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ความชุกและการระบุชนิดพยาธิใบไม้ *Echinostoma revolutum* ด้วย ND1 ในระยะเมตาเซอร์คาเรียและตัวเต็มวัย จากจังหวัดลำพูนและลำปางของประเทศไทย

PREVALENCE AND ND1 IDENTIFICATION OF TREMATODE, *Echinostoma revolutum* IN METACERCARIA AND ADULT STAGES FROM LAMPHUN AND LAMPANG PROVINCES, THAILAND

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บทคัดย่อ

ระยะเมตาเซอร์คาเรียเป็นระยะติดต่อของพยาธิใบไม้กลุ่ม echinostome ที่สามารถพบได้ในหอยน้ำจืด ลูกอ๊อดและปลา การศึกษาครั้งนี้ทำการเก็บเมตาเซอร์คาเรียในหอยขม *Filopaludina* จำนวน 3 ชนิด คือ *Filopaludina martensi martensi*, *F. dorliaris* และ *F. sumatrensis polygramma* จาก 4 อำเภอของจังหวัดลำพูนและลำปาง พบค่าความชุกของการติดพยาธิเท่ากับ ร้อยละ 27.05, 41.92 และ 48.05 ตามลำดับ นำตัวเต็มตัวที่ได้หลังจากการป้อนเมตาเซอร์คาเรียให้หนูแฮมเตอร์และลูกไก่ 14 วันมาระบุชนิดโดยการศึกษาลักษณะวิทยาภายใต้กล้องจุลทรรศน์แบบใช้แสงและกล้องจุลทรรศน์อิเล็กตรอน ร่วมกับเทคนิคทางอณูชีววิทยาโดยศึกษา Phylogenetic tree จากยีนไมโทคอนเตรียในตำแหน่ง ND1 (nicotinamide adenine dinucleotide dehydrogenase subunit 1) ตัวเต็มวัยของพยาธิที่พบมีลักษณะลำตัวยาว ส่วนท้องโค้งงอ head collar มี collar spines จำนวน 37 อัน และ testes มีรูปร่าง 2 แบบคือแบบ lobe และแบบกลม อย่างไรก็ตามการระบุชนิดได้พิจารณาจากลักษณะสัณฐานวิทยา การวัดทางสัณฐานและ phylogenetic tree จาก ND1 โดยสามารถระบุชนิดพยาธิที่พบเป็น *Echinostoma revolutum* ซึ่งมีหอยขม *Fliopaludina* spp. เป็นโฮสต์กึ่งกลางตามธรรมชาติ และเป็นการยืนยันว่ามีการระบาดของพยาธิชนิดนี้ในจังหวัดลำพูนและลำปาง

คำสำคัญ: หอยขม *Echinostoma revolutum* ลำพูน ลำปาง ยีนไมโทคอนเตรีย

Abstract

Echinostome metacercariae are the infective stage of echinostome flukes that can be found in fresh water snails, tadpoles, and fish. In this study, echinostome metacercariae from three species of *Filopaludina* snails; *Filopaludina martensi martensi, F. dorliaris* and *F. summatrensis polygramma* were collected from four districts of Lamphun and Lampang Provinces and the prevalence of metacercrial infection by the three species was 27.05%, 41.92% and 48.05% respectively. The adult flukes were covered from experimentally infected hamsters and chickens at the 14th day after infection and were identified to species using light microscopy (LM), scanning electron microscopy (SEM), and a phylogenetic tree for the mitochondrial gene ND1 (nicotinamide adenine dinucleotide dehydrogenase subunit 1). This fluke has an elongated body, entrally curved body , head collar with 37 prominent collar spines, and 2 type of testes shape including lobed and roudned. However, identification of *Echinostoma revolutum*in this present study has been based on morphology, morphometric data, and the phylogenetic tree from ND1 subunit The results confirmed status of *E. revolutum* in *Filopaludina* spp. snails as the natural second intermediate host and also demonstrated the occurrence of this intestinal flukes in Lamphun and Lampang provinces.

Keywords: *Filopaludina* snails, *Echinostoma revolutum*, Lamphun, Lampang, mitochondrial gene

Introduction

Echinostomes (family Echinostomatidae) are important intestinal parasitic trematodes in numerous vertebrate species, including humans (Chai, 2009). Human echinostomiasis caused by about 20 species and has been discovered worldwide (Lee et al., 1986; Lee et al., 2002; Cho et al., 2003; Park et al., 2004; Sohn et al., 2011; Chai et al., 2012; Khanna et al., 2016). The disease is caused by eating raw meat of fresh water snails, fresh water fish, tadpoles or amphibians, that are the second intermediate host of metacercariae (Chantima, 2013). Identification of echinostomes commonly uses morphologi calcharacteritics, such as the number of collar spines as taxonomic criteria. However a group with 37 “collar spines” or “revolutum” flukes is huge and complex due to their high morphological similarity and species-level identification of this group is difficult so many researchers use molecular techniques as an alternative method (Noikhong & Wongsawad, 2014; Nagataki et al., 2015).

