รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาความแตกต่างทางพันธุกรรมและโครงสร้างประชากรของพยาธิใบไม้ติดต่อผ่านปลาเพื่อเพิ่มผลสัมฤทธิ์ในวางแผนการควบคุมโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดี

โดย รองศาสตราจารย์จิตรา ไวคกุล

โครงการแล้วเสร็จเมื่อ เดือนธันวาคม 2554

สัญญาเลขที่ BRG5180005

โครงการ: การศึกษาความแตกต่างทางพันธุกรรมและโครงสร้างประชากรของพยาธิใบไม้ติดต่อผ่านปลาเพื่อเพิ่มผลสัมฤทธิ์ในวางแผนการควบคุมโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดี

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Abstract

Project Code: BRG5180005

Project Title: Genetic variation and population structure studies of fish-borne trematodes for increasing control impact of opisthorchiasis and cholangiocarcinoma

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Project Period: 3 years

This research project aimed to investigate morphological and genetic variations, population structures, and trends in population migration and gene flow, of opisthorchiid liver flukes (OLFs), *Opisthorchis viverrini,* and heterophyid intestinal flukes (HIFs), to strengthen opisthorchiasis surveillance in endemic areas of the Greater Mekong Sub-region (GMS). The study also aimed to gather public-health information on genetic variations among *O. viverrini,* for use in analyzing the relationship between opisthorchiasis and cholangiocarcinoma (CCA). The research project was divided into 7 sub-projects: 1) OLF and HIF metacercariae distributed in the GMS, 2) *Opisthorchis lobatus* in Lao PDR, 3) Variations in *O. viverrini* metacercarial infections among cyprinid fish, 4) population-genetics study of *O. viverrini* in the lower Mekong basin, 5) population-genetics study of *Haplorchis taichui* in the GMS, 6) molecular systematics of heterophyid minute intestinal flukes in the Subfamily Haplorchiinae (Trematoda:

Heterophyidae), based on the nuclear ribosomal DNA gene and ITS2 region, 7) molecular diagnosis of OLFs and HIFs, based on ribosomal ITS region. Freshwater fish were collected in Cambodia, Lao PDR, Thailand, and Vietnam during the period 2008-2011. We determined the prevalence of OLFs and HIFs, and also discovered a new species of *Opisthorchis, O. lobatus*, in the Mekong Basin. Using the samples collected, we studied the population structure of *O. viverrini* and *H. taichui*, and found that different dynamics drive the population structures of these two species. In the lower Mekong Basin, the *O. viverrini* populations showed low genetic differentiation between various geographical localities, while among the *H. taichui* populations, it was high. When studying HIFs, it is very difficult to classify parasites in the Heterophyidae by morphology alone. In the current study, we used morphology related to the genetic background of the worms, to reduce disagreement among taxonomists and obviate the need for molecular identification. We also developed a diagnostic method for patients' stool samples in areas of co-infection with *O. viverrini* and HIFs. The results showed high sensitivity and specificity.

Based on these seven research studies, we published five papers in international journals and at least 2 papers are in preparation for journal submission. The study was completed in 2011; however, the results of fish collection in 2010 were unsatisfactory, due to the unpredictability of the seasons in many GMS countries, especially Thailand. Climate change seems to be having an effect on the prevalence of OLF and HIF infections, so that the periods of high parasite prevalence in fish might change. Fish in the same endemic area should be collected continually over at least 4-

5 years, to amass reliable information for the construction of a mathematical model to predict periods of high parasite prevalence in fish.

Keywords: Opisthorchiid liver flukes, Heterophyid intestinal flukes, Greater Mekong Sub-region, Prevalence, Genetic variation.

บทคัดย่อ

รหัสโครงการ: **BRG5180005**

ชื่อโครงการ: การศึกษาความแตกต่างทางพันธุกรรมและโครงสร้างประชากรของพยาธิใบไม้ติดต่อผ่านปลาเพื่อเพิ่มผลสัมฤทธิ์ในวางแผนการควบคุมโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดี

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ระยะเวลาโครงการ: **3 ปี**

โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความแปรผันทางสัณฐานรูปร่างและพันธุกรรม โครงสร้างประชากร และแนวโน้มการแพร่กระจายย้ายถิ่นโดยกระบวนการยีนโฟว์ (gene flow) ของพยาธิใบไม้ตับ (opisthorchiid liver flukes; OLFs) โดยเฉพาะ *Opisthorchis viverrini* และพยาธิใบไม้ลำไส้ขนาดเล็ก (heterophyid intestinal flukes; HIFs) เพื่อเป็นข้อมูลในเพื่อการวางแผนเฝ้าระวังการแพร่กระจายของพยาธิกลุ่มเหล่านี้ในเขตลุ่มน้ำโขง จุดประสงค์ของงานวิจัยยังรวมถึงการวิเคราะห์ผลความเกี่ยวข้องสัมพันธ์ระหว่างความแปรผันทางพันธุกรรมของพยาธิ *O. viverrini* กับพยาธิสภาพของโรคติดเชื้อพยาธิ opisthorchiasis และ มะเร็งท่อน้ำดี (cholangiocarcinoma; CCA) โดยงานวิจัยแบ่งเป็นทั้งหมด 7 โครงการย่อย ได้แก่ 1) การศึกษาการแพร่กระจายของเชื้อระยะติดต่อของพยาธิใบไม้ปอดและลำไส้ขนาดเล็กในปลาน้ำจืดในเขตลุ่มน้ำโขง 2) การศึกษาสัณฐานวิทยาแลพันธุกรรมของพยาธิใบไม้ตับ

Opisthorchis lobatus ที่ได้ค้นพบครั้งแรกในเขตลุ่มน้ำโขง 3) การศึกษาความแปรผันของการติดเชื้อในปลาจำพวกปลาน้ำจืดจำพวก cyprinid 4) การศึกษาโครงสร้างประชากรของพยาธิใบไม้ตับในเขตลุ่มน้ำโขงตอนล่าง 5) การศึกษาโครงสร้างประชากรของพยาธิใบไม้ลำไส้ขนาดเล็ก *Haplorchis taichui* ในเขตลุ่มน้ำโขง 6) การศึกษาระเบียบวิทยาของพยาธิใบไม้ลำไส้ขนาดขนาดเล็กใน subfamily Haplorchiinae ด้วยวิธีทางอณูชีวโมเลกุล 7) เทคนิคทางอณูชีวโมเลกุลเพื่อการตรวจหาเพื่อการตรวจหาพยาธิใบไม้ติดต่อผ่านปลาในอุจจาระผู้ป่วย

ในงานวิจัยนี้ ปลาถูกเก็บจากท้องที่ในประเทศกัมพูชา ลาว ไทย และเวียดนาม ในระหว่างปี 2551 ถึง 2554 จากการเก็บตัวอย่างทำให้พบพยาธิใบไม้ตับชนิด *O. lobatus* ที่ไม่เคยมีรายงานในเขตลุ่มน้ำโขง และจากการศึกษาโครงสร้างประชากรของพยาธิใบไม้ตับ *O. viverrini* และลำไส้ขนาดเล็ก *H. taichui* ทำให้พบว่า โครงสร้างประชากรของ *O. viverrini* มีการแพร่กระจายโดยอิทธิพลจากกระบวนการ gene flow ในขณะที่โครงสร้างประชากรของ *H. taichui* มีลักษณะโครงสร้างประชากรที่มีพันธุกรรมแตกต่างชัดเจนระหว่างกลุ่มประชากรในแต่ละพื้นที่ นอกจากนี้โครงการวิจัยนี้ยังได้ทำการศึกษาพัฒนาองค์ความรู้ในการศึกษาระบบวิทยาในกลุ่มพยาธิใบไม้ลำไส้ขนาดเล็ก HIFs เพื่อใช้เป็นพื้นฐานในการปรับปรุงการศึกษาทางสัณฐานวิทยาในพยาธิกลุ่มยี้ซึ่งมีลักษณะรูปร่างภายนอกคล้ายคลึงกันมากและมักสร้างความสับสนแก่ผู้ศึกษาสัณฐานวิทยาอยู่เนื่องๆ โครงการวิจัยยังครอบคลุมและพัฒนาเทคนิคการตรวจหาพยาธิใบไม้ติดต่อผ่านปลาในอุจจาระของผู้ป่วย ทำให้ได้เทคนิคที่สามารถตรวจการติดเชื้อในผู้ป่วยได้อบ่างรวดเร็วและแม่นยำยิ่งขึ้น

จากงานวิจัยทั้งหมด คณะผู้วิจัยสามารถทำการตีพิมพ์ผลงานได้เป็นที่แล้วเสร็จจำนวน 5 ฉบับ ในวารสารวิชาการนานาชาติ และคาดว่าจะสามารถตีพิมพ์ผลงานต่อเนื่องได้อีกอย่างน้อย 2 ฉบับ อย่างไรก็ดี ในการทำวิจัยนี้พบปัญหาอุปสรรคในการลงพื้นที่เพื่อเก็บตัวอย่างจากปลาในท้องที่ โดยเฉพาะในปี 2553 ซึ่งมีปัญหาความแปรผันของสสภาพภูมิอากาศและปริมาณน้ำที่ไม่สามารถคาดเดาได้ ทำให้ผลจากการศึกษาการแพร่กระจายและการติดเชื้อของพยาธิใน

ปลามีความคลาดเคลื่อนไปบ้าง โดยผู้วิจัยเสนอแนะว่า เนื่องจากสภาพภูมิอากาศที่เปลี่ยนแปลงรุนแรง การเก็บผลการติดเชื้อและแพร่กระจายของเชื้อพยาธิใบไม้ติดต่อผ่านปลาจำเป็นต้องทำอย่างต่อเนื่องอีก 4 ถึง 5 ปีเพื่อให้ได้ข้อมูลซึ่งเป็นประโยชน์อย่างแท้จริงในการสร้างสมการเพื่อทำนายช่วงระยะเวลาที่พยาธิมีการติดเชื้อในปลาสูงสุดและต่ำสุดหรือความชุกในการติดเชื้อเพื่อใช้เป็นเครื่องมือในการเฝ้าระวังการติดเชื้อในคนซึ่งไม่สามารถปรับพฤติกรรมการกินปลาดิบ ๆสุก ๆ ได้โดยง่าย

คำหลัก: พยาธิใบไม้ตับ (Opisthorchiid liver flukes) พยาธิใบไม้ลำไส้ขนาดเล็ก (Heterophyid intestinal flukes) ลุ่มน้ำโขง ความชุก ความแปรผันทางพันธุกรรม

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INTRODUCTION

Opisthorchiid liver flukes (OLFs) and Heterophyid intestinal flukes (HIFs) are the causative agents of fish-borne trematodiases, long-known as major causes of serious public-health problems in Asia (WHO 1995, Chai *et al*., 2005). Chlolangiocarcinoma (CCA) is a major clinical manifestation associated with chronic OLF infection (Sripa, 2003). In the Greater Mekong Sub-region (GMS), *Opisthorchis viverrini* and *Clonorchis sinensis* have been reported as food-borne trematodes causing CCA; however, *C. sinensis* is usually found in northern Vietnam. The major OLF distributed throughout the lower Mekong sub-region is *O. viverrini* (Keiser and Utzinger, 2009). This parasite has apparently not been reported in Cambodia before. The current study is the first known report of *O. viverrini* in Cambodia (Touch *et al*., 2009). OLF infections are often mixed with HIF infections (Chai *et al*., 2005). Although HIFs are not considered the causative agent of CCA, they have been reported in the human heart, brain, and spinal cord (Africa *et al.,* 1940). The seriousness of parasitic diseases is usually related to parasite burden, which can be heavy, especially in fish-borne trematode infections (MacLean *et al.,* 1999). A major problem in the diagnosis of these groups of parasites (OLFs and HIFs) is the difficulty differentiating HIF eggs from OLF eggs in human stool by stool-microscopic examination, resulting in errors estimating the prevalence of both OLFs and HIFs (District *et al.,* 1992). Therefore, to determine recent prevalence and distribution, to identify current endemic areas for OLFs and HIFs, this study examined the prevalence of parasite metacercariae among freshwater fish in selected GMS sites, and their population structures based on appropriate DNA markers. In addition to current prevalence and population structures, the diversity of OLF & HIF parasites was investigated.

