

INTRODUCTION

World Health Organization (WHO) estimated about half of billion people infected with Foodborne Parasite Diseases, including 40 to 50 million infected with intestinal trematodes and at least 18 million people have fishborne zoonotic diseases. Seventy intestinal trematode species were found in many countries in the world, distributed in a number of families, including Heterophyidae and Echinostomatidae that have biggest number trematode species infecting humans recorded so far.

Up to now, the pathology of intestinal trematodiasis has not been completely studied. Small trematode infections in human can be actopic parasitized; and eggs and adults from the intestinal mucosa could penetrate the vessels then along the blood circulation to the heart, brain, spinal cord, leading to death of the host. The eggs of some small trematodes were also found in the form of a cyst in the brain of patients with neurological symptoms.

Intestinal trematode diseases have become a public health problem in countries such as China, Philippines, Taiwan, Thailand, Laos, Cambodia,... and they have been regarded as diseases transmitted from animals to humans (zoonotic diseases).

In recent years, several studies on mixed infections between small liver and small intestinal flukes in the human in many endemic areas of liver flukes in Laos, Thailand, Vietnam, Korea ... From those situations, there are many comments about the diagnosis, treatment and prevention of small fluke diseases. WHO has made recommendations to conduct mass drug treatment in the community for people who are high-risk infection with small flukes.

In Vietnam, intestinal flukes have long been found in intermediate hosts such as snails, freshwater fishes and in main hosts like dogs, cats, birds, chickens..... In recent years, a number of studies have also reported about problems of mixed infections by small liver and small intestinal flukes in

many endemic areas. These mixed infection problems give rise to attentions related to treatment strategies and prevention for small fluke diseases in the community. Besides, the data on morphological and molecular characteristics of small intestinal flukes are missing, scanty or have not been adequately studied in Vietnam.

Hence, study on morphological examination and molecular properties of mitochondrial and nuclear ribosomal genes of small intestinal flukes that infect human in different geographical localities, and proposal of their treatment in the community are essential. Reasonably we have conducted the study with the aims as:

1. To determine the species composition, descriptions of the morphological characteristic of small intestinal flukes in some provinces of Vietnam.
2. To identify the intestinal fluke species by molecular methods.
3. To assess treatment effects to small trematode infections by praziquantel with dose of 50mg/kg, in the community.

NEW CONTRIBUTIONS, SCIENCE AND PRACTICE OF THE THESIS

1. This study combined two approaches including morphological and molecular studies to determine, identify, and confirm the small intestine trematodes of Heterophyidae and Echinostomatidae in people in 9 provinces in Vietnam. On the other hand, the thesis indicated the interactive usage of correlative research using mitochondrial and nuclear ribosomal genetic markers. The results from molecular implementation of the mitochondrial and nuclear genetic markers indicated the complementary contribution to increase the reliability of identification of intestinal parasitic trematodes in the study.

2. As the results in this thesis, there have been four small trematode species of Heterophyidae, including *Haplorchis taichui*, *Haplorchis pumilio*, *Stellantchasmus falcatus*, *Centrocestus formosanus*, and a species,

Echinochasmus japonicus of Echinostomatidae to be determined, identified and confirmed at the taxonomic level by morphological and molecular application from a large number of samples that infected humans.

3. From the results of evaluating the effectiveness of treatment and the reduction rate of eggs after 2 weeks of the treatment for small trematodes in humans at high risk of infection in the community, who had been eating raw fishes, it could be a large application using therapy with praziquantel, 50 mg/kg dose, in the endemic localities of small trematodes in Vietnam.

THESIS STRUCTURE

The thesis consists of 138 pages: Introduction (2 pages), Literature review (37 pages), Research methods (24 pages), Results (42 pages), Discussions (30 pages), Comments and Suggestions (3 pages). References include 166 (26 documents in Vietnamese and 140 documents in English) and 3 appendices.

CHAPTER 1. OVERVIEW

1.1. SOME GENERAL CHARACTERISTICS OF FLUKES

General morphological features of a trematode were described by Ichiro Miyazaki, 1991 such as: body fluke flat, leaf-shaped, genital opening at the abdomen. The shape and size of the fluke are variable, depending on where the worms parasitize in their hosts. Trematodes have some organs well developed such as moving, digestion, excretory organs; nervous and reproductive systems.

Classification of small flukes: Kingdom: Animalia; Phylum: Platyhelminthes; Class: Trematoda; Subclass: Digenea; Order Opisthorchiida. There are many families consisting of plural number of species: 31 species in Heterophyidae, 21 species in Echinostomatidae, 4 species of Plagiorchiida and others.

The aim of this study was to focus on some small intestinal trematodes, confirmatorily found in humans in Southeast Asia and in Vietnam, belonging to the Heterophyidae and Echinostomatidae families.

1.2. The morphological identification characteristics of genera of Heterophyidae

Genus *Haplorchis*: abdominal genital sucker have spines, have one testis, dorsal pocket appeared. Genus *Procerovum*: abdominal genital sucker have very small spines, only one testis, not have dorsal pocket. Genus *Stellantchasmus*: no spines on the abdominal genital sucker have two testes, not have dorsal pocket.

1.3. The morphological identification characteristics of some species of Heterophyidae and Echinostomatidae

H. pumilio: Adult worms have pear-shaped; abdominal genital sucker has 32-40 spines; the spine is I-shaped or A-shaped, anterolateral lobes have some small spines, center dorsal lobe have small spines appearance.

H. taichui: Ventral sucker have 12-16 spines, some spines are of up to 30 μm in length (forms is similar bunch of bananas), bowel division 2 branches and they extend until testis location.

S. falcatus: Have two opposite testes; ventral sucker has 2 small spines in the outer group.

Echinostomatidae: Stick disk development, two testes laying one behind the other. Hooks boss a row, abdominal contour. Genital sucker does not exceed the ventral sucker. Uterus contains developed eggs.

E. japonicus: Head has 24 spines, ventral sucker is in middle of the body, that is bigger than oral sucker. The oval is located behind the body. The vitelline follicles are start shortly after ventral sucker.

