identify by the classical method[16]. This stage is very important in terms of the epidemiological study of trematodes because only one snail with a horde of cercariae can rapidly infect secondary intermediate hosts and increase the risk opportunities for humans and other definitive hosts in the infection area. Furthermore, it was helpful to discriminate between *E. revolutum* eggs and other trematode eggs that are similar in color and morphological appearances such as *Echinostoma* eggs, *Fasciola* eggs, *Fasciolopsis* eggs and *Gastrodiscoides* eggs [95, 96] and applied to detect *E. revolutum* eggs in Echinostomiasis patients with a high degree of accuracy. Consequently, the species-specific primers and epidemiological map obtained from this study can be helpful in related studies of trematodes, including medical and veterinary research, because of the shorter time requirement and higher specificity of results than the morphological method.

# REFERENCES


1. Sohn W-M, Chai J-Y, Yong T-S, Eom KS, Yoon C-H, Sinuon M, Socheat D, Lee S-H. *Echinostoma revolutum* infection in children, Pursat Province, Cambodia. Emerging Infectious Diseases. 2011;17(1):117-9.

2. Toledo R, Esteban JG. An update on human echinostomiasis. Transactions of The Royal Society of Tropical Medicine and Hygiene. 2016;110(1):37-45.

3. Noikong W, Wongsawad C, Chai J-Y, Saenphet S, Trudgett A. Molecular analysis of echinostome metacercariae from their second intermediate host found in a localised geographic region reveals genetic heterogeneity and possible cryptic speciation. PLoS Neglected Tropical Diseases. 2014;8(4):e2778.

4. Georgieva S, Faltýnková A, Brown R, Blasco-Costa I, Soldánová M, Sitko J, Scholz T, Kostadinova A. *Echinostoma*'*revolutum*'(Digenea: Echinostomatidae) species complex revisited: species delimitation based on novel molecular and morphological data gathered in Europe. Parasites & Vectors. 2014;7(1):520.

5. Morgan J, Blair D. Mitochondrial *ND*1 gene sequences used to identify echinostome isolates from Australia and New Zealand. International Journal for Parasitology. 1998;28(3):493-502.

6. Saijuntha W, Tantrawatpan C, Sithithaworn P, Andrews RH, Petney TN. Spatial and temporal genetic variation of *Echinostoma revolutum* (Trematoda: Echinostomatidae) from Thailand and the Lao PDR. Acta Tropica. 2011;118(2):105-9.

7. Chantima K, Chai J-Y, Wongsawad C. *Echinostoma revolutum*: freshwater snails as the second intermediate hosts in Chiang Mai, Thailand. The Korean Journal of Parasitology. 2013;51(2):183-9.

8. Sohn W-M, Chai J-Y, Na B-K, Yong T-S, Eom KS, Park H, Min D-Y, Rim H-J. *Echinostoma macrorchis* in Lao PDR: Metacercariae in *Cipangopaludina* snails and adults from experimentally infected animals. The Korean Journal of Parasitology. 2013;51(2):191-6.

9. Schär F, Inpankaew T, Traub RJ, Khieu V, Dalsgaard A, Chimnoi W, Chhoun C, Sok

D, Marti H, Muth S. The prevalence and diversity of intestinal parasitic infections in humans and domestic animals in a rural Cambodian village. Parasitology International. 2014;63(4):597-603.

10. Cortés A, Sotillo J, Muñoz-Antolí C, Martín-Grau C, Esteban JG, Toledo R. Resistance against *Echinostoma caproni* (Trematoda) secondary infections in mice is not dependent on the ileal protein production. Journal of Proteomics. 2016;140:37-47.

11. Noikong W, Wongsawad C. Epidemiology and molecular genotyping of echinostome metacercariae in *Filopaludina* snails in Lamphun Province, Thailand. Asian Pacific Journal of Tropical Medicine. 2014;7(1):26-9.

12. Afshan K, Fortes-Lima CA, Artigas P, Valero MA, Qayyum M, Mas-Coma S. Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. Geospatial Health. 2014;8(2):317-34.

13. Toledo R, Esteban JG, Fried B. Recent advances in the biology of echinostomes. Advances in Parasitology. 2009;69:147-204.

14. Kanev I, Fried B, Radev V. Collar spine models in the genus *Echinostoma* (Trematoda: Echinostomatidae). Parasitology Research. 2009;105(4):921-7.