This research project aimed to: 1) to investigate morphological and genetic variations, population structures, trends in the population migration and gene flow of *O. viverrini* and HIFs, to strengthen the surveillance of opisthorchiasis in selected endemic GMS areas, and 2) to establish public-health information about genetic variations in *O. viverrini,* to analyze the relationship between opisthorchiasis and CCA.

In research study #1-2, we collected freshwater fish from selected sites in Thailand, Lao PDR, Cambodia, and Vietnam. It was found that *Haplorchis* spp. were widely distributed in the GMS, except the study area in southern Cambodia. However, we discovered *O. viverrini* in this part of Cambodia (Touch *et al*., 2009). In previous reports about OLFs, only *O. viverrini* has been commonly reported in the GMS. Recently, our study reported another species of *Opisthorchis* (*O. lobatus*), found in *Channa limbata,* from Lao PDR (Thaenkham *et al*., 2012). This report is significant, because it alerts researchers to the genetic diversity of *Opisthorchis* spp. in the GMS, and genetic variation among *O. viverrini* populations from different localities. However, human infection, and the pathogenicity of this parasite to its definitive hosts, have not been reported.

Research #3 reported the prevalence and distribution of OLFs and HIFs among freshwater fish collected from many GMS areas, concluded and reported in the results section. However, it was difficult to collect data on parasite distribution and prevalence, because of climate-change impacts on the duration of rainy and dry seasons (Preparing manuscript).

Research #4: the population structure study of *O. viverrini* sensu lato, studied 6 parasite populations from Thailand, Lao PDR, and Cambodia. It may be concluded that *O. viverrini* populations in the lower Mekong Basin have a monophylitic relationship, without significant genetic diversity among populations (Thaenkham *et al*., 2010a). This study suggested that the genetic variation of *O. viverrini* might not be a major cause of different levels of pathogenicity or the development of CCA among *O. viverrini*-infected patients.

Research #5: population structure of HIFs. We selected *H. taichui* as the species candidate because it is widely distributed in the GMS. The study was conducted with 3 populations of *H. taichui* in Vietnam, as a pilot study (Dung *et al*., 2012). This study suggested that the population structure of *H. taichui* may differ from *O. viverrini*, since the *H. taichui* populations were genetically separate from each other, while the *O. viverrini* populations shared their genetics via the gene-flow process. However, we must confirm this hypothesis by studying other populations of *H. taichui* in the GMS, and from another distant populations (this study is awaiting samples from a distant population—an *H. taichui* population in Taiwan) (Preparing manuscript).

Research #6: genetic variation among human pathogenic HIFs (subfamily Haplorchiinae) was also revealed by phylogenetic relationships using DNA markers. We investigated whether the *Haplorchis taichui* that is widely distributed in the GMS is closely related to *Procerovum varium*, rather than other parasites in the same genus.

This study indicated that morphology only was inadequate for classifying parasites in this subfamily (Thaenkham *et al*., 2010b).

Research #7: a molecular diagnostic method for discriminating among OLFs and HIFs was also developed, based on the ribosomal ITS sequences of these parasites. We aimed to apply this method to detect infection, especially co-infection, among people in endemic areas for OLFs and HIFs in further work (Sato *et al*., 2009; 2010).

As outlined above, many tasks have been completed successfully. We published five papers reporting our results in international journals (Thaenkham *et al*., 2010a; Thaenkham *et al*., 2010b; Sato *et al*., 2010; Thaenkham *et al*., 2012; Dung *et al*., 2012). A further two papers, minimum, will be submitted for journal publication as soon as practicable. When the study has finished, we will use relevant information about parasite genetic variations and population structures, and integrate it with other factors, to strengthen the opisthorchiasis-control program.

MATERIALS AND METHODS

This section summarizes the materials and methods used in all research studies. Further details have been described in other publications.

Sample collection

Freshwater fish were collected from several areas of Cambodia, Lao PDR, Thailand, and Vietnam, during the period 2008-2011. All fish obtained from the field were examined and OLF-HIF metacercarial infections were recorded. The metacercariae found were separated from the fish bodies, fins and muscles, by pepsin digestion technique (WHO, 1995), and were then identified to species and infected into hamsters to obtain adult stages. OLFs take about 2 months to develop into adults, while HIFs need about 3-14 days. When the parasites were mature, the infected hamsters were scarified, and the adult worms obtained were separated into 2 groups: 1) one group was fixed with 10% hot formalin to study basic morphology, and 2) another group was preserved in 70% ethanol to study population structure and molecular systematics. Human stool samples were collected from OLF-HIF endemic areas, and used to develop a molecular diagnostic method for these two groups of parasites. Therefore, an additional objective was the molecular diagnosis of OLFs and HIFs from human stool.

Sample collection in Cambodia, for studying variations in metacercarial infections in different seasons

Seasonal variations in the prevalence of Opisthorchis metacercariae in cyprinid fish have been reported in Thailand (Sithtithaworn *et al.,* 1997). There is limited information on opisthorchiasis in Cambodia, so research into variations in liver-fluke infections was conducted. Eight sites at and around Lake 500--an endemic area reported in our previous study, in 2006-2007 (Touch *et al.,* 2009)--were selected for fish collection between August 2008-July 2009 (Fig. 1). The third Saturday of each month, a fisherman was hired to catch cyprinid fish using a cast net for about 30 mins per site. The fish were packed in an ice box and transported to Bangkok. At the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, the fish were identified to species, based on a taxonomic reference (Rainboth *et al.,* 1996). The pectoral fins, tails, and muscles, were examined by tissue compression technique under a stereomicroscope (Touch *et al.,* 2009). Metacercariae were isolated by digestion method and identified using morphological criteria (size and shape of cyst, folding body displaying vigorous movement within the cyst, prominent and clearly visible oral and ventral suckers) (Kaewkes, 2003; Scholz *et al.,* 1991; Vajrasthira *et al.,* 1960). Species of liver fluke metacercariae from this region were confirmed as *Opisthorchis viverrini* from experimental animal infections and COI sequencing in our previous study (Touch *et al.,* 2009). In the current study, all metacercariae that agree morphologically with the liver fluke will be identified as *O. viverrini*. Only *O. viverrini* metacercariae were recorded and analyzed in this study. The positive rate of opisthorchiid metacercariae will be analyzed descriptively according to fish species and fish-collection sites. Variations in the positive rates of *Opisthorchis viverrini* infection over the year of examination will be expressed as mean ± standard error.

Morphological identification

The fixed adult worms were stained by acetic carmine and mounted in Canada balsam. The worms were identified following the trematode identification keys for the Families Opisthorchiidae and Heterophyidae (Pearson, 1899; Bray, 2008).

DNA analyses for population study

PCR and DNA sequencing

The adult worms were extracted by Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan). The PCR amplicons (DNA markers) were amplified by specific primers under optimized conditions. The population-structure study of *O. viverrini* used the mitochondrial *nad*1 gene as a DNA marker (Thaenkham *et al*., 2010a), while for *Haplorchis taichui* (the representative species for HIFs) the mitochondrial *cox*1 gene was used as a DNA marker (Dung *et al*., 2012). The PCR amplicons were sequenced under BigDyeIM terminator cycling conditions and run with an automatic sequencer, model 3730xl (Macrogen Inc, Korea). DNA sequences were checked manually through electropherogram and aligned using BioEdit v. 7.0 (Hall, 1999). The BLAST program (McGinnis and Madden, 2004) was used to confirm that the sequence belonged to the DNA marker needed.

Clustering dendrogram and haplotype network

DNA sequences were employed to construct a clustering dendrogram using the neighbor-joining method with the MEGA 4.0 program (Kumar *et al*., 2008). A haplotype network was also constructed using the Network program (Bandelt *et al*., 1999), to reveal the genetic relationships among haplotypes within/among populations, which can indicate the population structure of the parasite population.

Neutrality tests

The evolutionary neutrality of the DNA sequences was evaluated with Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989) tests, using Arlequin version 3.1 (Excoffier *et al*., 2005), and Fu and Li's F* and D* statistics (Fu and Li, 1993), using DnaSP version 4.0 (Rozas *et al*., 1992). Fu's Fs test is powerful for the detection of population growth and genetic hitchhiking (Fu, 1997), while Fu and Li's F* and D* tests are best for detecting background selection (Fu and Li, 1993). In Fu's Fs test, significance suggests that a population is growing and hitchhiking, because of population expansion and selective sweep (Fu, 1997).Tajima's D is significantly positive when there is a population bottleneck, and significantly negative with expansion (Aris-Brosou et al., 1996).

Analysis of Molecular Variance (AMOVA)

To clarify any potential genetic differentiation in the populations, DNA sequences underwent AMOVA analysis. This analysis evaluated the feasibility of genetic differentiation by regional separation, and the Arlequin program, version 3.1 was used to estimate groupings by population-grouping test (Exocffier *et al.,* 2005). The parasite populations were also tested for genetic differentiation, without regional separation, by global AMOVA.

Phylogenetic analysis for molecular-systematic study

Phylogenetic trees were reconstructed for the molecular-systematics study of the Haplorchiinae, a subfamily of the Family Heterophyidae, using 18S, 28S rDNA and ITS2 as DNA markers. This subfamily consists of many species of human pathogenic parasites. To construct the preliminary tree, a clustering dendrogram was developed by neighbor-joining (NJ) method, in PAUP* 4.0b10 (Swofford, 2001), to evaluate the congruence between gene partitions. The uncorrected distance (p-distance) was used to calculate genetic distances. Bootstrap values were conducted for 1000 replicates. Partition homogeneity testing was then conducted using the PAUP* program (Swofford, 2001), to confirm the congruency of each DNA marker before generating the combined data set to reconstruct the phylogenetic tree. Significant incongruence after multiple comparisons of DNA partitions was determined at P>0.02.