A summary by Jitra Waikagul demonstrated that there were 14 species of small intestinal trematodes recorded in Thailand, while 12 species in the Philippines; 8 species in Indonesia and 4 species in Malaysia, respectively. Many species of small intestinal trematodes were found in other countries such as China, Korea, Taiwan, Laos PDR, and Cambodia.

1.4. Research on small intestinal flukes in human in Vietnam

In 2006, in Vietnam there was a report announcing some of small intestinal flukes infected in humans, including *H. pumilio*, *H. taichui*, *H.*

yokogawai, *S. falcatus*, *Procerovum* sp. and *Echinostoma* spp. These species were found in some provinces such as Ha Tay, Nam Dinh, Yen Bai, Thanh Hoa, Lam Dong and Thua Thien Hue, with prevalence based on the results of stool examination, from 0.2% to 6.6%. *H. pumilio* particularly was found in most the provinces.

Another study in Nghe An, Nam Dinh and An Giang provinces showed that the prevalence of fishborne trematodes were 0.06%, 64.9% and 0.29%, respectively. One survey in a mountainous commune of Phu Tho province recorded that the prevalence of small liver flukes was 16.4%, small intestinal fluke infection was 4.3%. A study in two communes of Nghia Hung district, Nam Dinh province where people have a habit of eating raw fishes, the prevalence of small trematode infections was up to 64.9%, and the prevalence in men was 68.7% and 23.1% in women.

In 2007, a survey to determine the situation of small fluke infections in Nam Dinh showed that 37% of men infected with small flukes, while the rate for women was 25.7%. Some fluke samples have been evaluated by molecular techniques using ITS2 and 18S genetic markers, comparing the nucleotide sequences components of ITS2 and 18S genes of *H. pumilio* and *H. taichui* of Vietnam and Thailand.

1.5. Diagnosis of small trematode infections

Diagnosis of small fluke infections was based on the detection of parasite eggs in the feces, of which the result depended much on the determination of a carefully observed and measuring size of the collected eggs. The ability to distinguish between the eggs in faeces are complicated and difficult in the cases of mixed infections with both small liver and small intestinal trematodes.

Besides, serologic test can be used for diagnosis such as ELISA technique, that is useful in case of a negative stool tests. Nowadays, molecular methods have been initially applied to the diagnosis of small intestinal flukes in humans.

1.6. Treatment for small trematode infections

Praziquantel is the drug of choice for all heterophyid infections in human, with a single dose of 10-20/kg, successfully treated for 95-100% of the cases. The treatment of *Echinostoma* infection required using praziquantel from 10 to 25 mg/kg in a single oral dose. Some authors had recommendations that, doses to treat small intestinal flukes might be higher as of 25 mg/kg dose with 3 times/day.

In recent years, several studies have found the mixed infections with small liver and small intestinal flukes in humans in many endemic loci of liver flukes in Laos, Thailand, Vietnam, Korea.

Many authors have mentioned that, eggs of the small liver flukes (Opisthorchiidae) can not be identified with small eggs of intestinal flukes (Heterophyidae) by using the traditional Kato-Katz examination method. Therefore, WHO has made recommendations to conduct mass drug treatment in the community for human who are of high-risk infection with small trematodes.

1.7. The applications of molecular biology in small trematode research

Some species of small intestinal trematode infections in humans in Vietnam were identified by morphological method such as *H. taichui* and *H. pumilio* species, which are common in Southeast Asian countries. Those species have been assessed by molecular biology using ITS2 and 18S genes. By using sequencing method and comparative analysis of nucleotide sequences of ITS2 and 18S sequences, *H. pumilio* and *H. taichui* of Vietnam and Thailand are at the similarity rate of 99%, respectively.

The Thai author, Urusa Thaenkham, successfully distinguished two small trematodes, *O. viverrini* and *H. taichui* based on mitochondrial cytochrome c oxidase subunit I (COI) gene with high sensitivity for different developmental stages including adult, metacercaria, and egg. The small and large subunit of ribosomal DNA genes (18S rDNA and 28S rDNA) and internal transcribed spacer subunit 2 (ITS2) were used as molecular markers

for the subfamily Haplorchiinae. Maximum Likelihood and Bayesian analyses using combined rDNAs and ITS2 sequences indicated a close relationship between the genera of *Haplorchis* and *Procerovum*, while these two genera were distinct from *Stellantchasmus falcatus*.

A study for classification of *Haplorchis* and *Opisthorchis* using approach with PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to identify the exact species of each metacercaria for each of *H. taichui*, *H. pumilio*, *H. yokogawai*, *P. varium*, *S. falcatus*, và *C. formosanus*, compbined with the 28S ribosomal RNA gene as the genetic marker.

CHAPTER 2: SUBJECTS AND METHODS

2.1. Subjects, study sites and time of the study

2.1.1. Subjects of the study

2.1.1.1. Study on morphology and molecular biology of small intestinal trematode adults

Subjects of this study were the small intestinal trematode adult samples of Heterophyidae and Echinostomatidae that obtained from patients after treatment in some provinces of Vietnam. These patients were identified by cross-sectional surveys to determine the prevalence, intensity infection with small trematode and recovery adult worms after treatment.

2.1.1.2. Study on treatment effects for small trematode infections in human in the community.

People from 6 years old and <80 of age who have lived and ever-eating raw fish at the study sites.

2.1.2. The study site

2.1.2.1. The study site of morphological and molecular biology studies

- The provinces including Ha Giang, Hoa Binh, Phu Tho, Ha Noi, Quang Ninh, Nam Dinh, Ninh Binh, Thanh Hoa and Quang Tri.
- The study was conducted at the Laboratory of the National Institute of Malariology Parasitology and Entomology; at the Institute of Biotechnology

in Vietnam and at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

2.1.2.2. The study site of research on assessment the treatment effect for small trematodes in human in the community.

- Purpose choosing two communes including Nghia Hong commune of Nghia Hung district, and Nghia Hoa commune of Hai Hai Hau district, Nam Dinh province where people have habit of eating raw fish and they are endemic of small liver fluke disease.