15. Chai J-Y, Sohn W-M, Na B-K, Van De N. *Echinostoma revolutum*: metacercariae in Filopaludina snails from Nam Dinh Province, Vietnam, and adults from experimental hamsters. The Korean Journal of Parasitology. 2011;49(4):449-55.

16. Nagataki M, Tantrawatpan C, Agatsuma T, Sugiura T, Duenngai K, Sithithaworn P, Andrews RH, Petney TN, Saijuntha W. Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: *Echinostoma revolutum* and *E. miyagawai*. Infection, Genetics and Evolution. 2015;35:56-62.

17. Fried B, Graczyk TK. Recent advances in the biology of *Echinostoma* species in the “revolutum” group. Advances in Parasitology. 2004;58(58):139-95.

18. Morgan J, Blair D. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial *CO*1 and *ND*1 genes for distinguishing among *Echinostoma* species

(Trematoda). Parasitology. 1998;116(3):289-97.

19. Won EJ, Kim SH, Kee SJ, Shin JH, Suh SP, Chai JY, Ryang DW, Shin MG. Multiplex Real-Time PCR assay targeting eight parasites customized to the Korean population: potential use for detection in diarrheal stool samples from gastroenteritis patients. PloS One. 2016;11(11):e0166957.

20. Onsurathum S, Pinlaor P, Charoensuk L, Haonon O, Chaidee A, Intuyod K, Laummaunwai P, Boonmars T, Kaewkes W, Pinlaor S. Contamination of *Opisthorchis viverrini* and *Haplorchis taichui* metacercariae in fermented fish products in northeastern Thailand markets. Food Control. 2016;59:493-8.

21. Chontananarth T. Multiplex PCR assay for discrimination of *Centrocestus caninus* and *Stellantchasmus falcatus*. Asian Pacific Journal of Tropical Biomedicine. 2017;7(2):103-6.

22. Detwiler JT, Bos DH, Minchella DJ. Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America. Molecular Phylogenetics and Evolution. 2010;55(2):611-20.

23. Saijuntha W, Sithithaworn P, Duenngai K, Kiatsopit N, Andrews RH, Petney TN. Genetic variation and relationships of four species of medically important echinostomes (Trematoda: Echinostomatidae) in South-East Asia. Infection, Genetics and Evolution. 2011;11(2):375-81.

24. Georgieva S, Selbach C, Faltýnková A, Soldánová M, Sures B, Skírnisson K, Kostadinova A. New cryptic species of the 'revolutum'group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data. Parasites & Vectors. 2013;6(1):64.

25. Kostadinova A, Herniou E, Barrett J, Littlewood DTJ. Phylogenetic relationships of *Echinostoma Rudolphi,* 1809 (Digenea: Echinostomatidae) and related genera re-assessed via DNA and morphological analyses. Systematic Parasitology. 2003;54(3):159-76.

26. Chai J-Y. Echinostomes in humans. The biology of echinostomes: Springer; 2009. p. 147-83.

27. Kanev I. Life-cycle, delimitation and redescription of *Echinostoma revolutum* (Froelich, 1802)(Trematoda: Echinostomatidae). Systematic Parasitology. 1994;28(2):125-44.

28. Esteban JG, Muñoz-Antoli C. Echinostomes: Systematics and life cycles. The Biology of Echinostomes: Springer; 2009. p. 1-34.

29. Kanev I, Eisenhut U, De Nunez MO, Gonzalez MM, Tzolov D, Dimitrov V, Radev V. Redescription of the tail and fin folds of *Echinostoma revolutum* cercariae from its type locality (Trematoda: Echinostomatidae). Annales de Parasitologie Humaine et Comparée. 1993;68(3):125-7.

30. Anazawa K. On a human case of Echinostoma revolutum and its infection route. Taiwan Igahkai Zasshi. 1929;288(288):221-41.

31. Rondelaud D, Vignoles P, Dreyfuss G. Larval trematode infections in *Galba truncatula* (Gastropoda, Lymnaeidae) from the Brenne Regional Natural Park, Central France. Journal of Helminthology. 2016;90(3):256-61.

32. Sohn W-M, Chai J-Y. Infection status with helminthes in feral cats purchased from a market in Busan, Republic of Korea. The Korean Journal of Parasitology. 2005;43(3):93-100.

33. Hembram A, Panda M, Mohanty B, Pradhan C, Dehuri M, Sahu A, Behera M. Prevalence of gastrointestinal helminths in *Banaraja* fowls reared in semi-intensive system of management in Mayurbhanj district of Odisha. Veterinary World. 2015;8(6):723-6.