The phylogenetic trees of both individual DNA markers (18S rDNA, 28S rDNA, and ITS2 sequences) and combined 18S and 28S rDNA sequences, were constructed using maximum likelihood (ML) and Bayesian (BI) methods. The appropriate model of nucleotide substitution was identified via a hierarchical likelihood-ratio test with the jModelTest 0.1.1 program (Posda, 2008), to determine the best-fit model of 88 different evolutionary models. The Akaike information criterion (AIC) implements the best-fit model by calculating the likelihood of proposed models (Posda, 2008). The best-fit model was used to construct the ML tree using PhyML version 3.0 (Guindon and Gascuel, 2003). Topology search was conducted by simultaneous nearest neighbor interchange (NNI). Values supported for the ML trees were estimated by approximate likelihood ratio test (aLRT). Calculation of non-parametric branch support was based on the Shimodaira-Hasegawa-like procedure (SH-like).

Bayesian posterior probabilities (BPP) were calculated for separate and combined partitions of DNA markers, using MrBayes 3.1 (Ronquist *et al.*, 2005). Using MrBayes implements of the DNA substitution model, the default parameter values--topology branch lengths, nucleotide frequencies, nucleotide substitution rate, proportion of invariable sites, and γ-distribution--were used for the priors. Four simultaneous Markov Chains were run for 1,000,000 metropolis-coupled generations, starting with random initial trees and sampling every 100 generations. Convergence between runs was estimated visually by estimating the plot “number of generations versus loglikelihood value” and using the potential scale reduction factor (PSRF) provided in the “sump” output of MrBayes 3.1. The trees were constructed after “burn in” to discard the chain state. The remaining trees, with posterior probabilities plotted on each node, were generated by majority rule-consensus.

Molecular diagnosis

Stool collection and microscopic examination

The study protocol was approved by Mahidol University Ethical Review Committee (Approval numbers: MUTM 2006-040 and 2006-065). Written and oral informed consent was obtained from the participants. Adult worms were collected from human hosts; *Clonorchis sinensis* from Nam Dinh Province, Vietnam, *Opisthorchis viverrini* from Sakaew Province, Thailand, and *Haplorchis taichui* from Nan Province, Thailand. Metacercariae of *Haplorchis pumilio* were collected from *Trichogaster trichopterus* caught in Nakhon Pathom Province, Central Thailand. Twenty-one human fecal specimens, diagnosed as having small trematode eggs by modified thick-smear method, were collected in Sakaew Province. Adult worms and stool samples were preserved in 70% ethanol and metacercariae in 20% ethanol, then stored at -20 °C until used.

DNA preparation

Adult *O. viverrini* and *C. sinensis* worms were digested with lysis buffer containing 25mM Tris buffer (pH 7.5), 0.5% sodium dodecyl sulfate, 100m sodium chloride, 10mMEDTA and 0.1% Proteinase K at 56 °C in a water bath overnight. The lysate was extracted by phenol/isoamyl alcohol/chloroform method, and precipitated with ethanol. Finally, the DNA was resuspended with 200µl of distilled water (DW). The *H. pumilio* metacercariae and adult *H. taichui* worms were ground and put into a PCR tube with 10µl sterile DW, then used directly in PCR. Ten samples were examined per species.

0.5 ml of fecal sample per experiment were washed twice in DW to remove fixative, and frozen at -80 °C. The frozen feces were crushed three times with a glass bar to break any eggs. DNA was extracted from the broken eggs by Qiagen Stool kit (Qiagen) with half a tablet of inhibitEX (Stensvold *et al.,* 2006). The DNA was resuspended with 50 µl DW and used as a template.

PCR

The primers, targeting complete ITS regions, were designed manually from the sequences of intestinal flukes, *H. taichui* ribosomal RNA (GenBank accession no. AY245705), and *H. pumilio* ribosomal RNA (GenBank accession no. AY245706). The reaction was carried out reported by Sato *et al.,* 2009. Amplicons were electrophoresed in 1.0% agarose gel or/and 2.0% agarose gel.

Statistical analysis

The results of modified thick smear and PCR methods using fecal samples were analyzed by kappa test, to measure the agreement between the two methods.

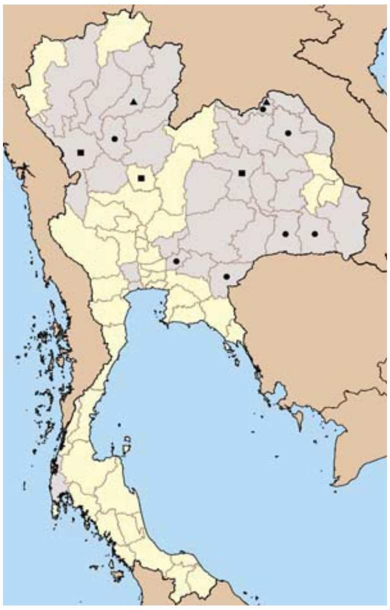
RESULTS

Research #1

OLF and HIF metacercariae distributed in the GMS

Liver and intestinal fluke metacercariae in fish in Thailand

In October 2009-March 2011, 50 species of freshwater fish were collected from 25 provinces in central, eastern, northern, and northeastern Thailand.



A total of 11,763 fish were examined for trematode metacercariae utilizing fish as the second intermediate host. Of the 11,763 samples, 1,704 (14.49%) were positive for metacercariae (Table 1). Among the metacercariae found, 9 other species, including unknown species, were recovered. Here, we report only metacercariae of the liver fluke (*Opisthorchis viverrini*), and minute intestinal flukes (*Haplorchis* spp.) found in fish from the Thai provinces studied.

Table 1 Infection rates of fluke metacercariae in fish collected in Thailand, October 2009-March 2011

Region	Fish examined	Fish positive	%
North	2,243	370	16.50
Northeast	7,056	984	13.95
Central	268	20	7.46
East	2,196	330	15.03
Total	11,763	1,704	14.48

The fish species examined and the infection rates for *Opisthorchis* and *Haplorchis* metacercariae are summarized in Table 2. Fish intermediate hosts of liver flukes comprised 14 species and an overall infection rate of 6.86%. Fish intermediate hosts for *Haplorchis* spp. comprised 7 species and an overall infection rate of 1.96%. Only 3 fish species were infected with both groups of metacercariae. High liver-fluke infection rates were found in the cyprinids *Puntioplites falcifer* and *Henicorhynchus lineatus.* The major second intermediate host of intestinal flukes was not a cyprinid, but the anabantoid, *Trichogaster trichopterus.*

Table 2. Percentage of fish positive for liver and intestinal fluke metacercariae, Thailand, October 2009-March 2011

Fish species	**Local name**	**No. examined**	**Ov positive**	**%**	***Haplorchis* positive**	**%**
Amblyrhynchichthys truncatus	PlaTa min	48			7	14.58
Barbonymus altus	Pla Tapien Thong	1	1	100		
Barbonymus brevis	Pla Tapien Sai	1371	91	6.64		
Barbonymus gonionotus	Pla Tapien Khao	143	9	6.29	17	11.89
Cyclocheilichthys armatus	Pla Saiton Ta Khao	432	44	10.19	28	6.48
Cyclocheilichthys enoplos	Pla Ta Kok	496	23	4.64		
Esomus metallicus	Pla Seaw Nuad	436	8	1.83	10	2.29
Hampala dispar	Pla Sood	24			3	12.50
Henicorhynchus lineatus	Pla Soi Lhung Khon	26	11	42.3		
Henicorhynchus siamensis	Pla Soi Khao	351	48	13.6		
Labiobarbus siamensis	Pla Kui Lam	261	3	1.15		

Luciosoma bleekeri	Pla Seaw Aow	20	4	20.0		
Mystus mysticetus	Pla Khayaeng Khanglai	16	2	12.5		
Pristolepis fasciatus	Pla Mor Chang Yeab	46			2	4.35
Puntioplites falcifer	Pla Sa Kang	5	3	60.0		
Puntioplites proctozysron	Pla Ka Mang	36	7	19.4		
Systomus orphoides	Pla Kaem Cham	98	8	8.16		
Trichogaster trichopterus	Pla Kra Dee	8			8	100
Total		3818	262	6.86	75	1.96

Among the 25 provinces where fishes were found positive with metacercariae of liver and/or intestinal flukes, fish were found positive during the period of the study, and only among the samples of fish studied. This does not imply that other provinces reported no metacercaria-positive fish, or that fish in these areas were negative for these human parasites.

Liver and intestinal fluke metacercariae in fish from Lao PDR

Two hundred and twenty-nine (229) fish of 23 species from Bor Keaw province were examined, and no metacercariae from liver or intestinal flukes were found. Two hundred and forty-six (246) fish of 4 species from Champasak Province were examined, and the metacercariae of liver and intestinal flukes were found, with co-infections in 39.4% (13/33) *Barbodes gonionotus.* From Vientiane City, two fish species--8.3% of *B. gonionotus,* and 2.4% of *Channa limbata*--were positive for liver-fluke metacercariae. It was found that *B. gonionotus* was infected with *Opisthorchis viverrini,* and *C. limbata* was infected with *Opisthorchis lobatus* (Table 3). The distribution of *O. lobatus* in the Mekong region was recorded for the first time by our study. Marked 3 species of small liver fluke existing in the Mekong basin (Thaenkham *et al.,* 2011).

Our study found only 2 species of fish were 2nd intermediate hosts of *O. viverrini* in Laos--*Barbodes gonionotus* in the central and southern regions, but no infection was found in the north of Laos.

Table 3. Metacercariae found in freshwater fish collected in Naxon Village, Vientiane City, Lao PDR, 8-17 October, 2008

Species of fish		**Number of fish examined**	**Metacercaria identification [Number of positive fish (%)]**			
Scientific name	Common name in Laos		Small liver fluke	Clinostome	Unknown	Total
Anabas testudineus	Pa Kheng	6	0	0	0	0
Barbodes gonionotus	Pa Pak Na	24	2 (8.3)	0	4 (16.7)	6(25)
Channa limbata	Pa Kho Kang	85	2 (2.4)	7 (8.2)	0	9(10.6)
Channa striata	Pa Kho	9	0	0	0	0
Esomus metallicus	Pa Seaw	24	0	0	5 (20.8)	5 (20.8)
Hampala dispar	Pa Sood Jam	9	0	0	0	0

Hampala macrolepidota	Pa Sood Kan	1	0	0	0	0
Henicorhynchus siamensis	Pa Soi	15	0	0	2 (13.3)	2 (13.3)
Labiobarbus siamensis	Pa Kui Lam	3	0	0	0	0
Luciosoma bleekeri	Pa Seaw	103	0	0	14 (13.6)	14 (13.6)
Trichogaster trichopterus	Pa Ka Dird	24	0	0	4 (16.7)	4 (16.7)
Total		303	4 (1.3)	7 (2.3)	29 (9.6)	40(13.2)

Intestinal fluke metacercariae in fish from Central Vietnam

Fish were purchased from markets in Hue and Danang provinces, Central Vietnam, in 17-20 August 2009. A total of 259 fish of 5 species were examined--rohu, tilapia, climbing perch, snakehead, and eel. No metacercariae of trematodes that can infect humans were found in 3 species of fish, but were found in climbing perch (*Anabas testudineus:* 92% (59/64), from Hue) and rohu (*Labeo rohita:* 100% (7/7) from Danang) were positive for *Haplorchis taichui* metacercariae. The metacercariae were recovered from the muscles of the pectoral and tail fins. One clinostome (un-encysted) metacercaria was found in 0.5% (1/18) rohu bought at Moi Market in Danang. A clinostome can cause laryngitis in humans if swallowed alive. *Haplorchis taichui* is a minute fluke that lives in the small intestines of humans.