2.1.3. Time of the study

From January, 2010 to December, 2013

2.2. Study methods

2.2.1. Study design

2.2.1.1. The descriptive analysis based on the methods for identifying morphological and molecular biology characteristics

2.2.1.2. Intervention studies evaluating treatment effects for small fluke at the community, not controlled subjects.

2.2.2. Sample size

2.2.2.1. Sample size for morphological study

- Total 45,621 small intestinal fluke adults were collected in the provinces, which were taxonomically determined based on morphological characteristics with direct observation method with microscopy.

- Some slides of adult intestinal flukes after staining with Semichon's acetic carmine that were of pretty, clear body part, visually observed, measured, described organs were purposely selected to determine morphological characteristics.

2.2.2.2. Sample size for molecular research

At least one sample for each species of small trematodes in each province, which was clearly identified by morphological method, was purposely selected to assess the molecular characteristics.

A total of eighty-four (84) adult intestinal flukes have been studied on the molecular characteristics using *cox1* and 28S genes in molecular biology research.

2.2.2.3. Sample size to assess the effects of treatment for small trematodes in human at the community

The participants in the entire cross-sectional study were purposely selected to collect adult fluke samples, as research subjects for intervention studies evaluating the effectiveness of treatment; and a total of 396 people were involved in the study.

2.3. Content of the morphological study on intestinal fluke adults

2.3.1. The direct determination of small intestinal trematodes by microscopy

2.3.2. The determination by Semichon's acetic carmine staining method

2.3.3. The basis of the determination of adult fluke by morphological method

- Based on morphological type keys of Pearson and Ow-Yang (1982) for small intestinal trematodes of Heterophyidae.
- For the small intestinal fluke species of Echinostomatidae, the determination based on the morphological type keys described by Nguyen Thi Le and Jitra Wikagul.

2.4. The contents of molecular research of some small trematode species

2.4.1. Total DNA extraction method from trematode samples

+ Extraction of DNA: Total genomic DNA of small intestinal flukes were extracted by AccuPrep® Genomic DNA Extraction Kit (BIONEER, Korea).

2.4.2. PCR technique

The primers were designed based on conserved sequence of genes in GenBank including JB3F; JB4,5R; U28SF; U28SR; COI-Ov-Hap-F; COI-Ov-Hap-R (Table 2.1).

Table 2.1. Primer sequences using for genetic study of *cox1* and 28S genes

Name of primer	Sequences (5' – 3')	No. nucleotide	PCR products
JB3F	TTTTTTGGGCATCCTGAGGTTTAT	24	0.44 kb
JB4,5R	TAAAGAAAGAACATAATGAAAATG	24	
U28SF	CTAACAAGGATTCCTTAGTAAC	23	1.2 kb
U28SR	GTCTTTCGCCCCTATACTCAC	21	
COI-F	GGG TTY GGT ATR RTK AGW CAC	21	3.75 kb
COI-R	AAA CCA AGT RTC ATG MAA CAA AG	23	

2.4.3. Checking PCR products on agarose gel by electrophoresis

2.4.4. PCR product purification method

2.4.5. Sequencing method

2.5. The contents of studies to assess treatment effects on small trematodes in human in the community

- Determination of the prevalence of small trematode infections before treatment, after treatment for 2 weeks, 3 months, 6 months and 12 months.
- Evaluation on treatment efficacy after 2 weeks of treating on participants.
- Determination of the egg reduction rate (%).

2.6. Ethical issues in the study

The content of research and ethical issues in intervention studies were accepted and allowed to conduct research by Scientific Committee and the Ethics Committee of the National Institute of Malariology, Parasitology and Entomology, Ministry of Health.

The study was conducted under the provisions of the Ethics issues in biomedical research.

2.7. Data analyses

Using the Excel software and SPSS software version 10.0 and Stata version 16.0. Using bioinformatic software as GENDOC2.7; MEGA6:06, BioEdit 7.0 to evaluate the research results.

CHAPTER 3. RESULTS

3.1. The results of cross-sectional survey to assess the situation helminth infections

A total of 4,731 people have been tested, there were 1,243 people infected with small flukes including liver and small intestinal flukes for 26.3% (When using Kato-Katz test, it can not be distinguished between liver fluke eggs and eggs of small intestinal flukes of Heterophyidae).

3.2. The results of the recovery adult small intestinal flukes in the studied provinces

Table 3.2. The results of recovery adult flukes from patients after treatment.

Name of provinces	No. patient	The trematode species recovery					
		<i>H. taichui</i>	<i>H. pumilio</i>	<i>S. falcatus</i>	<i>C. formosanus</i>	<i>E. japonicus</i>	<i>Small liver fluke</i>
Ha Giang	5	91	41	0	54	0	0
Phu Tho	1	6	5	0	0	43	0
Hoa Binh	6	279	178	0	0	16	436
Ha Noi	3	54	75	0	0	15	136
Quang Ninh	5	259	0	30	0	0	0
Nam Dinh	6	250	208	26	0	0	18
Ninh Binh	7	115	855	0	0	0	47
Thanh Hoa	8	3,534	178	0	0	0	1
Quang Tri	4	39,179	130	0	0	0	1
Total	45	43,767	1,670	56	54	74	639

A total of 46,260 adult flukes were collected from 45 patients. The adult worms were identified by morphological method using optical microscopy and selected some for staining by Semichon's acetic carmin method to measure and describe morphological characteristics.

According to Table 3.3, the purposely chosen stained adult intestinal flukes from the provinces were 733, which were beautifully staining flukes,

for observation, description, and measurement for determining the morphological characteristics. There were 346 *H. taichui* from 9 provinces, 295 *H. pumilio* from 8 provinces, 20 *S. falcatus* flukes from Quang Ninh province, 16 *S. falcatus* from Nam Dinh province. *E. japonicus* adults stained from Phu Tho, Hoa Binh and Hanoi were 19, 9 and 7 worms, respectively. From Ha Giang province, 21 samples of *C. formosanus* adult worms were stained to analyze the morphological characteristics.