Metacercariae of *Echinostomare volutum* which is the most common species of echinostomes in Northern Thailand, has been reported to infect many species of freshwater snails, for example, *Filopaludina* spp., *Bithynia* spp., *Pila* spp., *Corbicula mareletiana*, *Eyriesia eyriesi* and *Cleahelena*. *Filopaludina* snails are the favorite local food and can be found in all regions of Thailand (Chanthima et al., 2013). In other to determine the presence of echinostomes, a survey of echinostomes in freshwater snails especially *Filopaludina* spp. was conducted in four districts of Lampun and Lampang Provinces. Species identification of echinostomes was done by standard morphology with molecular confirmation.

Materials and methods

1. Study area, sampling and identification of snails

A total of 1,724 *Filopaludina* spp. snails were sampled from Ban-Thi, Muang Lamphun, Hang-Chat and Muang Lampang districts (Figure 1) during November 2016 - October 2017. The collected snails were transferred to our laboratory and identified to species based on the key of Brandt (1974).

2. Collecting and Identification of metacercariae

Snails were individually examined by a crushing method under a light microscope. The prevalence and intensity of infected metacercariae were calculated following Margolis et al. (1982). The prevalence is the number of hosts infected with 1 or more individuals of a particular parasite species divided by the number of hosts examined for that parasite species. The intensity of infection is the number of individuals of a particular parasite species in a single infected host.

Specimens of *Echinostoma revolutum* metacercariae were separated into 2 groups: the first group was fed to hamsters (*Mesocricetusauratus*) and domestic chickens (*Gallus gallusdomesticus*), and the second group was washed with distilled water and preserved in 70% ethanol for molecular study. Use of laboratory animals followed the Ethical Priciples and Guidelines for the Use of Animals, Institute for Animals for Sciencetific Purpose Developmrnt (IAD), National Research Council of Thailand (NRCT) under supervision of researcher ID U1-03301-2559.

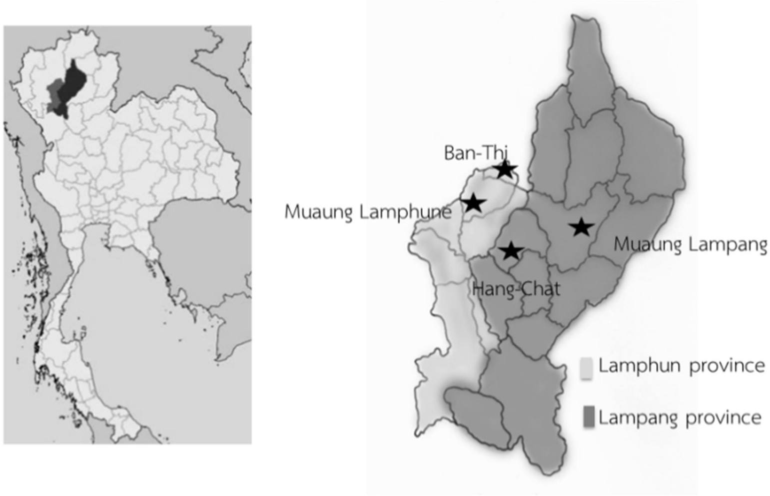

Figure 1 Map of snail sampling sites in four districts of Lamphun and Lampang provinces, Thailand.

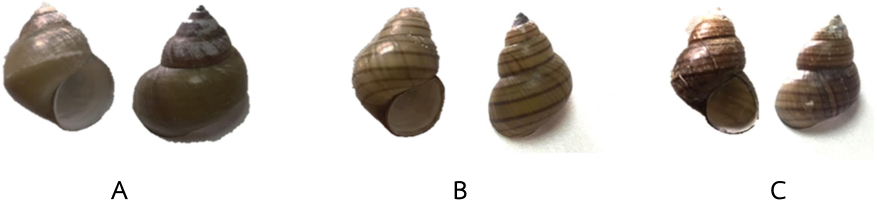

Figure 2 External morphology of *Filopaludina* snails
A) Filopaludina summatrensis polygramma B) *F. dorliaris*, and C) *F. martensi martensi*

3. Electron microscopic study

The tegument surface of adult worms prepared for scanning electron microscope (SEM) studies were examined by JEOL JSM-5910LV SEM at an accelerating voltage of 15 kV.