Liver-fluke metacercariae in fish from Cambodia

In 2010, 223 fish were collected from 2 provinces of Cambodia; 52.47% (117/223) were positive for liver-fluke metacercariae. Two species of fish were collected from Kandal Province, southern Cambodia, *Puntius bimaculatus* (ta-pian sai) (33.33%; 10/30) and sai tan (18.75%; 3/16). Ten species of fish were collected from Prey Veng Province, northeastern Cambodia; 58.75% (104/177 samples) were found positive for liver-fluke metacercariae. Cyprinids from both provinces were heavily infected with liver-fluke metacercariae. However, in Cambodia, information on human infection was limited; opisthorchiasis may be under-reported, and more extensive investigations of opisthorchiasis are recommended for Cambodia.

Research #2

The fish for this study were collected from Naxon Village, Vientiane City, Lao PDR, in October 2008. Small-liver-fluke metacercariae were found in some fish, in two (8.3%) *Barbodes gonionotus* (Family Cyprinidae) and two (2.3%) *Channa limbata* (Family Channidae) (Table 2).

Three weeks' post-infection, fecal samples from infected hamsters were examined twice a week, and at 4 weeks' post-infection, small fluke eggs were found. Adult *O. viverrini* were recovered from one hamster infected with cyprinoid-fish metacercariae; the adult recovery rate was 54% (28 worms/50 metacercariae). The adults recovered from another two hamsters, infected with the metacercariae from snakehead fish, were dissimilar to *O. viverrini*; the adult recovery rates were 26% (13 worms/50 metacercariae), and 46% (23 worms/50 metacercariae). After careful study of the stained specimens, the small liver fluke from the snakehead fish was identified as *Opisthorchis lobatus.*

Opisthorchis lobatus Description (Figs 1A &B)

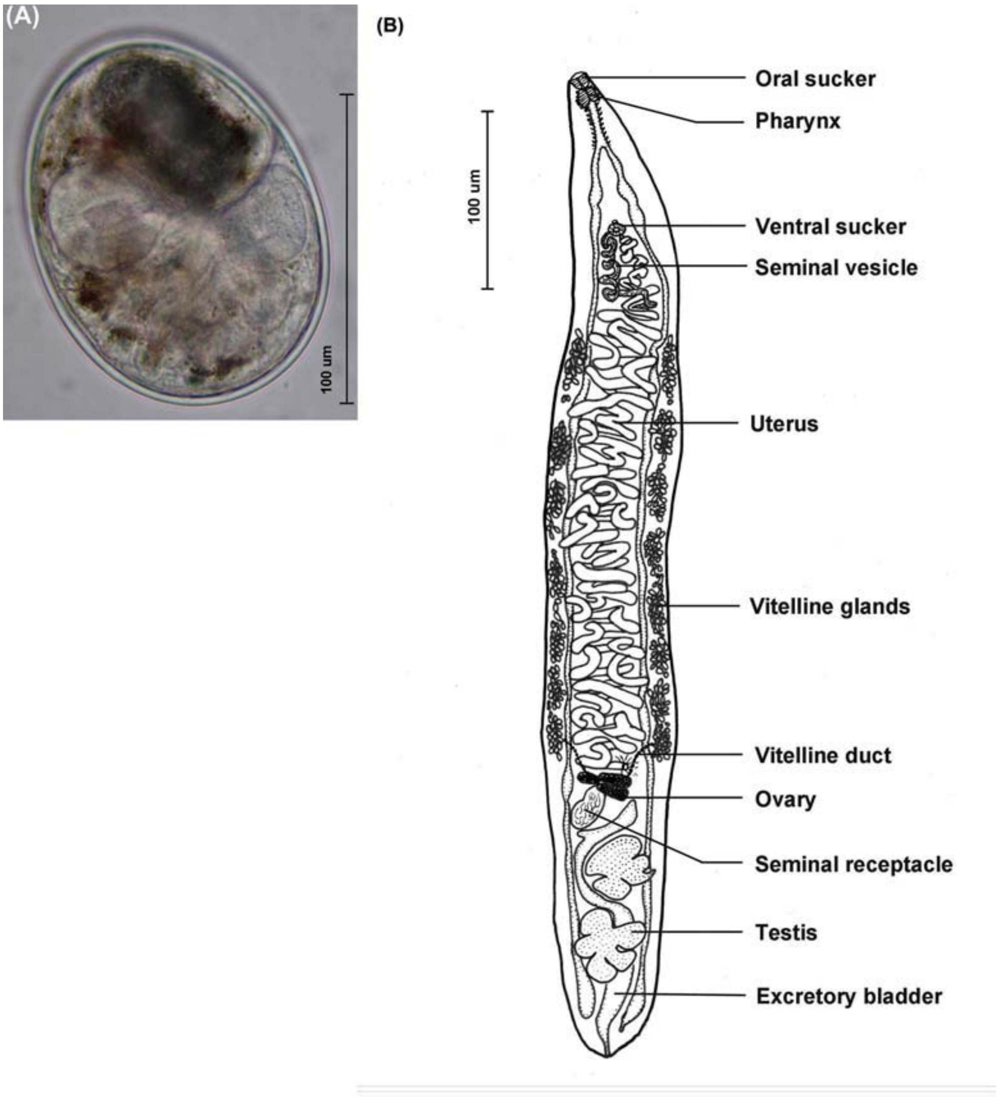


Figure 1. Metacercaria (A) and adult worm (B) of *Opisthorchis lobatus*

Metacercariae

The encysted metacercariae were oval, moved vigorously in a thin and transparent, single-layered cyst wall; 150 (100-200) µ long and 80 (70-90) µ wide (15 cysts) (Fig. 1A). Excysted metacercariae were elongated-oval in shape, 432 (320-480) µ long and 72 (60-80) µ wide. An oral sucker was terminate, 48(45-50) µ long and 37(35-37)µ wide. No pre-pharynx. Pharynx small, 22(20-25) µ in diameter; esophagus long, slender, bifurcate at anterior one third of the body; ceca long, slender, terminating at the posterior end, lateral to the excretory bladder. Ventral sucker global, 51(50-53) µ in diameter, immediately post-bifurcation. Pigment granules densely scattered throughout the entire body. Excretory bladder large, oval, and located in the posterior third of the body.

Host: Red-tailed snakehead fish, *Channa limbata*

Location: Muscle of the tail-fin

Locality: Vientiane City, Lao PDR, N 18° 05.311´, E 102° 58.998´

Adult

Body spatulate, oval to elongate, thin and transparent, anterior end broad to pointed, posterior end broad, 4(4-6) mm long and 0.9(0.7-1.1) mm wide (Fig. 1B). Oral sucker terminal, very small, 94 (60-100) µ in diameter; prepharynx absent; pharynx small, 105 (80-110)µ long and 90 (60-100)µ wide; short esophagus, 89 (20-200)µ. Narrow ceca, extended to the posterior body, ending lateral to the excretory bladder. Pharynx and ceca bifurcation densely covered with unicellular glands, with short ducts opening into the lumen of the covered area. Small ventral sucker, 91(70-125) µ in diameter, located in the mid-body of the anterior third. Two testes, tandem, large, irregularly lobed, located at the posterior end; posterior testis 481 (400-500) µ by 364 (380-460) µ, anterior testis 474 (380-550)µ by 376 (310-520)µ. The sperm duct from each testis runs anteriorly and joins to form a seminal vesicle immediately anterior to the vitelline follicles. Seminal vesicle tubular, coiled and running forward dorsally to the ventral sucker and immediately opening as a genital pore at the anterior border of the ventral sucker. Ovary with 3-4 finger-like lobes, closed and anterior to the seminal receptacle, (230-380) µ by (100-230) µ; oviduct runs forward shortly to open into an ootype covered with numerous Mehlis' glands; uterus runs transversely forward, forming transverse irregular folds in the mid-body line between the ceca, with some folds overlapping the ceca. The metraterm dorsal to the ventral sucker joins with the ejaculatory duct, and immediately opens into a genital pore. The seminal receptacle is large, voluminous, pre-testicular; the duct from the seminal receptacle opens into the ootype. The Laurer's canal branches off the duct of the seminal receptacle, is relatively long, and opens dorsally anterior to the anterior wall of the excretory bladder. The vitelline follicles are relatively large, forming into 7-8 groups on each lateral side of the middle third of the body; extra cecal zone; the posterior groups do not extend beyond the ovary; vitelline ducts are not always clearly visible, vitelline chamber is triangular ventral to ovary; a common vitelline duct runs forward to open into the ootype. Uterine eggs numerous, 24 (22-28) µ long and 11 (10-12) µ wide, thick-walled, distinct shoulder, large operculum, posterior end sometimes provided with an abopercular knob. The excretory bladder is tubular, winding between two testes and the seminal receptacle.

Host: Golden hamster (experimental)

Location: Bile ducts

Deposition: Natural History Museum, UK (NHM 2010.9.15.1); National Parasite collection, USA (USNPC 103739); Meguro Parasitological Museum, Japan (MPM 18912); Unit of Parasitology, Faculty of Basic Science, University of Health Science, Lao PDR (UHSPC 2010-1/2); Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand (MUTMHM 2553001/2).