Table 3.3. The numbers of stained worms were selected for observing and measuring the morphological characteristics in the provinces

Trematode Species Name of pro.	<i>Number of worms stained by Semichon's acetic carmine</i>				
	<i>H. taichui</i>	<i>H. pumilio</i>	<i>S. falcatus</i>	<i>E. japonicus</i>	<i>C. formosanus</i>
Ha Giang	31	20	0	0	21
Phu Tho	3	2	0	19	0
Hoa Binh	34	33	0	9	0
Ha Noi	32	34	0	7	0
Quang Ninh	39	0	20	0	0
Nam Đinh	37	39	16	0	0
Ninh Binh	46	35	0	0	0
Thanh Hoa	59	61	0	0	0
Quang Tri	65	71	0	0	0
Total = 733	346	295	36	35	21

3.3. The morphological characteristics of some adult intestinal flukes species in the study

The images of adult flukes parasites in humans that were stained by Semichon's acetic carmine method (A), the images of spines in the ventral genital sucker and spines at oral sucker (B and C).

The trematode species were identified by morphological methods; some of important characteristics such as number and type of spines; the arranged of

spines at the mouth and ventral genital sucker; number of testes; position; shape of some organs were observed and described by using the morphological keys for determining the species of the small intestinal fluke obtained.

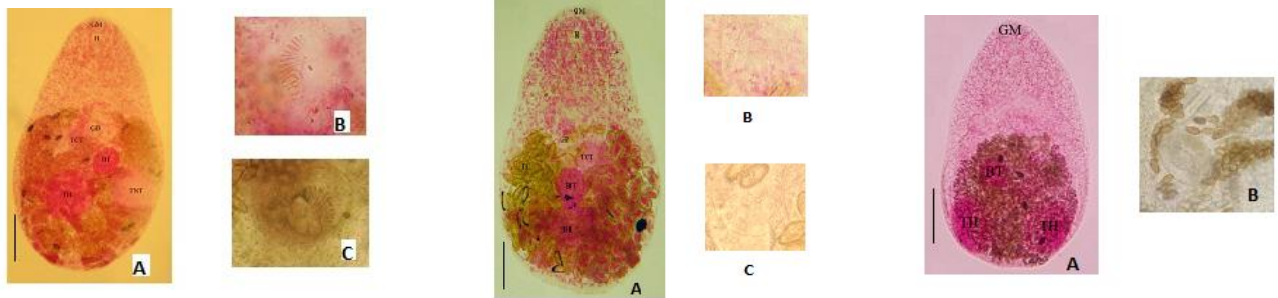


Figure 3.2. *H. taichui*

Figure 3.3. *H. pumilio*

Figure 3.4. *S. falcatus*

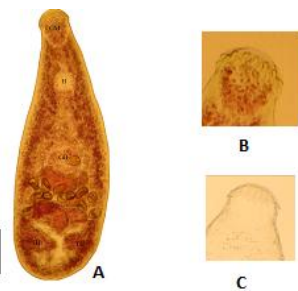


Figure 3.5. *C. formosanus*

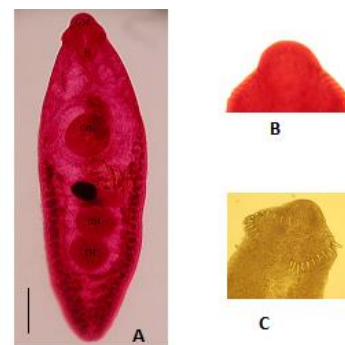


Figure 3.6. *E. japonicus*

3.4. The results of identification of small intestinal flukes of Heterophyidae and Echinostomatidae using genetic markers of mitochondrial *cox1* and 28S ribosome

3.4.1. Obtaining *cox1* and 28S ribosomal gene sequences from the samples

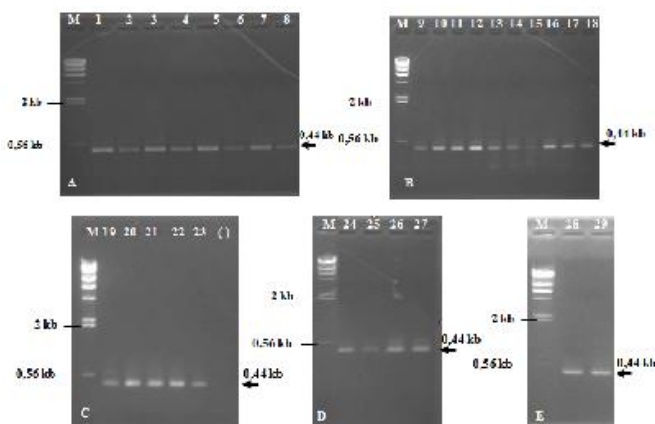


Figure 3.7 PCR products of *cox1* checked by electrophoresis

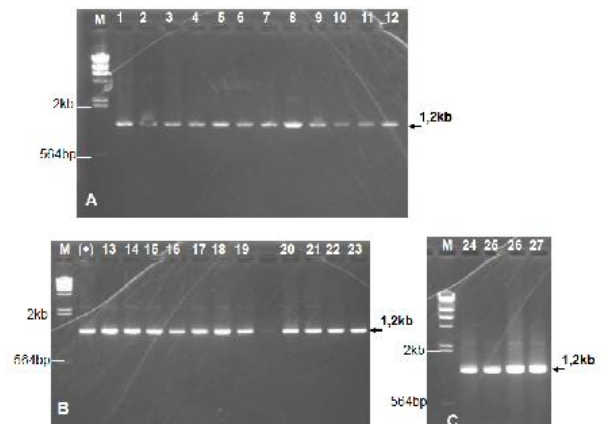


Figure 3.8. PCR products of 28S checked by electrophoresis

Figure 3.7 indicated that all 29 DNA templates of small intestinal trematodes produced clear mitochondrial DNA *cox1* products with JB3F-JB4,5R primer. PCR bands were appeared completely bright, clearly, approximately 0.44 kb. This result demonstrated that the designing primers, DNA extraction, composition and thermal cycling conditions of PCR reactions were correct.