4. Molecular study

Genomic DNA of metacercariae and adults was extracted using Chelex solution (Fluka, Sigma-Aldrich, St. Louis, Missouri, USA). The ND1 subunit was amplified by using the primers, JB11 (5′- AGA TTC GTA AGG GGC CTA ATA -3′) and JB12 (5′-ACC ACT AAC TAA TTC ACT TTC -3′) (Morgan & Blair, 1998). PCR comprised of the initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, anealing at 55°C for 45 s, separation the amplicons by 1.5% agarose gel electrophoresis, staining with DNA Dye Non-Tox and recording by using a digital camera. Amplicons were purified by using the GF-1 PCR Kit (Vivantis, Malaysia) and sequenced by Bio Design Pathum Thani, Thailand.

ND1 sequences of *E. revolutum* metacercariae and adult stages were compared with previously available sequences in the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was reconstructed by using Maximum Likelihood method with 1,000 bootstrap replicates of multiple sequence alignments created by MEGA version 7.0.

Results

Prevalence of echinostome infection

Metacercariae of *E. revolutum* were found in three species of *Filopaludina* snails, specifically *Filopaludina martensi martensi*, *F. dorliaris* and, *F. summatrensis polygramma* (Figure 2). The prevalence and intensity of infection with *E. revolutum* metacercariae are shown in Table 1. The highest prevalence was in *F. summatrensis polygramma* followed by *F. dorliaris*, and *F. martensi martensi* respectively.

Table 1 Prevalence and intensity of infection with *E. revolutum* metacercariae in *Filopaludina* snails from Lamphun and Lampang provinces.

Species of snails	No. of snails examined	No. of infected snails	Prevalence %	Intensity (metacercariae /snail)
F. martensi martensi	599	162	27.05	6.77
F. dorliaris	613	257	41.92	10.09
F. sumatrensis polygramma	512	246	48.05	7.28

Light and Scanning electron microscopic studies

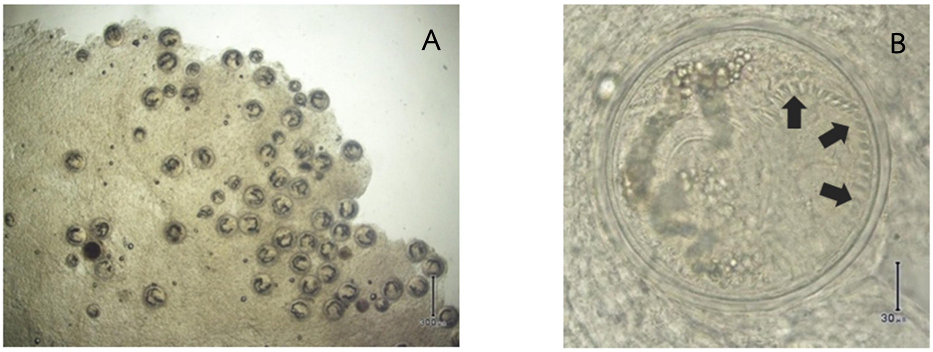

Figure 3 A) Metacercariae found in pericardial sac of *Filopaludina* snails
B) Encystedmetacercaria showing the collar spines (black arrows)

The metacercariae are spherical in shape with a bilayer of wall comprising a thick outer wall and a thin inner wall. The head collar isobviously visible with 37 collar spines. Oral sucker, ventral sucker, and many rounded excretory granules are distinct (Figure 3B).

Description of *E. revolutum* adult

Type I testes lobed : *E. revolutum* has 7.48 (7.45-7.50) x 1.49(1.43-1.55) mm in body size, testes are tandem slightly lobed; anterior testes 0.83 (0.43-1.23) x 0.94 (0.45-1.43) mm and posterior testes 1.01 (0.51-1.50) x 0.76 (0.46-1.05) mm (Figure 4A).

Type II testes rounded : *E. revolutum* has 6.85 (4.48-14.23) x 1.04 (0.68-1.48) mm in body size, testes are tandem smooth rounded, anterior testes 0.44 (0.30-0.66) x 0.39 (0.31-0.56) mm and posterior testes 0.51 (0.30-0.90) x 0.4 (0.33-0.62) mm (Figure 4B).