34. Saijuntha W, Duenngai K, Tantrawatpan C. Zoonotic echinostome infections in free-grazing ducks in Thailand. The Korean Journal of Parasitology. 2013;51(6):663-7.

35. Kechemir N, Jourdane J, Mas-Coma S. Life cycle of a new African echinostome species reproducing by parthenogenesis. Journal of Natural History. 2002;36(15):1777-84.

36. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H, editors. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor symposia on quantitative biology; 1986: Cold Spring Harbor Laboratory Press.

37. Bartlett JM, Stirling D. A short history of the polymerase chain reaction. PCR protocols: Springer; 2003. p. 3-6.

38. Hertel J, Haberl B, Hamburger J, Haas W. Description of a tandem repeated DNA sequence of *Echinostoma caproni* and methods for its detection in snail and plankton samples. Parasitology. 2003;126(5):443-9.

39. Tantrawatpan C, Saijuntha W, Manochantr S, Kheolamai P, Thanchomnang T, Sadaow L, Intapan PM, Maleewong W. A singleplex real-time fluorescence resonance energy transfer PCR with melting curve analysis for the differential detection of *Paragonimus heterotremus, Echinostoma malayanum* and *Fasciola gigantica* eggs in faeces. Transactions of The Royal Society of Tropical Medicine and Hygiene. 2016;110(1):74-83.

40. Mostafa OM, Taha HA, Ramadan G. Diagnosis of *Fasciola gigantica* in snail using the polymerase chain reaction (PCR) assay. Journal -Egyptian Society of Parasitology. 2003;33(3):733-42.

41. Tantrawatpan C, Intapan PM, Thanchomnang T, Sanpool O, Janwan P, Lulitanond V, Sadaow L, Maleewong W. Development of a PCR assay and pyrosequencing for identification of important human fish-borne trematodes and its potential use for detection in fecal specimens. Parasites & Vectors. 2014;7(1):88.

42. Haag-Liautard C, Coffey N, Houle D, Lynch M, Charlesworth B, Keightley PD. Direct estimation of the mitochondrial DNA mutation rate in *Drosophila melanogaster*. PLoS Biology. 2008;6(8):e204.

43. Monteiro FA, Peretolchina T, Lazoski C, Harris K, Dotson EM, Abad-Franch F, Tamayo E, Pennington PM, Monroy C, Cordon-Rosales C. Phylogeographic pattern and extensive mitochondrial DNA divergence disclose a species complex within the Chagas disease vector *Triatoma dimidiata*. PloS One. 2013;8(8):e70974.

44. Koga A, Inagaki H, Bessho Y, Hori H. Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. Molecular and General Genetics MGG. 1995;249(4):400-5.

45. JS M. Cathepsin B. In: ND Rawlings GS, editor. Handbook of proteolytic enzymes: Elsevier; 2013. p. 3413-8.

46. Cai G, Bae Y, Zhang Y, He Y, Jiang M, He L. Expression and characterization of

two tyrosinases from the trematode *Schistosoma japonicum*. Parasitology Research. 2009;104(3):601-9.

47. Jex AR, Young ND, Sripa J, Hall RS, Scheerlinck J-P, Laha T, Sripa B, Gasser RB. Molecular changes in *Opisthorchis viverrini* (Southeast Asian liver fluke) during the transition from the juvenile to the adult stage. PLoS Neglected Tropical Diseases. 2012;6(11):e1916.

48. Smyth J, Clegg J. Egg-shell formation in trematodes and cestodes. Experimental Parasitology. 1959;8(3):286-323.

49. Bae Y-A, Cai G-B, Kim S-H, Sohn W-M, Kong Y. Expression pattern and substrate specificity of *Clonorchis sinensis* tyrosinases. International Journal for Parasitology. 2013;43(11):891-900.

50. Smooker PM, Jayaraj R, Pike RN, Spithill TW. Cathepsin B proteases of flukes: the key to facilitating parasite control? Trends in Parasitology. 2010;26(10):506-14.

51. Harrop SA, Sawangjaroen N, Prociv P, Brindley PJ. Characterization and localization of cathepsin B proteinases expressed by adult *Ancylostoma caninum* hookworms. Molecular and Biochemical Parasitology. 1995;71(2):163-71.