Using U28SF/U28SR primers, the PCR products of about 1.2 kb were obtained from 27 samples of intestinal trematodes including *Haplorchis spp.*, *Stellantchasmus spp.*, *Centrocestus spp.* và *Echinochasmus spp.*

3.4.2. Comparison and nucleotide analysis of cox1 sequences of small intestinal flukes with corresponding sequences of the identified species

The nucleotide sequence of *cox1* gene contains 350 nucleotides of the 29 small intestinal fluke samples (including 9 sample belonging to *H. taichui*; 8 *H. pumilio*; 3 of *S. falcatus*; 3 *C. formosanus*, and 6 strains of the *E. japonicus* species) collected from provinces/localities in Vietnam has been compared, analyzed for comparison with the corresponding gene sequences of eight trematode species representing different varieties.

- The strains of *H. taichui* have similarity from 98% to 100%, compared with each other and with the reference strains of *H. taichui* of Thailand.
- With the strains of *H. pumilio* in research, there was a high identity rate at nucleotide level (98-100%) with the corresponding sequence of *H. pumilio* species of Thailand.
- For *Stellantchasmus falcatus*, two strains from Quang Ninh and another strain from Nam Dinh province have the same nucleotide sequence, 99-100% identity, with the reference strain collected in Vietnam that has been studied in Thailand with registration code (Sf-TH KF044301) in GenBank.
- Similarly, the three strains of *C. formosanus* that were collected in Ha Giang and Nam Dinh, have 99% identity to each other.
- There was no difference (100% identity) between strains of *E. japonicus*, which were studied in this research.

- There were higher differences between the strains of one to another species. Between species within a group in Heterophyidae, identity rate reached only 79-83%.

- Between the *E. japonicus* and species of other groups like *H. taichui*, *H. pumilio*, *C. formosanus* and *S. falcatus*, identity rates ranged from 68-78%.

3.4.3. The species relationship between strains of small intestinal flukes by phylogenetic analysis based on *cox1* sequences

The phylogenetic analysis showed that, 37 small intestinal trematode species of Vietnam and the global strains were divided into two main groups:

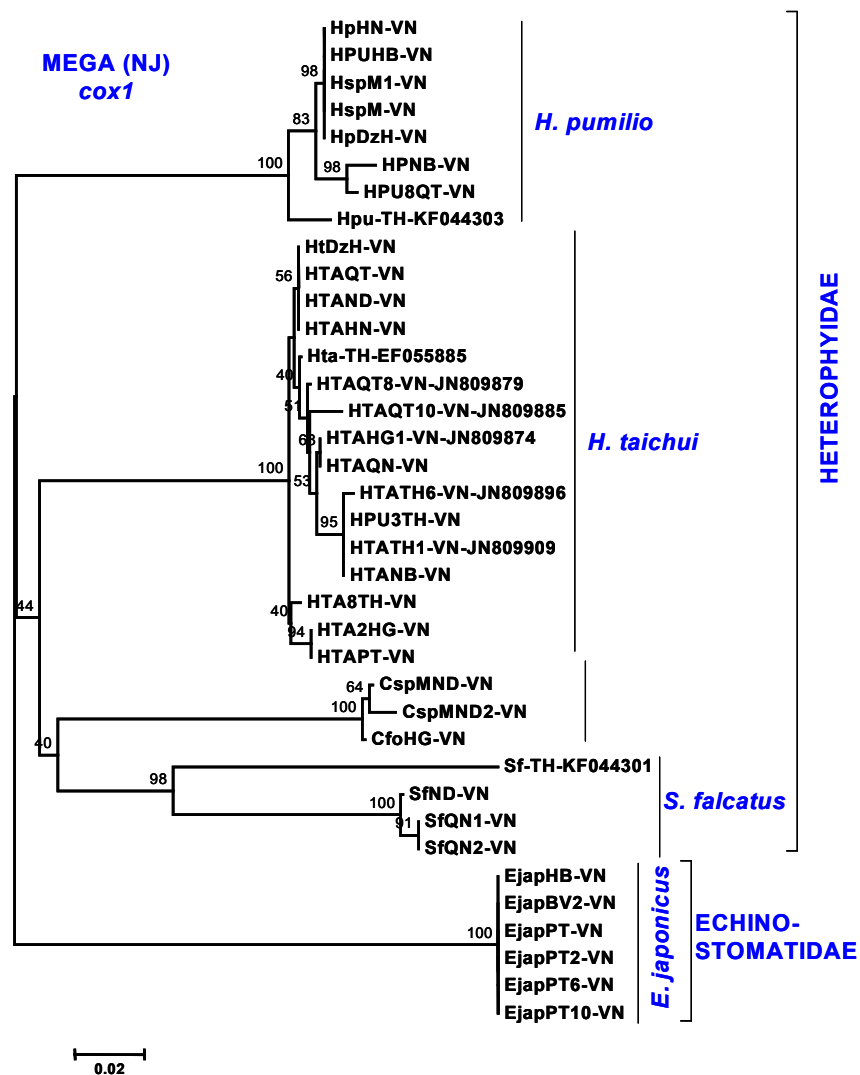


Figure. 3.9. Phylogenetic tree of species relationships of small intestinal fluke species based on partial *cox1* nucleotide sequences (350 bp).

- The first group consists of 31 species of Heterophyidae, that divided into four branches including strains belonging to *H. taichui*, *H. pumilio*, *C. formosanus* and *S. falcatu*, respectively.
- The second group, completely separated from the group 1, consisting of 6 strains of *E. japonicus* of Echinostomatidae that infected humans.

3.4.4. The nucleotide sequence analysis of 28S ribosomal sequences of the studied strains with the identified strains

The nucleotide sequence of 28S gene (1028-1053 nucleotides) of 27 strains of the studied species including *H. taichui*; *H. pumilio*; *C. formosanus*; *S. falcatus* and *E. japonicus* were identified morphologically, that were sorted and compared the sequence with four reference strains of each species in GenBank.