Relative-morphological comparison between *O. lobatus* and *O. viverrini*

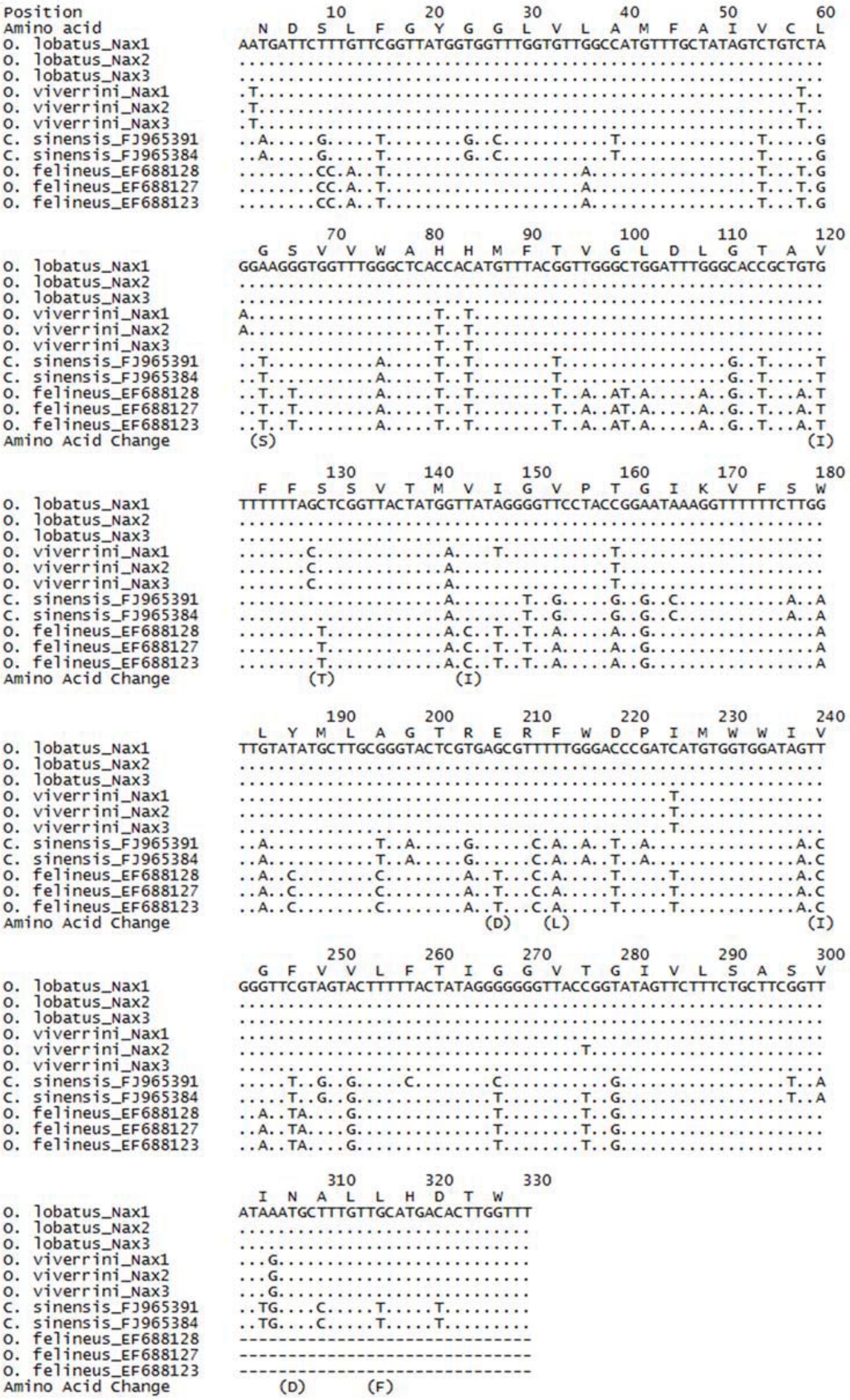
The metacercarial cyst of *Opisthorchis lobatus* is smaller than *O. viverrini*, but the excysted metacercaria has a larger body (Table 4). The adult *O. lobatus* is about 1.6 times more slender than *O. viverrini*. The oral sucker of *O. lobatus* is about 2 times smaller than *O. viverrini*. The size of the oral sucker is quite similar to the pharynx in *O. lobatus* (oral sucker: pharynx is 0.9: 1), while the size of the oral sucker is larger than the pharynx in *O. viverrini* (oral sucker: pharynx is 1.7: 1). The oral sucker of *O. lobatus* is also about two times smaller than *O. viverrini* (Table 4). However, the oral sucker in both species is smaller than the ventral sucker; the ratio of the oral sucker: ventral sucker of *O. lobatus* is 0.9: 1 and of *O. viverrini* 0.8: 1. Beside the differences in body and sucker sizes, the two species also differ in other characteristics. The esophageal glands are larger and more numerous in *O. lobatus*, while they are small and scattered in *O. viverrini*. The vitelline follicles are located in the middle third of the body and do not extend beyond the ovary in *O. lobatus,* but in *O. viverrini* they are in the middle two-fifths of the body, and extend beyond the ovary to the anterior testis. In *O. lobatus*, the vitelline ducts are not clearly visible, but they are fairly clearly visible in *O. viverrini*. The uterus and seminal vesicle do not extend beyond the ventral sucker in *O. lobatus*, but they sometimes do in *O. viverrini*. The lobes of the testes are irregular in shape and size in *O. lobatus*, but more regular in *O. viverrini*.

Table 4. Measurement and ratios of diagnostic characters for *Opisthorchis viverrini* and *O. lobatus*

Diagnostic characters		***O. viverrini* (μm)**	***O. lobatus* (μm)**	**Ratio**
Metacercariae (n=15)				
Encysted	L	200	150	1.3
metacercaria	W	160	100	1.6
Excysted	L	320	432	0.8
metacercaria	W	60	72	0.8
Oral sucker	L	37	47	0.8
	W	37	37	1.0
Ventral sucker	L	46	51	0.9
	W	45	51	0.9
Adults (n=20)				
Body	L	5,256	4,385	1.2
	W	1,456	917	1.6
Oral sucker	L	198	94	**2.1**
	W	184	82	**2.2**
Pharynx	L	118	105	1.1
	W	114	90	1.3
Esophagus	L	230	89	**2.6**
Ventral sucker	L	242	103	**2.3**
	W	222	91	**2.4**
Posterior testis	L	372	481	0.8
	W	336	364	0.9
Anterior testis	L	396	474	0.8
	W	324	367	0.9
Ovary	L	334	376	0.9
	W	224	156	1.4
Uterine egg	L	18	24	0.8
	W	16	11	1.5

DNA and phylogenetic analyses

The percentages of fixed differences in the nucleotide variations of the partial COI gene (282 bp) were determined using pairwise comparison between the nucleotide sequences of *O. lobatus* and *O. felineus*, and of *O. lobatus* and *C. sinensis*; they were 17.37 and 13.82%, respectively. Pairwise comparison between the partial COI genes (330 bp) of *O. lobatus* and *O. viverrini* indicated 3.03% nucleotide fixed differences. After COI-sequence translation, the numbers of amino-acid changes between *O. lobatus* and *O. viverrini* were the same as between *O. lobatus* and *C. sinensis* (3 amino acids changed; 2.72%), while the numbers of amino-acid changes between *O. lobatus* and *O. felineus* comprised 6 positions (6.38%) (Fig. 2). Using the ITS2 region as a DNA marker, the percentage of fixed-different positions was smaller than the COI gene. The percentages of nucleotide fixed-differences of ITS2 sequences (264 bp), by pairwise comparison between *O. lobatus* and *O. felineus*, was 3.03, while *O. lobatus* and *C. sinensis* (337 bp) were 2.37. A pairwise comparison between the partial ITS2 region (347 bp) of *O. lobatus* and *O. viverrini* showed 0.86 bp nucleotide fixed differences (Figure not shown).



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Position                         10        20        30        40        50        60
Amino acid               N  D  S  L  F  G  Y  G  G  L  V  L  A  M  F  A  I  V  C  L
O. lobatus_Nax1         AATGATTCTTTGTTCGGTTATGGTGGTTTGGTGTTGGCCATGTTTGCTATAGTCTGTCTA
O. lobatus_Nax2         ............................................................
O. lobatus_Nax3         ............................................................
O. viverrini_Nax1       .T.......................................................T..
O. viverrini_Nax2       .T.......................................................T..
O. viverrini_Nax3       .T.......................................................T..
C. sinensis_FJ965391    ..A.....G.....T........G..C...........T..............T.....G
C. sinensis_FJ965384    ..A.....G.....T........G..C...........T..............T.....G
O. felineus_EF688128    ........CC.A..T....................A.................T...T.G
O. felineus_EF688127    ........CC.A..T....................A.................T...T.G
O. felineus_EF688123    ........CC.A..T....................A.................T...T.G

                                 70        80        90       100       110       120
                         G  S  V  V  W  A  H  H  M  F  T  V  G  L  D  L  G  T  A  V
O. lobatus_Nax1         GGAAGGGTGGTTTGGGCTCACCACATGTTTACGGTTGGGCTGGATTTGGGCACCGCTGTG
O. lobatus_Nax2         ............................................................
O. lobatus_Nax3         ............................................................
O. viverrini_Nax1       A...................T..T....................................
O. viverrini_Nax2       A...................T..T....................................
O. viverrini_Nax3       ....................T..T....................................
C. sinensis_FJ965391    ..T...........A.....T..T........T.................G..T.....T
C. sinensis_FJ965384    ..T...........A.....T..T........T.................G..T.....T
O. felineus_EF688128    ..T..T........A.....T..T........T..A..AT.A.....A..G..T...A.T
O. felineus_EF688127    ..T..T........A.....T..T........T..A..AT.A.....A..G..T...A.T
O. felineus_EF688123    ..T..T........A.....T..T........T..A..AT.A.....A..G..T...A.T
Amino Acid Change        (S)                                                      (I)

                                130       140       150       160       170       180
                         F  F  S  S  V  T  M  V  I  G  V  P  T  G  I  K  V  F  S  W
O. lobatus_Nax1         TTTTTTAGCTCGGTTACTATGGTTATAGGGGTTCCTACCGGAATAAAGGTTTTTTCTTGG
O. lobatus_Nax2         ............................................................
O. lobatus_Nax3         ............................................................
O. viverrini_Nax1       .......C.............A....T...........T.....................
O. viverrini_Nax2       .......C.............A................T.....................
O. viverrini_Nax3       .......C.............A................T.....................
C. sinensis_FJ965391    .....................A.......T..G.....G..G..C...........A..A
C. sinensis_FJ965384    .....................A.......T..G.....G..G..C...........A..A
O. felineus_EF688128    ........T............A.C..T..T..A.....A..G.................A
O. felineus_EF688127    ........T............A.C..T..T..A.....A..G.................A
O. felineus_EF688123    ........T............A.C..T..T..A.....A..G.................A
Amino Acid Change              (T)          (I)

                                190       200       210       220       230       240
                         L  Y  M  L  A  G  T  R  E  R  F  W  D  P  I  M  W  W  I  V
O. lobatus_Nax1         TTGTATATGCTTGCGGGTACTCGTGAGCGTTTTTGGGACCCGATCATGTGGTGGATAGTT
O. lobatus_Nax2         ............................................................
O. lobatus_Nax3         ............................................................
O. viverrini_Nax1       ............................................T...............
O. viverrini_Nax2       ............................................T...............
O. viverrini_Nax3       ............................................T...............
C. sinensis_FJ965391    ..A...........T..A.....G.....C..A..A..T..A...............A.C
C. sinensis_FJ965384    ..A...........T..A.....G.....C..A..A..T..A...............A.C
O. felineus_EF688128    ..A..C........C........A..T..C..A.....T.....T............A.C
O. felineus_EF688127    ..A..C........C........A..T..C..A.....T.....T............A.C
O. felineus_EF688123    ..A..C........C........A..T..C..A.....T.....T............A.C
Amino Acid Change                                (D)   (L)                        (I)

                                250       260       270       280       290       300
                         G  F  V  V  L  F  T  I  G  G  V  T  G  I  V  L  S  A  S  V
O. lobatus_Nax1         GGGTTCGTAGTACTTTTTACTATAGGGGGGGTTACCGGTATAGTTCTTTCTGCTTCGGTT
O. lobatus_Nax2         ............................................................
O. lobatus_Nax3         ............................................................
O. viverrini_Nax1       ............................................................
O. viverrini_Nax2       ...................................T........................
O. viverrini_Nax3       ............................................................
C. sinensis_FJ965391    .....T..G..G.....C........C...........G.................T..A
C. sinensis_FJ965384    .....T..G..G..............T........T..G.................T..A
O. felineus_EF688128    ..A..TA....G..............T........T..G.....................
O. felineus_EF688127    ..A..TA....G..............T........T..G.....................
O. felineus_EF688123    ..A..TA....G..............T........T..G.....................