52. Skelly P, Shoemaker C. *Schistosoma mansoni* proteases Sm31 (*cathepsin B*) and Sm32 (*legumain*) are expressed in the cecum and protonephridia of cercariae. Journal of Parasitology. 2001;87(5):1218-21.

53. Park H, Kim S-I, Hong K-M, Kim M-J, Shin C-H, Ryu J-S, Min D-Y, Lee J-B, Hwang UW. Characterization and classification of five cysteine proteinases expressed by *Paragonimus westermani* adult worm. Experimental parasitology. 2002;102(3-4):143-9.

54. Na B-K, Lee H-J, Cho S-H, Lee H-W, Cho J-H, Kho W-G, Lee J-S, Lee J-S, Song K-J, Park P-H. Expression of cysteine proteinase of *Clonorchis sinensis* and its use in serodiagnosis of clonorchiasis. Journal of Parasitology. 2002;88(5):1000-6.

55. Sripa J, Laha T, To J, Brindley PJ, Sripa B, Kaewkes S, Dalton JP, Robinson MW. Secreted cysteine proteases of the carcinogenic liver fluke, *Opisthorchis viverrini:* regulation of *cathepsin F* activation by autocatalysis and trans-processing by *cathepsin B*. Cellular Microbiology. 2010;12(6):781-95.

56. Young ND, Jex AR, Li B, Liu S, Yang L, Xiong Z, Li Y, Cantacessi C, Hall RS, Xu X. Whole-genome sequence of *Schistosoma haematobium*. Nature Genetics. 2012;44(2):221-5.

57. Meemon K, Grams R, Vichasri-Grams S, Hofmann A, Korge G, Viyanant V, Upatham ES, Habe S, Sobhon P. Molecular cloning and analysis of stage and tissue-specific expression of *cathepsin B* encoding genes from *Fasciola gigantica*. Molecular and Biochemical Parasitology. 2004;136(1):1-10.

58. McGonigle L, Mousley A, Marks NJ, Brennan GP, Dalton JP, Spithill TW, Day TA, Maule AG. The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduces gut penetration. International Journal for Parasitology. 2008;38(2):149-55.

59. Parson W, Pegoraro K, Niederstätter H, Föger M, Steinlechner M. Species identification by means of the *cytochrome b* gene. International Journal of Legal Medicine. 2000;114(1-2):23-8.

60. Kato H, Gomez EA, Martini-Robles L, Muzzio J, Velez L, Calvopiña M, Romero-Alvarez D, Mimori T, Uezato H, Hashiguchi Y. Geographic distribution of *Leishmania* species in Ecuador based on the *cytochrome B* gene sequence analysis. PLoS Neglected Tropical Diseases. 2016;10(7):e0004844.

61. Bensch S, Hellgren O, PÉREZ-TRIS J. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial *cytochrome b* lineages. Molecular Ecology Resources. 2009;9(5):1353-8.

62. Cook J, Aydin-Schmidt B, González IJ, Bell D, Edlund E, Nassor MH, Msellem M, Ali A, Abass AK, Mårtensson A. Loop-mediated isothermal amplification (LAMP) for point-of-care detection of asymptomatic low-density malaria parasite carriers in Zanzibar. Malaria Journal. 2015;14(1):43.

63. Xu W, Morris U, Aydin-Schmidt B, Msellem MI, Shakely D, Petzold M, Björkman A, Mårtensson A. SYBR Green real-time PCR-RFLP assay targeting the *Plasmodium* cytochrome B gene–a highly sensitive molecular tool for malaria parasite detection and species determination. PloS One. 2015;10(3):e0120210.

64. Hüttner M, Nakao M, Wassermann T, Siefert L, Boomker JD, Dinkel A, Sako Y, Mackenstedt U, Romig T, Ito A. Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. International Journal for Parasitology. 2008;38(7):861-8.

65. Wickström L, Haukisalmi V, Varis S, Hantula J, Fedorov V, Henttonen H. Phylogeography of the circumpolar *Paranoplocephala arctica* species complex (Cestoda: Anoplocephalidae) parasitizing collared lemmings (*Dicrostonyx* spp.). Molecular Ecology. 2003;12(12):3359-71.

66. Xiao N, Qiu J, Nakao M, Li T, Yang W, Chen X, Schantz PM, Craig PS, Ito A. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. International Journal for Parasitology. 2005;35(6):693-701.