- The nucleotide sequence of the 28S gene for 8 *Haplorchis spp* isolates that were collected in the provinces of Vietnam had absolute similarity (100%) when compared with the corresponding sequence of the Vietnam strain with each other and with the reference strains of *H. taichui* from Thailand.

- Similarly, the nucleotide sequences of the 28S genes of 10 strains of *Haplorchis pumilio* that were identified by morphological method have very high similarity rate (99-100%) with the reference strains of *H. pumilio* of Thailand (HpNP1-TH, GenBank No. HM004186) and compared with the corresponding sequences of the Vietnamese strain together.

- For *S. falcatus*, two isolates were collected in Quang Ninh (SfQN1-VN; SfQN2-VN) has been identified based on the comparing results using the nucleotide sequences of the 28S gene with the reference strain, SfalVN1-VN (HM004174). This strain was originated from Vietnam, and taxonomically identified as belonging to *S. falcatus* of Heterophyidae by Thailand experts with the registration number in GenBank (HM004174).

- The nucleotide sequence of the 28S gene of three strains *Centrocestus spp* collected in Ha Giang province (CfoHG-VN) and strain (CspmND2-VN)

collected in Nam Dinh province were compared with the reference strain of Thailand (Cfor-TH, HQ874609) showed 100% identity to each other.

- For *E. japonicus*, sequences of 28S for four isolates collected in Hoa Binh (EjapHB-VN); Ba Vi, Hanoi (EjapBV2-VN) and Phu Tho (EjapPT-VN; EjapPT10-VN) were compared with each other. The results showed identity rate of 100% for these nucleotide sequences.

3.4.5. The species relationships between strains of small intestinal flukes based on phylogenetic analysis of 28S ribosomal sequences

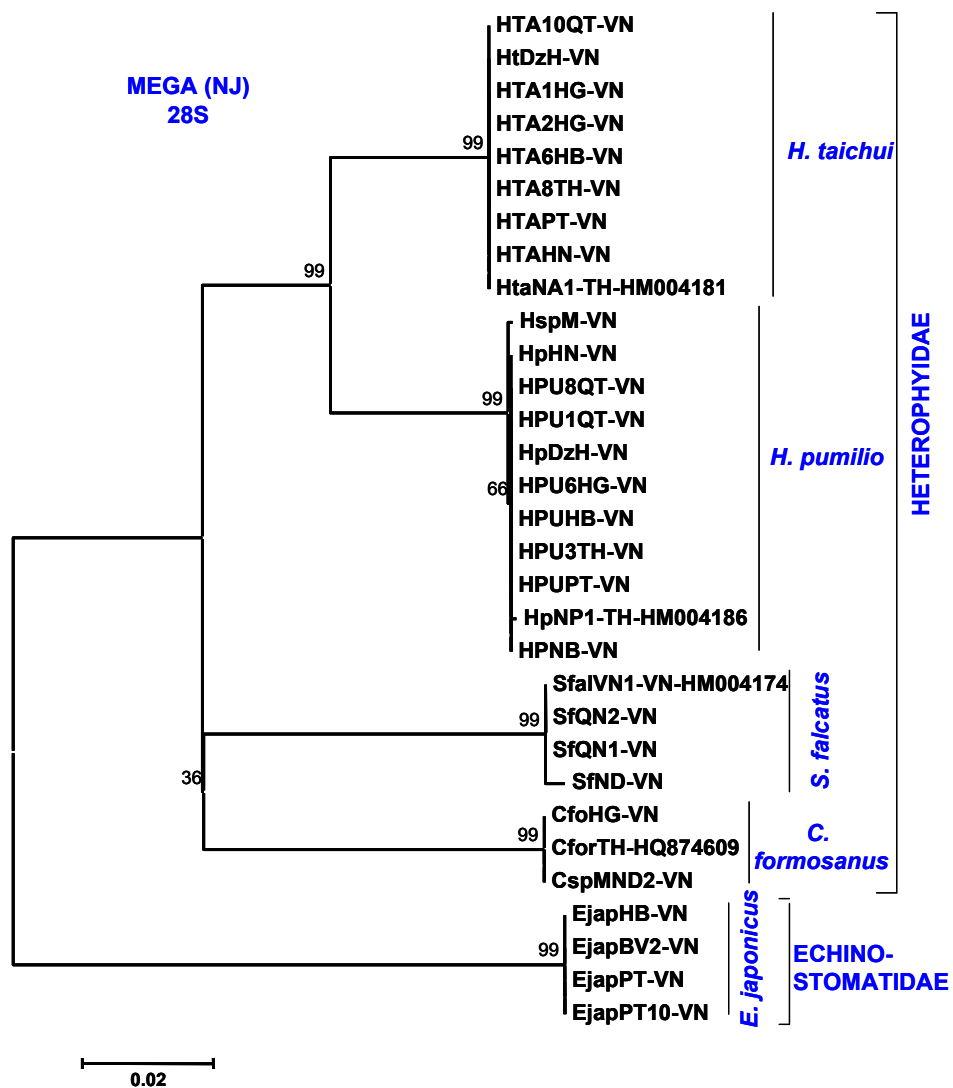


Figure. 3.10. Phylogenetic tree of species revealing relationships of small intestinal fluke species based on partial 28S nucleotide sequences.

Partial 28S ribosomal nucleotide sequences (1028-1053 nucleotides) of 27 strains of the studied species, including *H. taichui*, *H. pumilio*, *S. falcatus*, *C. formosanus* and *E. japonicus* were used for constructing phylogenetic tree.

According to Figure 3.10, the phylogenetic tree of the 31 strains belonging to 5 species of small intestinal flukes, including 27 species of Vietnam and 4 reference strains were divided into five separate groups for *H. taichui*, *H. pumilio*, *C. formosanus*, *S. falcatus* and *E. japonicus*.