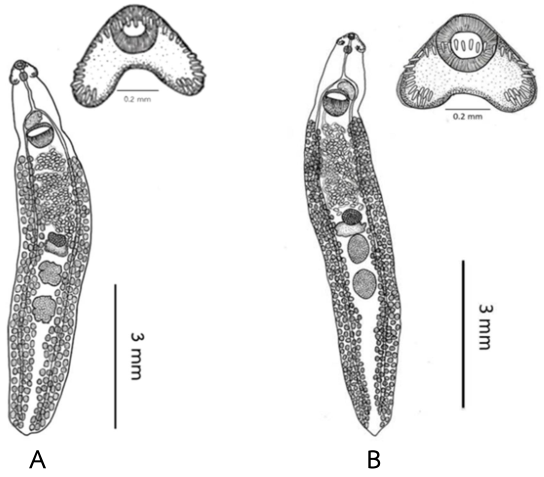

Figure 4 LM studies, morphology of *E. revolutum* adults from experimental host A) *E. revolutum* Type I B) *E. revolutum*type II

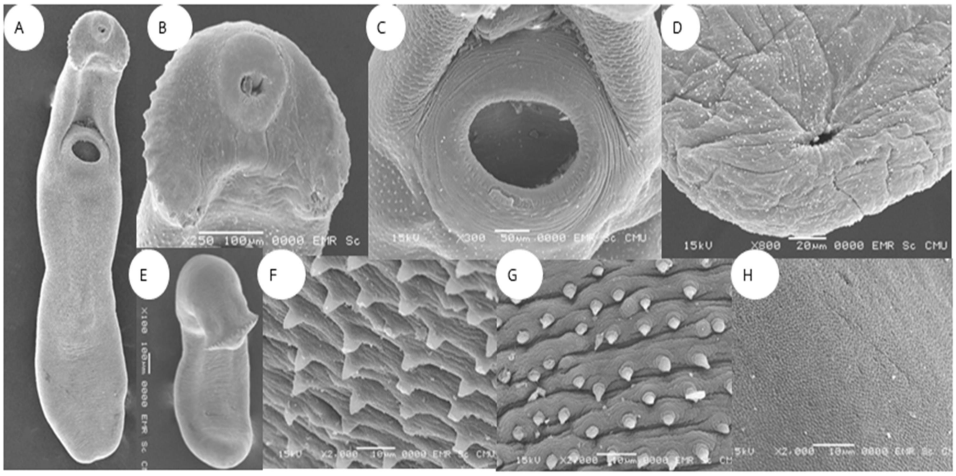

Figure 5 SEM view of *E. revolutum* adult; A) Ventral view of the whole fluke showing elongated body and anterior tip with head collar, collar spines, oral sucker and ventral sucker; B) A close-up of head collar showing oral sucker and collar spines surrounded the horse-shoe shaped head collar; (C) A close-up of the rounded ventral sucker and cirrus arm; (D) Posterior tip of the body showing excretory pore; (E) Ventral view of the whole fluke showing its anterior body and showing the dorsal collar spines; (F) Ventral view of the anterior tugumental surface showing the peg-shaped tegument spines; (G) Middle portion of the anterior tugumental surface showing the cone-shaped tegument spines; (H) Posterior portion of the body tegument showing the smooth skin without spines.

Phylogenetic relationships based on ND1 subunit

The phylogenetic tree demonstrated the relationships of metacercariae and adults of *E. revolutum* and other echinostome species from Gen Bank. Adult of *Paragonimus westermani* and *P. miyazanii* were used as out groups of the phylogenetic tree based on ND1 data (Figure 6).