67. Wicht B, Yanagida T, Scholz T, Ito A, Jiménez JA, Brabec J. Multiplex PCR for differential identification of broad tapeworms (Cestoda: *Diphyllobothrium*) infecting humans. Journal of Clinical Microbiology. 2010;48(9):3111-6.

68. Hanelt B, Schmidt-Rhaesa A, Bolek MG. Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution. 2015;82:211-8.

69. Blasco-Costa I, Cutmore SC, Miller TL, Nolan MJ. Molecular approaches to trematode systematics:'best practice'and implications for future study. Systematic Parasitology. 2016;93(3):295-306.

70. Jing L, Tian K, Huang JZ. Stratified feature sampling method for ensemble clustering of high dimensional data. Pattern Recognition. 2015;48(11):3688-702.

71. Brandt R. The non-marine aquatic Mollusca of Thailand: Archiv. Molluskenkunde; 1974. p. 1-423.

72. Sinuon M, Krailas D. Morphological and molecular identification of the liver fluke *Opisthorchis viverrini* in the first intermediate host *Bithynia* snails and its prevalence in Kampong Cham Province, Cambodia. Parasitology International. 2017;66:319-23.

73. Leary SL, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Bran S, McCrackin M, Meyer R, editors. AVMA guidelines for the euthanasia of

animals: 2013 edition2013: American Veterinary Medical Association Schaumburg, IL.

74. Allen A, Ridley D. Further observations on the formol-ether concentration technique for faecal parasites. Journal of Clinical Pathology. 1970;23(6):545-6.

75. Anucherngchai S, Chontananarth T. *Echinostoma revolutum*: Development of a high performance DNA-specific primer to demonstrate the epidemiological situations of their intermediate hosts. Acta Tropica. 2019;189:46-53.

76. Abd-Elsalam K. Bioinformatic tools and guideline for PCR primer design. African Journal of Biotechnology. 2003;2(5):91-5.

77. Beaver PC. Experimental studies on *Echinostoma revolutum* (Froelich) a fluke from birds and mammals. Illinois Biological Monographs 1937;34(15):1-96.

78. Lee S-H, Sohn W-M, Chai J-Y. *Echinostoma revolutum* and *Echinoparyphium recurvatum* recovered from house rats in Yangyang-gun, Kangwon-do. The Korean Journal of Parasitology. 1990;28(4):235-40.

79. Kostadinova A, Gibson D, Biserkov V, Ivanova R. A quantitative approach to the evaluation of the morphological variability of two echinostomes, *Echinostoma miyagawai* Ishii, 1932 and *E. revolutum* (Frölich, 1802), from Europe. Systematic Parasitology. 2000;45(1):1-15.

80. Kostadinova A, Gibson D, Biserkov V, Chipev N. Re-validation of *Echinostoma miyagawai* Ishii, 1932 (Digenea: Echinostomatidae) on the basis of the experimental completion of its life-cycle. Systematic Parasitology. 2000;45(2):81-108.

81. Grover M, Dutta R, Kumar R, Aneja S, Mehta G. *Echinostoma ilocanum* infection. Indian Pediatr. 1998;35(35):549-52.

82. Sohn W-M, Kim H-J, Yong T-S, Eom KS, Jeong H-G, Kim J-K, Kang A-R, Kim M-R, Park J-M, Ji S-H. *Echinostoma ilocanum* infection in Oddar Meanchey Province, Cambodia. The Korean Journal of Parasitology. 2011;49(2):187-90.

83. Lie K, Nasemary S. Studies on Echinostomatidae (Trematoda) in Malaysia. XVI. The life history of *Echinostoma ilocanum* (Garrison, 1908). Proceedings of the Helminthological Society of Washington. 1973;40(1):59-65.

84. Sohn W-M. Life history of *Echinoparyphium recurvatum* (Trematoda:

Echinostomatidae) in Korea. The Korean Journal of Parasitology. 1998;36(2):91-8.

85. Toledo R, Muñoz-Antolí C, Perez M, Esteban JG. Redescription of the adult stage of *Hypoderaeum conoideum* (Bloch, 1782)(Trematoda: Echinostomatidae) and new record in Spain. Research and Reviews in Parasitology. 1996;56(4):195-201.

86. Kostadinova A, Gibson D. The systematics of the echinostomes. Echinostomes as Experimental Models for Biological Research Edited by: Fried B, Graczyk TK. 2000. Dordrecht: Kluwer Academic Publishers; 2000. p. 37-57.