3.5. The research on *cox1* of *H. taichui* in 3 provinces Ha Giang, Thanh Hoa and Quang Tri

A total of 49 samples from three populations of *H. taichui* in different geographical areas, ie. Ha Giang (HG), Thanh Hoa (TH) and Quang Tri (QT) provinces of Vietnam were used obtaining of 375 bp *cox1* sequence for each variant using primers binding in *cox1* and subjected to comparative analysis (GenBank numbers: JN809861-JN809909).

3.5.1. The result of comparison and analysis of the *cox1* nucleotide sequence of *H. taichui* obtained in 3 provinces of Quang Tri, Thanh Hoa and Ha Giang

The comparative analysis for *cox1* gene sequences (375 bp) of 49 species of small intestinal flukes of Vietnam, including 19 strains in Quang Tri (QT6-QT19), 15 strains in Ha Giang (HG1 - HG15) and 15 strains in the Thanh Hoa (TH1- Th15) were conducted by the BioEdit 7.0 program.

As results, *cox1* nucleotide sequences of the strains belonging to three different geographical regions had very high identity at the nucleotide level.

3.5.2. Analysis of the relationship of 49 strains of small intestinal fluke *H. taichui* obtained in 3 provinces of Quang Tri, Thanh Hoa and Ha Giang

- Phylogenetic tree of 3 groups for 3 populations of *H. taichui* with 15 nucleotide sequences obtained from *H. taichui* in Ha Giang, 15 strains obtained from Thanh Hoa and 19 from small intestinal fluke *H. taichui* in Quang Tri province in Vietnam is shown in Figure 3.11.

- The average evolutionary divergence over pairwise sequences within groups was around 0.003 (HG = 0.004, TH = 0.001 and QT = 0.002), while the average over pairwise sequences between populations were around 0.014.

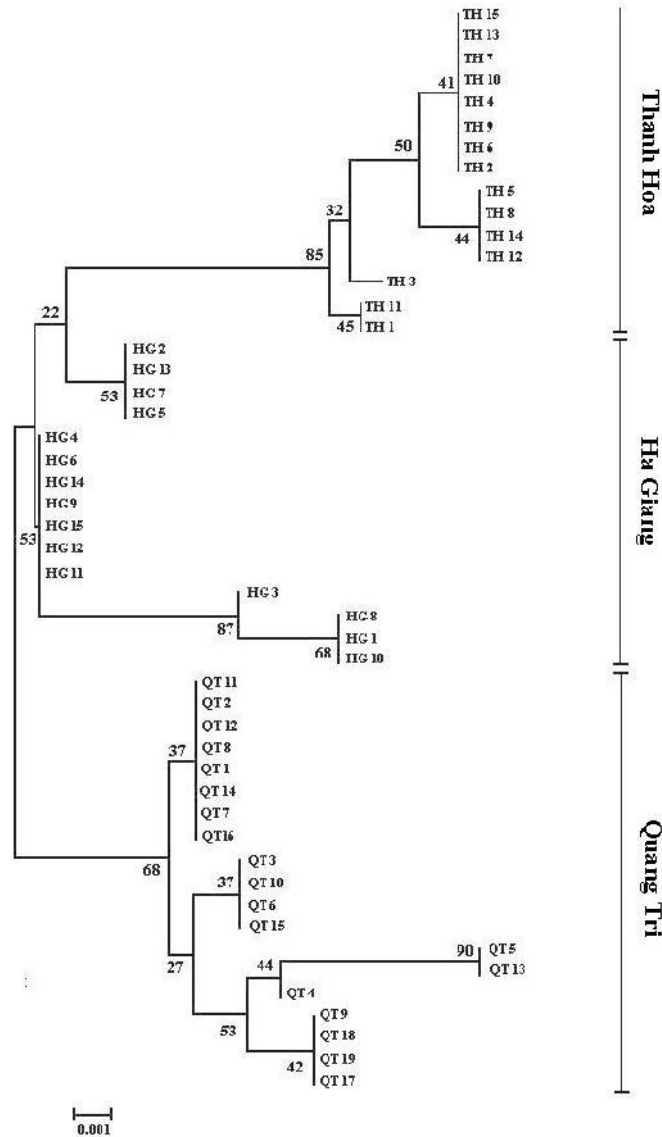


Figure 3.11. Phylogenetic tree expression relationship three populations of *H. taichui* built by Neighbor-joining method.

- Phylogenetic tree showing the relationship of 49 species of small intestinal fluke *H. taichui*, using partial nucleotide sequences (375 bp) *cox1* was constructed by MEGA5.5 program. The results demonstrating relationships of 49 strains of small intestinal flukes in Figure 3.11 showed that the small intestinal fluke strains of *H. taichui* in Vietnam were divided into three main

groups and classified according to three various geographic regions from Thanh Hoa, Ha Giang and Quang Tri provinces.

3.6. The results of assessment of the treatment effectiveness for small trematode in the community

Total 396 people participated in the study, of which were 186 people in Hong Nghia commune (47%), including 131 men (70.4%) and 55 female (29.6%); while in Hai Hoa commune, there were 210 (53%) involved in the study, including 111 men (52.9%) and 99 female (47.1%).

Table 3.15. The infection rate of small trematodes before treatment, after treatment in 2 weeks, 4 months, 7 months and 15 months by gender for the two communes.

Time of examination	Sex	No. exam	No. (+)	%	OR (95%CI)
Before treatment	Women	154	31	20.1	4.4 (2.7-7.3)
	Men	242	128	52.9	
	Total	396	159	40.2	
After treatment 2 weeks	Women	154	1	0.7	5,2 (0.6-42.7)
	Men	242	8	3.3	
	Total	396	9	2.3	
After treatment 4 months	Women	154	4	2.6	7.2 (2.5-21.1)
	Men	242	39	16.1	
	Total	396	43	10.9	
After treatment 7 months	Women	154	13	8.4	3.5 (1.8-6.7)
	Men	242	59	24.4	
	Total	396	72	18.2	
After treatment 15 months	Women	154	30	19.5	2.4 (1.5-3.8)
	Men	242	88	36.4	
	Total	396	118	29.8	

Table 3.15 shows that, total 396 people were involved in the entire research process including 154 women (38.9%) and 242 male (61.1%). The infection rate of small trematode in men was many times higher than that in women.