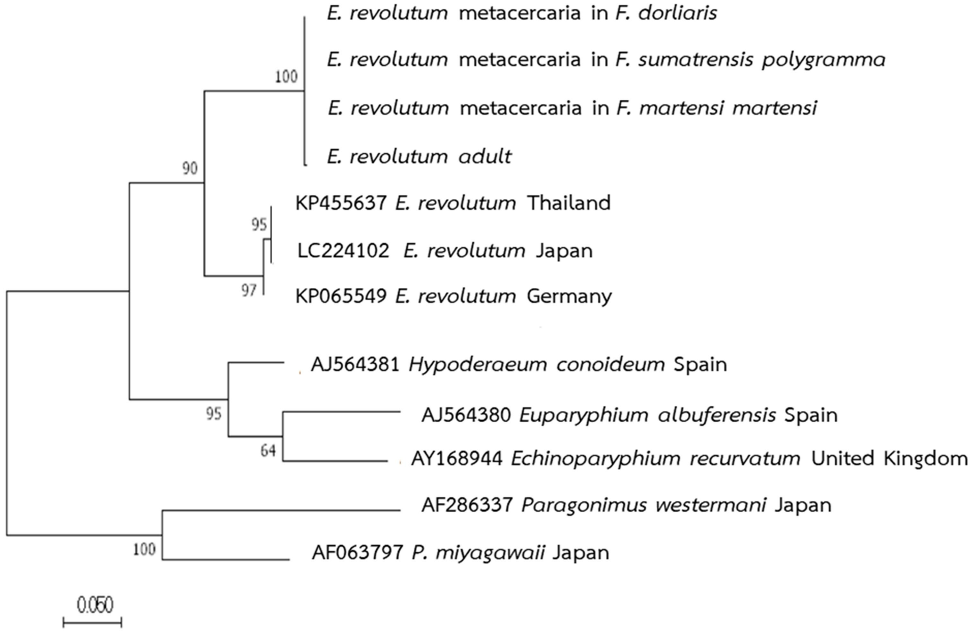

Figure 6 Maximum-likelihood (ML) bootstrap consensus tree with 1,000 bootstrap replicates of ND1.

The obtained phylogenetic trees were reconstructed using ML methods. The chosen consensus tree showed the relationship of *E. revolutum* metacercariae from three *Filopaludina* spp. snails and adults of *E. revolutum*. The phylogenetic tree was divided into 2 main clades: the first clade was a group of *Echinostoma* having 37 collar spines and the second clade was group of flukes having more than 37 collar spines. Metacercariae and adult of *E. revolutum* in this study were grouped together with other *E. revolutum* from other province of Thailand and other countries.

Discussion

In this study, *E. revolutum* metacercaria were collected from three species of *Filopaludina* snails. The total prevalence of metacercarial infection in *F. summatrensis polygramma* was higher than in *F. dorliaris* and *F. martensimartensi*. According to the results of Noikhong & Wongsawad (2014), *Echionstoma* metacer carialin fection in *F.*

dorliaris was higher than in *F. martensimartensi* because of different host specificity in individual host species. Prevalence of metacercarial infection in *F. dorliaris* is similar to *F. sumatrensis polygramma* and maybe caused by the morphology and size of those snails being very similar, and their usually being found together in this field. Based on LM studies, echinostome metacercariae in this study could be identified into the genus *Echinostoma* by the number of collar spines (37 collar spines), and by other morphological characters that are also similar (Georgieva et al., 2014; Nagataki et al., 2015). Also, the morphology in the adult stage was identical to *E. revolutum*, except, for some variations of testes shape. Testes of adult *E. revolutum*were divided into 2 patterns: slightly lobed and smooth rounded. Lobed testes observed in this study were similar to those reported by Lee et al. (1990) and Chai et al. (2011). Whereas, smooth rounded testes were similar with the results of Chantima et al. (2013). In agreement with Sohn et al. (2011), that found 2 patterns of testes in *E. revolutum*from children were discovered in Pursat province of Cambodia.

SEM observations showed the surface morphology of *E. revolutum* in the adult stage. Results of the present study revealed the arrangement of collar spines on the head collar and many types of tegumental spines on the whole body of fluke (except the posterior end of the body) were similar to previous studies (Chai et al., 2011; Chantima, 2014).

Molecular methods have been reported to distinguish to species level among morphologically similar echinostomes. There are several records in using data derived from genetic markers for phylogenetic tree reconstruction. For example, application of CO1 and ND1 genes to discriminate *Echinostoma* species in Australia (Morgan & Blair, 1998), utilizing ND1 and 28S rRNA genes for identification of *E. revolutum*in Europe (Georgieva et al., 2014). In Thailand, Chantima (2014) studied in ITS2 and ND1 genes of *E. revolutum* from Chiang Mai province and found that the isolates of this species in Thailand were closely related to those in Australia and Europe. Nagataki et al. (2015) focused on CO1 and ND1 of *E. revolutum* from the northeast region, middle and lower north part of Thailand and discovered that species in Thailand clustered together with the European isolates based on a ML tree of ND1. According the tophylogenetic tree inferred from ND1 sequence of the present study, it could be concluded that two major clusters of *E.revolutum* metacercariae and adult stages from Lumphun and Lampang provinces were closely related to the same species in Europe and agreed with reports of Chantima (2014) and Nagataki et al. (2015).

Conclusions

Echinostoma revolutum found in this study was separated into 2 types : slightly lobed and smooth rounded shapes of testes. Molecular study of ND1 subunit could be also used to indicate species of metacercariae and adult in *E.revolutum.* Additionally, the results of this study confirmed the distribution of echinostome, the cause of echinostomiasis in humans, previously recorded in Lamphun province and newly discovered Lampang province, in the northern part of Thailand.

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