                                310       320       330
                         I  N  A  L  L  H  D  T  W
O. lobatus_Nax1         ATAAATGCTTTGTTGCATGACACTTGGTTT
O. lobatus_Nax2         ..............................
O. lobatus_Nax3         ..............................
O. viverrini_Nax1       ...G..........................
O. viverrini_Nax2       ...G..........................
O. viverrini_Nax3       ...G..........................
C. sinensis_FJ965391    ..TG....C.....T.....T.........
C. sinensis_FJ965384    ..TG....C.....T.....T.........
O. felineus_EF688128    ------------------------------
O. felineus_EF688127    ------------------------------
O. felineus_EF688123    ------------------------------
Amino Acid Change           (D)      (F)
```

Figure 2. Genetic variation positions among *O. lobatus, O. viverrini, C. sinensis*, and *O. felineus*, using the mitochondrial *cox*1 gene as DNA marker.

The phylogenetic tree showed that *O. lobatus* is closely related to *O. viverrini*, when the COI gene and the ITS2 region were used as DNA markers. The phylogenetic trees reconstructed from the amino acids of both COI genes and ITS2 sequences revealed a close genetic relationship between *O. lobatus* and *O. viverrini*, genetically closer to *Clonorchis sinensis* than *Opisthorchis felineus* (Figs. 3A & 3B).

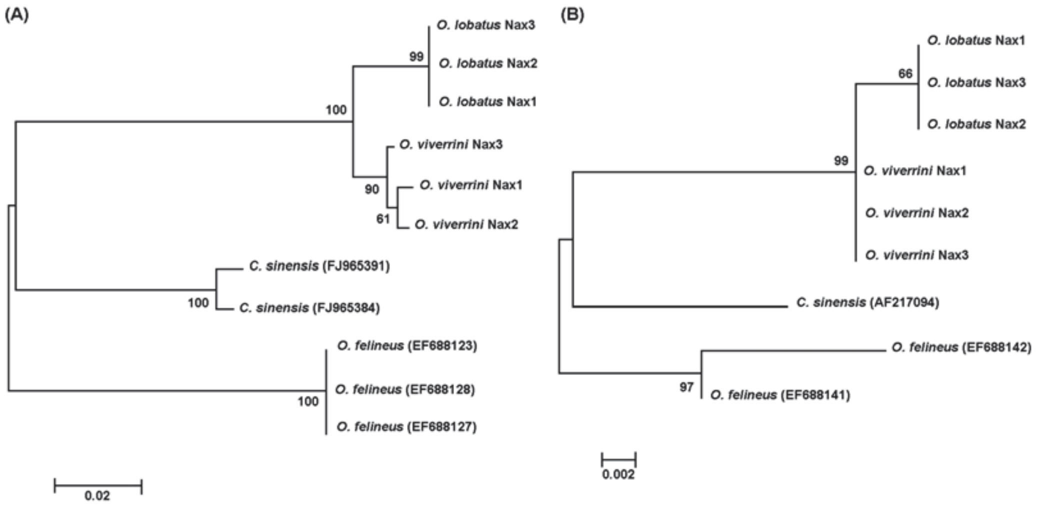


Figure 3. Phylogenetic tree for *O. lobatus, C. sinensis*, and *O. felineus,* using (A) mitochondrail cox1 gene (B) ribosomal ITS2 regions as genetic marker.

Research #3

Variations in *Opisthorchis viverrini* metacercarial infection in Cyprinid fish

A total of 1874 fish were collected; the numbers varied monthly, from 0 to 343 fish per month, with the lowest in August and the highest in November (Fig. 4). The total number of fish positive for liver-fluke metacercariae was 624; the minimum number positive was 1 fish in July, and the maximum 160 in March; the mean number of positive fish ± standard error was 53.5 ± 14.9. The average monthly positive rate was 34.3% (range 2.0-76.2%); the mean positive rate per month ± standard error was 28.5 ± 6.4.

A total of 17 species of 1874 cyprinid fish were examined; every species harbored liver-fluke metacercariae; the average percentage was 34.3% (range 5.6-100%); the mean rate ± standard error was 38.3 ± 6.6 (Table 5). *Henicorhynchus siamensis, Puntius brevis*, *Cyclocheilichthys lagieri, Larbiobarbus siamensis, Puntioplites proctozysron,* and *Hampala dispar* were commonly collected. Over 50% of *Cyclocheilichthys lagieri*, *Amblyrhynchichthys truncates, Barbonymus schwanenfeldi, Cyclocheilichthys armatus, Larbiobarbus siamensis,* and *Thynnichthys thynnoides* were positive with liver-fluke-morphology metacercariae. The result showed *Cyclocheilichthys lagieri* (Trey Srawka Kdam) and *Larbiobarbus siamensis* (Trey Ach Kok) played a leading role in opisthorchiasis transmission in southern Cambodia.

Between 44 and 579 cyprinid fish were collected from 8 different sites around Lake 500, in Kandal Province. The number of positive fish from each collection site varied between 0-214. The average positive percentage was 34.3% (range 0-57.2%), and the mean ± standard error was 26.9 ± 8.6 (Table 5).

Small-liver-fluke infection is caused by a fish-borne trematode, and is the major causative agent of cholangiocarcinoma in the Greater Mekong Sub-region. In this paper, we described variations in metacercarial infection by *Opisthorchis viverrini* in cyprinid fish caught in endemic areas of southern Cambodia, in 2008-2009. One thousand, eight hundred and seventy-four (1874) fish of 17 species were collected from 8 sites in Kandal Province. The pectoral fins, tail fins and muscles of the fish were examined by tissue compression technique. Opisthorchiid metacercariae were found in all species of fish, within the range 5.6% (1/18) in *Crossocheilus reticulates* (Cambodian name: Trey Changwa Chunh Chuak) to 100% (10/10) in *Amblyrhynchichthys truncates* (Cambodian name: Trey Kambot Chramos). The prevalence of *Opisthorchis viverrini* among the sample fish in Kandal Province was 34.3% (642/1874). The highest infection was found in fish caught from O Roley Bridge, with an infection rate of 57.2% (214/374). Metacercariae appeared during the entire examination period except August, when the area was dry and fish could not be caught, with varying positive rates; the average was 34.3% (range 2.0-76.2%), with a peak in March and a trough in July.

Table 5. Prevalence of *O. viverrini* metacercariae in fish species from Kandal Province, August 2008-July 2009

Fish species	**Cambodian name**	**No. examined**	**No. positive**	**%**
Amblyrhynchichthys truncates	Kambot Chramos	10	10	100.0
Barbonymus altus	Kahe Kror Horm	20	6	30.0
Barbonymus gonionotus	Chpin Prak	93	15	16.1
Barbonymus schwanenfeldi	Kahe	53	35	66.0
Crossocheilus reticulates	Changwa Chuhn Chuak	18	1	5.6
Cyclocheilichthys apogon	Srawka Kdam	84	27	32.1
Cyclocheilichthys armatus	Pka Kor	39	33	84.6
Cyclocheilichthys lagieri	Srawka Kdam	268	156	58.2
Hampala dispar	Khmann	115	36	31.3
Henicorhynchus lobatus	Riel Angkam	3	1	33.3
Henicorhynchus siamensis	Riel Top	338	37	10.9
Larbiobarbus siamensis	Ach Kok	199	102	51.3
Oseochilus hasselti	Kros	66	4	6.1
Puntioplites proctozysron	Chara Keng	135	62	45.9
Puntius brevis	Angkat Prak	292	49	16.8
Systomus orphoides	Ampil Tum	69	25	36.2

Thynnichthys thynnoides	Linh	72	43	59.7
Total		**1874**	**642**	**34.3** **(mean± SE = 38.3±6.6)**

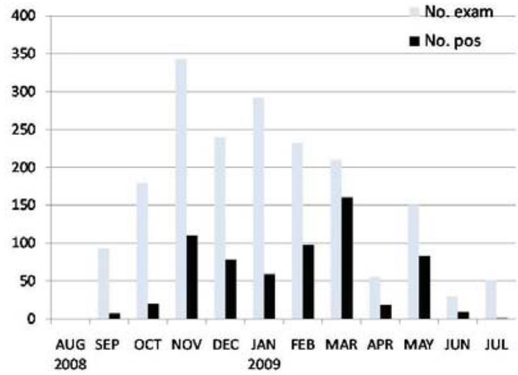


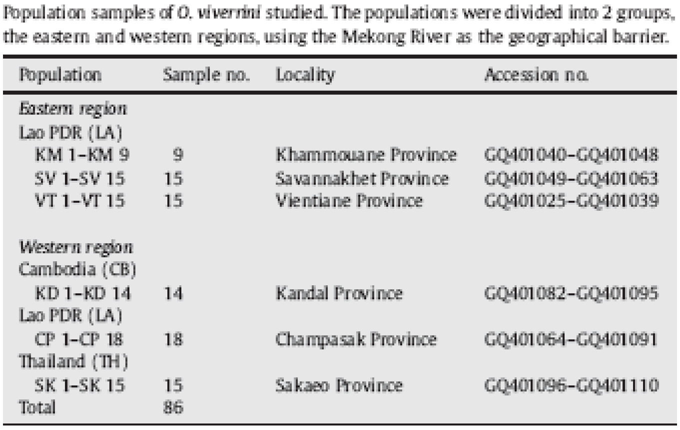
Figure 4. Histogram comparing number of examinations and numbers of fish infected with *O. viverrini* from Kandal Province, August 2008-July 2009.

Research #4

Population-genetic study of *Opisthorchis viverrini* in the lower Mekong Basin

O. viverrini were collected from 6 geographically different localities (6 populations); for each population, samples were used from between 9-18 adult worms (Table 6).

Table 6. Population samples of *O. viverrini* studied.



Population samples of *O. viverrini* studied. The populations were divided into 2 groups, the eastern and western regions, using the Mekong River as the geographical barrier.

Population	Sample no.	Locality	Accession no.
Eastern region			
Lao PDR (LA)			
KM 1–KM 9	9	Khammouane Province	GQ401040–GQ401048