87. Johnston TH, Angel LM. The life history of *Echtnosioma revoluium* in South Australia. Transactions of the Royal Society of South Australia. 1941;65(2):317-22.

88. Humphries JE, Reddy A, Fried B. Infectivity and growth of *Echinostoma revolutum* (Froelich, 1802) in the domestic chick. International Journal for Parasitology. 1997;27(1):129-30.

89. Ali ME, Razzak MA, Hamid SBA, Rahman MM, Al Amin M, Rashid NRA. Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Food Chemistry. 2015;177:214-24.

90. Ali ME, Rahman MM, Hamid SBA, Mustafa S, Bhassu S, Hashim U. Canine-specific PCR assay targeting *cytochrome b* gene for the detection of dog meat adulteration in commercial frankfurters. Food Analytical Methods. 2014;7(1):234-41.

91. Staats M, Arulandhu AJ, Gravendeel B, Holst-Jensen A, Scholtens I, Peelen T, Prins TW, Kok E. Advances in DNA metabarcoding for food and wildlife forensic species identification. Analytical and Bioanalytical Chemistry. 2016;408(17):4615-30.

92. Kaewpitoon N, Kootanavanichpong N, Kompor P, Chavenkun W, Kujapun J, Norkaew J, Ponphimai S, Matrakool L, Tongtawee T, Panpimanmas S. Review and current status of *Opisthorchis viverrini* infection at the community level in Thailand. Asian Pacific Journal of Cancer Prevention. 2015;16(16):6825-30.

93. Wongsawad C, Wongsawad P, Chai J-Y, Paratasilpin T, Anuntalabhochai S. DNA quantities and qualities from various stages of some trematodes using optical and HAT-RAPD methods. Southeast Asian Journal of Tropical Medicine and Public Health. 2006;37:62-8.

94. Krailas D, Chotesaengsri S, Dechruksa W, Namchote S, Chuanprasit C, Veeravechsukij N, Boonmekam D, Koonchornboon T. Species diversity of aquatic mollusks and their cercarial infections; Khao Yai National Park, Thailand. J Trop Med Parasitol. 2012;35(35):37-47.

95. Qureshi AW, Tanveer A, Mas-Coma S. Epidemiological analysis of human fascioliasis in northeastern Punjab, Pakistan. Acta Tropica. 2016;156:157-64.

96. Sah R, Khadka S, Hamal R, Poudyal S. Human echinostomiasis: a case report. BMC Research Notes. 2018;11(1):17.

# VITA

| | |
|---|---|
| NAME | Sothorn Anucherngchai |
| DATE OF BIRTH | 24 November 1992 |
| PLACE OF BIRTH | Nakhon Pathom |
| INSTITUTIONS ATTENDED | Elementary School (2542-2547): Wat Phrapathomchede School<br>Secondary/High School (2548-2553): The Demonstration School of Nakhon Pathom Rajabhat University (Science - Mathematics program)<br>Bachelor's degree (2554-2557): Srinakharinwirot University (Biology) |
| PUBLICATION | Anucherngchai, S., Tejangkura, T., & Chontananarth, T. (2016). Epidemiological situation and molecular identification of cercarial stage in freshwater snails in Chao-Phraya Basin, Central Thailand. Asian Pacific Journal of Tropical Biomedicine, 6(6), 539-545.<br><br>Anucherngchai, S., Tejangkura, T., & Chontananarth, T. (2017). Molecular confirmation of trematodes in the snail intermediate hosts from Ratchaburi Province, Thailand. Asian Pacific Journal of Tropical Disease, 7(5), 286-292.<br><br>Chontananarth, T., Anucherngchai, S., & Tejangkura, T. (2018). The rapid detection method by polymerase chain reaction for minute intestinal trematodes: Haplorchis taichui in intermediate snail hosts based on 18s ribosomal DNA. Journal of parasitic diseases, 42(3), 423-432. |

Anucherngchai, S., & Chontananarth, T. (2019). Echinostoma revolutum: Development of a high performance DNA-specific primer to demonstrate the epidemiological situations of their intermediate hosts. Acta tropica, 189, 46-53.

Anucherngchai, S., Chontananarth, T., Tejangkura, T., & Chai, J. Y. (2019). The study of Cytochrome B (CYTB): species-specific detection and phylogenetic relationship of Echinostoma revolutum,(Froelich, 1802). Journal of Parasitic Diseases, 43(1), 66-74.