Table 3.16. Treatment efficacy of praziquantel dosage 50mg/kg body weight on the small fluke treatment in the community

No. examination	Before treatment		After treatment in two weeks		Treatment efficacy
	No. (+)	%	No. (+)	%	
396	159	40.2	9	2.3	94.3%

Table 3:16 shows small fluke infection rates in human in the community before treatment was 40.2%, 159/396 persons positive with stool tests; while the infection rate after treatment 2 weeks was 2.3% (9/396). Treatment effects were calculated comparing before and after 2 weeks of treatment reached 94.3% and in the treatment process it was safe without any side effects of the drug.

The infection rate with small trematodes increased from 2.3% to 10.9% after 4 months of treatment, increased to 18.2% after 7 months and to 29.8% after 15 months of treatment.

Average eggs number/gram of feces in two communes were measured at different times of before and after treatment. There was difference of this index before and after 2 weeks of treatment (87.7 eggs/gram before treatment and 0.94 eggs/gram feces after 2 weeks of treatment). Treatment effects were also shown by egg reduction rate was 98.9%. The average egg number start increased after period 4, 7 and 15 months of the treatment.

CONCLUSION

1. The determination of species composition and description of morphological characteristics of small intestinal flukes

This study has demonstrated that five small intestinal fluke species of two families, Echinostomatidae and Heterophyidae, that parasitize in human, have been identified. The morphological characteristics and their distribution in the provinces were described as follows:

- *Haplorchis taichui* were collected and determined as small intestinal fluke of Heterophyidae, that infected in human in Vietnam based on the specific characteristics such as ventral genital sucker having 10-21 spines of banana shape and bunch arrangements of bananas. They were identified as parasites on the people in the provinces, namely Ha Giang, Phu Tho, Hoa Binh, Ha Noi, Quang Ninh, Nam Dinh, Thanh Hoa, Ninh Binh and Quang Tri.

- *Haplorchis pumilio* belongs to Heterophyidae, parasitic on the human in Ha Giang, Phu Tho, Hoa Binh, Hanoi, Nam Dinh, Thanh Hoa, Ninh Binh and Quang Tri provinces, they were determined based on a number of morphological characteristics such as ventral genital sucker having 31 (ranging from 26-35) spines, arranged in circle and the spines were I or A letter shape.

- Small intestine fluke *Stellantchasmus falcatus* of Heterophyidae has been identified based on morphological characteristics such as size and characteristics of the ventral genital sucker, with genital pore, with 2 large testicles in opposite sides in the lower one of third body; this species has been found in the Nam Dinh and Quang Ninh province.

- Small intestinal fluke *Centrocestus formosanus* was identified in Heterophyidae. They were determined based on the characteristics of small size body, optical characteristic spines around oral sucker with 32 spines arranged in two rows, this species was found in Ha Giang province.

- *Echinochasmus japonicus* was intestinal flukes species of Echinotomatidae only, that were detected and morphological studies on the

specific characteristics, ventral sucker bigger than oral sucker; the oral sucker have a surrounded spines with 24 spines; the spines formed a line of 12 dorsal spines interrupted, having 2 testes in third aligned under body and they parasitize in people in Hoa Binh, Phu Tho province and Ha Noi city.

2. The identify of small intestinal fluke by molecular biology

- The small intestinal flukes, *H. taichui*, *H. pumilio*, *S. falcatius*, *C. formosanus* (Heterophyidae) and a species *E. japonicus* (Echinostomatidae) have been studied accurately by each species, belonging to 2 families, using nucleotide sequences of mitochondrial *cox1* and nuclear 28S ribosomal genes as molecular markers, which contributed to clarify the classification system of the trematode species in the class Trematoda.
- The small intestinal fluke isolates obtained in the study have the similarity rate of 98% to 100%, between themselves and the reference strains at the nucleotide level of mitochondrial *cox1* gene and 28S ribosomal sequences.
- The molecular findings were of complete correlation with the results of morphological studies for accurate classification confirmed for family and species of the small intestinal flukes in the study.

3. The treatment effectiveness of small flukes in the community by praziquantel dose 50mg/kg

- The effectiveness of the treatment for small trematodes by praziquantel 50mg/kg/day/2 times for the raw fish-eating people in the community was 94.3%, the egg reduction rate after 2 weeks of treatment was 98.9%.
- The infection rate with small trematodes increased from 2.3% to 10.9% after 4 months of treatment, increased to 18.2% after 7 months and to 29.8% after 15 months of treatment.
- The praziquantel drug almost had no expression of side effects during treatment of the raw fish-eating people in the community.

RECOMMENDATIONS

1. The need is for collection of more small intestinal fluke samples in different geographic regions of Vietnam, such as the Coastal Central provinces, West Central, East and South West, for identification and classification, as well as the need to deeply study on both epidemiology and the true impact of small intestinal flukes in public health in Vietnam, to offer the treatment and prevention activities.

2. Sequencing of near or entire mitochondrial genome, as well as a number of other nuclear genes of trematodes isolated in Vietnam, for comparison with strains of the same genus, and/or the same Heterophyidae, Echinostomatidae and other pathogen liver flukes, intestinal flukes and zoonotic parasites, providing essential information for the prevention and treatment.

3. It can be applied for the treatment of small flukes in high risk communities with therapy of praziquantel 50 mg/kg body weight divided in two doses with 4-6 hours apart in the endemic regions. Because the infection rate after treatment was high, so the treatment strategy should be combined with the provision of information, propagandizing communication and education in the community. The strategy of interventions on the species of intermediate hosts such as snails, fish and pathogen reservoir hosts such as dogs, cats, pigs... to increase understanding about disease prevention is also applied, as well as to reduce the risk of spread of pathogens into the environment, to cut off the link in the development cycle of the small trematodes.