SV 1–SV 15	15	Savannakhet Province	GQ401049–GQ401063
VT 1–VT 15	15	Vientiane Province	GQ401025–GQ401039
Western region			
Cambodia (CB)			
KD 1–KD 14	14	Kandal Province	GQ401082–GQ401095
Lao PDR (LA)			
CP 1–CP 18	18	Champasak Province	GQ401064–GQ401091
Thailand (TH)			
SK 1–SK 15	15	Sakaeo Province	GQ401096–GQ401110
Total	86		

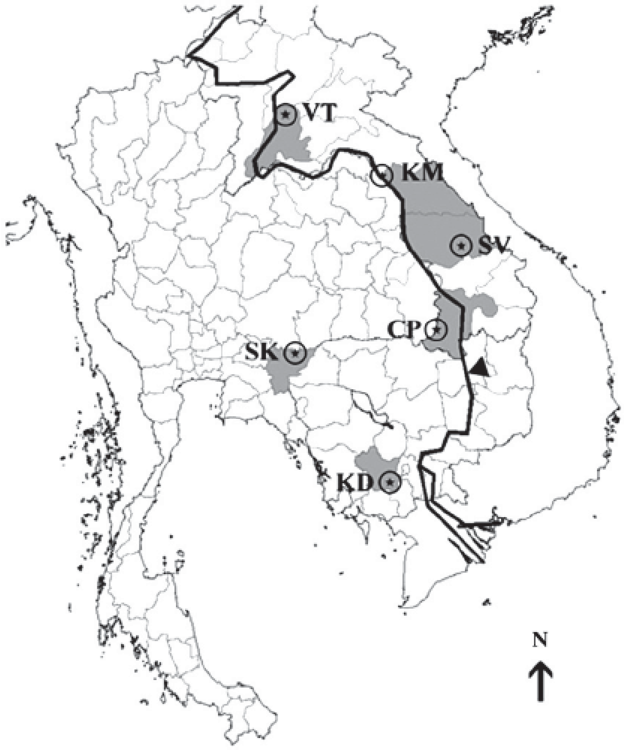
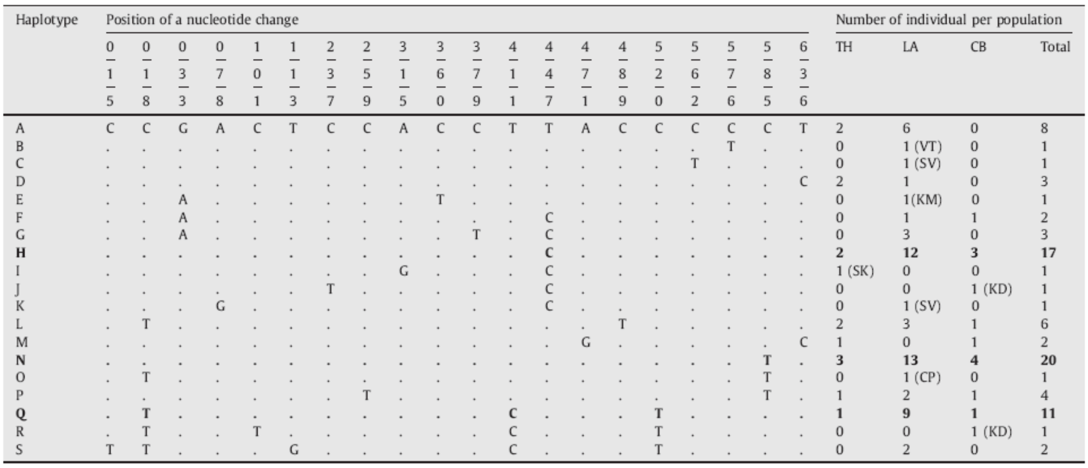


Figure 5. The 6 populations of *O. viverrini* collected in the Lower Mekong Basin provinces: VT=Vientiane; KM=Khammouane; SV=Savannakhet; CP=Champasak, in Lao PDR. KD=Kandal, in Cambodia. SK=Sakaew, in Thailand. The geographic coordinates are 19° 23′N and 102° 31′E for VT, 18° 17′N and 104° 38′E for KM, 16° 39′N and 106° 09′E for SV, 14°57′N and 105° 44′ for CP, 11°28′N and 104°56′E for

KD, and 14°07′N and 102° 51′E for SK. The closed triangle points to the Mekong River.

The 86 sequences of the *O. viverrini* 645 bp partial nad1 gene were analyzed for genetic variance. The results showed 19 haplotypes and 20 variable positions with base compositions: C=57.91%, T=21.98%, A=15.12% and G=5.00%. Haplotypes N, H, and Q had high frequencies, of 24.42, 19.77, and 12.79%, respectively, while haplotypes B, C, E, I, J, K, O, and R were unique (singletons) (Table 7).

Table 7. Variation sites of 645 bp of nad1 gene from the sequence study. Haplotypes A to S belonged to *O. viverrini* from 6 geographically different populations. The bold type indicates highly frequent haplotypes among the populations. The unique haplotypes (singletons) are shown with their populations. “TH” = “Thai” population; “LA” = Lao population; CB = Cambodian population.



Haplotype	Position of a nucleotide change																				Number of individual per population			
	015	018	033	078	101	113	237	259	315	360	379	411	447	471	489	520	562	576	585	636	TH	LA	CB	Total
A	C	C	G	A	C	T	C	C	A	C	C	T	T	A	C	C	C	C	C	T	2	6	0	8
B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	0	1 (VT)	0	1
C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	0	1 (SV)	0	1
D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	2	1	0	3
E	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	0	1(KM)	0	1
F	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	0	1	1	2
G	.	.	A	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	0	3	0	3
H	.	.	.	.	.	.	.	.	.	.	.	.	**C**	.	.	.	.	.	.	.	**2**	**12**	**3**	**17**
I	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	1 (SK)	0	0	1
J	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	0	0	1 (KD)	1
K	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	0	1 (SV)	0	1
L	.	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	2	3	1	6
M	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	1	0	1	2
N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**T**	.	**3**	**13**	**4**	**20**
O	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	0	1 (CP)	0	1
P	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	1	2	1	4
Q	.	**T**	.	.	.	.	.	.	.	.	.	**C**	.	.	.	**T**	.	.	.	.	**1**	**9**	**1**	**11**
R	.	T	.	.	T	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	0	0	1 (KD)	1
S	T	T	.	.	.	G	.	.	.	.	.	C	.	.	.	T	.	.	.	.	0	2	0	2

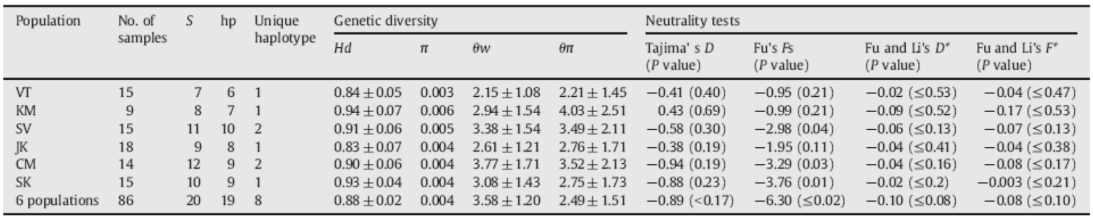
Singletons were found in all geographical populations—haplotype B in VT, E in KM, C and K in SV, O in CP, J and R in KD, and haplotype I in SK. Regarding the genetic diversity of the 6 populations of *O. viverrini* studied, average haplotype diversity (*Hd*) was 0.882±0.019, and nucleotide diversity (π) was 0.0040±0.0003. As shown in Table 8, the highest level of genetic diversity was observed in the KM population in Lao PDR (*Hd* = 0.944±0.070 and π = 0.006±0.001), while the lowest was in the VT population, also in Lao PDR (*Hd* = 0.84±0.05, π = 0.003±0.001). The theta-*w* value (θw = 3.58±1.20) was larger than the theta-π value ($\theta\pi$ =2.49±1.51) (Table 8), suggesting purifying selection, resulting in selective removal of the deleterious allele from the *O. viverrini* population under study.

The results of neutrality testing for the 6 populations were significantly negative by Fu's *Fs* ($-$6.30, $P \leq 0.02$) tests, whereas the Tajima's *D* values were not significant

(一0.89, Pb0.17) (Table 8). The Fu's *Fs* values suggested population expansion and selective sweep, although Tajima's *D* could exhibit neither population bottleneck nor expansion. In other words, the *O. viverrini* populations in the Lower

Mekong Basin might have expanded, advantageous mutations might be fixed, and previous polymorphisms might be removed. The non-significant values for Fu and Li's D^* (一0.10, $P \leq 0.08$) and F^* (一0.08, $P \leq 0.10$) tests (Table 8) provided evidence that *O. viverrini* populations progressed without background selection, which might not purge a non-deleterious allele from the genome due to strong negative selection. Fu's *Fs* test supported the supposition that the *O. viverrini* population might have expanded, reducing heterozygosity and increasing homozygosity.

Table 8. Indexes of genetic diversity and test of population equilibrium. *S* = no. of segregation sites (polymorphic), *hp* = no. of haplotypes, *Hd* = haplotype diversity, π = nucleotide diversity, θw = Watterson's theta, based on S and $\theta\pi$ = the theta based on π. Significance: $P>0.05$.



Population	No. of samples	*S*	hp	Unique haplotype	Genetic diversity				Neutrality tests			
					Hd	π	θw	$\theta\pi$	Tajima' s *D* (*P* value)	Fu's *Fs* (*P* value)	Fu and Li's D^* (*P* value)	Fu and Li's F^* (*P* value)
VT	15	7	6	1	0.84 ± 0.05	0.003	2.15 ± 1.08	2.21 ± 1.45	−0.41 (0.40)	−0.95 (0.21)	−0.02 (≤0.53)	−0.04 (≤0.47)
KM	9	8	7	1	0.94 ± 0.07	0.006	2.94 ± 1.54	4.03 ± 2.51	0.43 (0.69)	−0.99 (0.21)	−0.09 (≤0.52)	−0.17 (≤0.53)
SV	15	11	10	2	0.91 ± 0.06	0.005	3.38 ± 1.54	3.49 ± 2.11	−0.58 (0.30)	−2.98 (0.04)	−0.06 (≤0.13)	−0.07 (≤0.13)
JK	18	9	8	1	0.83 ± 0.07	0.004	2.61 ± 1.21	2.76 ± 1.71	−0.38 (0.19)	−1.95 (0.11)	−0.04 (≤0.41)	−0.04 (≤0.38)
CM	14	12	9	2	0.90 ± 0.06	0.004	3.77 ± 1.71	3.52 ± 2.13	−0.94 (0.19)	−3.29 (0.03)	−0.04 (≤0.16)	−0.08 (≤0.17)
SK	15	10	9	1	0.93 ± 0.04	0.004	3.08 ± 1.43	2.75 ± 1.73	−0.88 (0.23)	−3.76 (0.01)	−0.02 (≤0.2)	−0.003 (≤0.21)
6 populations	86	20	19	8	0.88 ± 0.02	0.004	3.58 ± 1.20	2.49 ± 1.51	−0.89 (<0.17)	−6.30 (≤0.02)	−0.10 (≤0.08)	−0.08 (≤0.10)

Mismatch distribution analysis of the 6 isolated populations showed unimodal distribution, for the sum of squared deviation, but without significance (0.0032, $P \geq 0.74$) (Fig. 6). This result indicates recent growth of the *O. viverrini* populations.

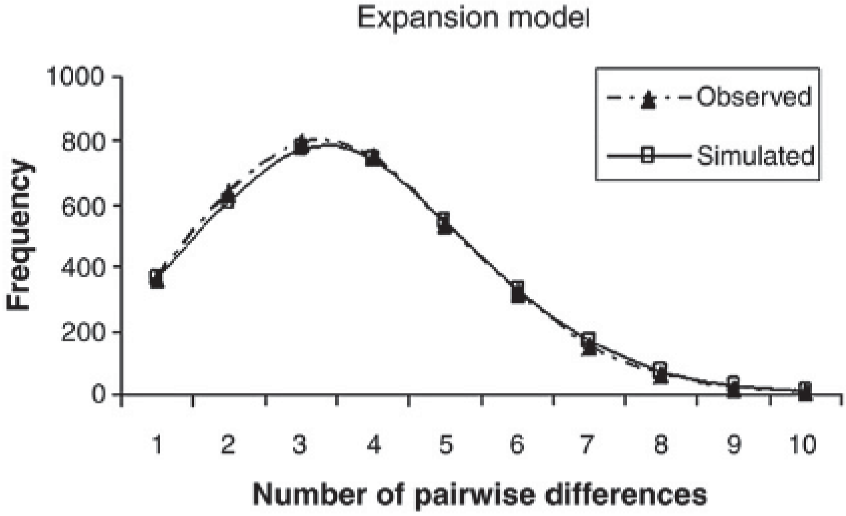
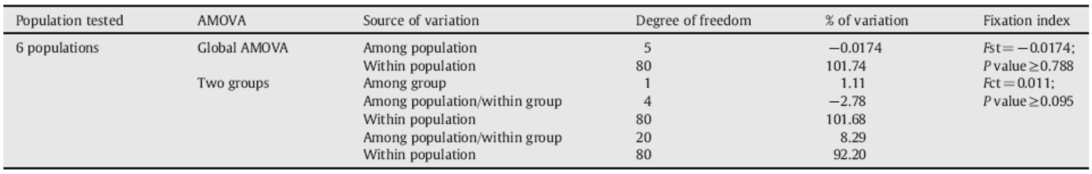


Figure 6. Mismatch distribution of the 86 partial nad1 sequences from 6 populations of *O. viverrini* from the Lower Mekong Basin. Significance: $P > 0.05$.

The aforementioned analyses were confirmed by a genetic differentiation study using the F*st* value from the global AMOVA test. The F*st* value indicated no significant difference in genetic variation among the *O. viverrini* populations ($F_{st}=-0.017$, $P \geq 0.788$). If the Mekong River is the geographical barrier for the hosts, *O. viverrini* populations east and west of the river might be significantly different by genetic variation; this hypothesis was tested by calculating the F*ct* values. The results indicated no significant difference between the groups of *O. viverrini* populations studied, along both sides of the Mekong River ($F_{ct}=0.011$, $P \geq 0.095$)

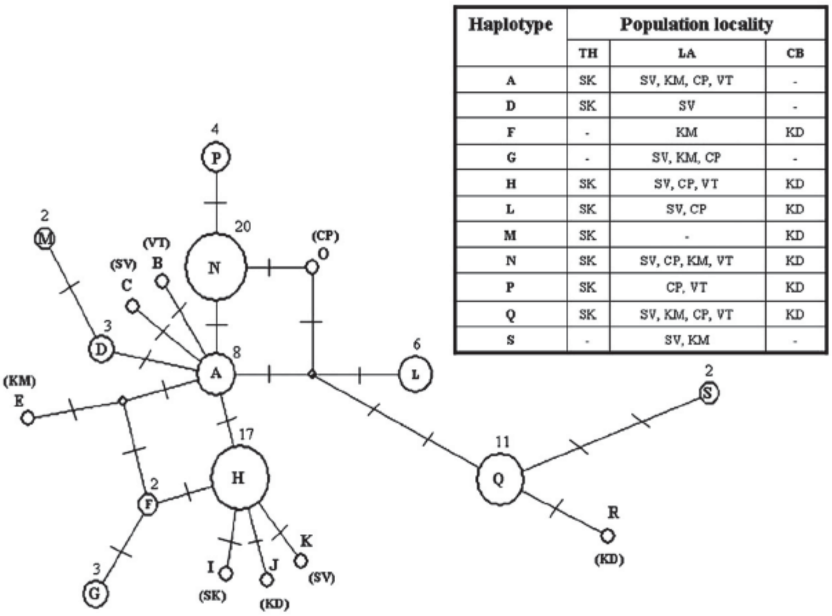
(Table 9). Taken together, these data suggested that the *O. viverrini* populations in the Lower Mekong Basin might have been expanding. Table 9. AMOVA and fixation-index values for the global AMOVA for all *O. viverrini* populations studied, and grouping test for populations using the criterion of the Mekong River as a geographical barrier between the eastern and western river-banks. Significance: P>0.05.

Table 9. AMOVA test of genetic differentiation among 6 populations of *O. viverrini* in the lower Mekong Basin



Population tested	AMOVA	Source of variation	Degree of freedom	% of variation	Fixation index
6 populations	Global AMOVA	Among population	5	−0.0174	*F*st = −0.0174;
		Within population	80	101.74	*P* value ≥ 0.788
	Two groups	Among group	1	1.11	*F*ct = 0.011;
		Among population/within group	4	−2.78	*P* value ≥ 0.095
		Within population	80	101.68	
		Among population/within group	20	8.29	
		Within population	80	92.20	

The phylogenetic network of 19 haplotypes from 86 *nad*1 gene sequences of 6 geographically different populations showed no distinct geographic structure. A star-like cluster network represented the demographic expansion event of *O. viverrini* populations in the Lower Mekong Basin region. The haplotype network indicated no genetic divergences between the *O. viverrini* populations from the eastern and western sides of the Mekong River. Moreover, specimens in the same population, or those from geographically near isolates, did not group together (Fig. 7).



Haplotype	Population locality		
	TH	LA	CB
A	SK	SV, KM, CP, VT	-
D	SK	SV	-
F	-	KM	KD
G	-	SV, KM, CP	-
H	SK	SV, CP, VT	KD
L	SK	SV, CP	KD
M	SK	-	KD
N	SK	SV, CP, KM, VT	KD
P	SK	CP, VT	KD
Q	SK	SV, KM, CP, VT	KD
S	-	SV, KM	-

Figure 7. The phylogenetic network of 19 haplotypes (A–S) from 6 geographically different populations in the Lower Mekong Basin. Each open circle represents each haplotype, with the number of individuals per haplotype outside the circle. The 8 singletons (haplotypes B, C, E, I, J, K, O, and R) are shown as the smallest circle. The solid line shows the network indicating the phylogenetic relationship of the haplotypes. The frequency of nucleotide change between haplotypes is shown as a small solid line. Each singleton is marked with the locality of the sample population. The small table shows the members of each haplotype belonging to the 3 countries (TH = Thailand, LA = Laos, and CB = Cambodia).

Research #5

Population-genetic study of *Haplorchis taichui* in the GMS

The *H. taichui* samples were collected from 8 different geographical localities in the GMS (Fig. 8)

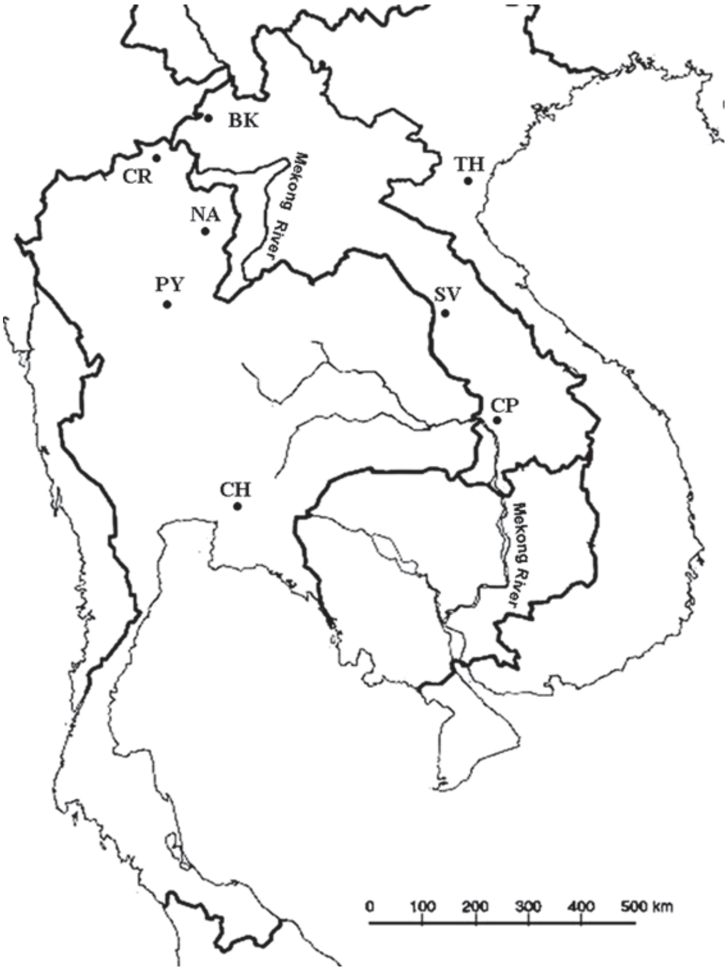


Figure 8. The 8 different geographical locations of the *H. taichui* populations studied.

Estimates of genetic diversity

From the 375 bp COI gene sequences of the 127 individuals, 14 segregation sites and 19 haplotypes; their GC content was 41%. The total haplotype diversity (*Hd*) and nucleotide diversity (π) were 0.908 ± 0.011, and 0.010 ± 0.001, respectively. Calculating the *Hd* and π of each population showed a much wider range of genetic diversity (*Hd*= 0.23-0.86; π = 0.0006-0.0063), except the population of Savannakhet (SV), having no nucleotide variation. The population of Boe Keaw (BK) had the highest haplotype diversity (0.86 ± 0.05), while the population of Champasak (CP) had the lowest (0.23 ± 0.13). The Phayao (PY) population had the highest nucleotide diversity (0.78 ± 0.06), while the population of CP was also the lowest (0.0006 ± 0.0003). The results of $\theta\pi$ - θw of each population were positive, except the CP population. However, considering all individuals (not separate populations), $\theta\pi$ was larger than θw and the result of $\theta\pi$ - θw was also positive.

Neutrality tests

The estimated Tajima'*D* values of all populations studied were not significantly different from zero. The *D* values of most tested populations (all individuals; no population) were positive, except the CP population. The Fu & Li's *F** and *D** were non-significant when tested with each population; nevertheless, the values were significant when tested with all individuals (Table 10).

Table 10. Molecular diversity index and neutrality tested values; * Statistical significance $P < 0.05$

Population	N	No. of haplotypes	Haplotype diversity ($Hd \pm$ SE)	Nucleotide diversity ($\pi \pm$ SE)	θ_{π} - θ_{w}	Tajima's D	Fu &Li's F*	Fu & Li's D*
CR	16	3	0.70±0.05	0.0024±0.0003	0.30	1.262	1.14	0.91
NA	16	2	0.33±0.13	0.0009±0.0003	0.02	0.156	0.63	0.69
PY	16	4	0.78±0.06	0.0063±0.0007	0.84	1.844	1.59	1.21
CH	16	2	0.53±0.56	0.0014±0.0002	0.22	1.473	1.01	0.69
BK	16	6	0.86±0.05	0.0035±0.0004	0.42	1.349	1.29	1.04
SV	16	1	N/A	N/A	N/A	N/A	N/A	N/A
CP	16	2	0.23±0.13	0.0006±0.0003	-0.07	-0.448	0.45	0.69
TH	15	4	0.67±0.09	0.0022±0.0004	0.20	0.872	1.03	0.92

Mismatch distribution analyses

Mismatch distribution analyses indicated no genetic signal of a sudden population expansion by rejection of *SSD* values. Non-sudden expansion evidence was confirmed by the comparison of observed and simulated frequency distribution of pairwise nucleotide differences among individuals. Each population showed multimodal distribution of pairwise differences (Fig. 9).

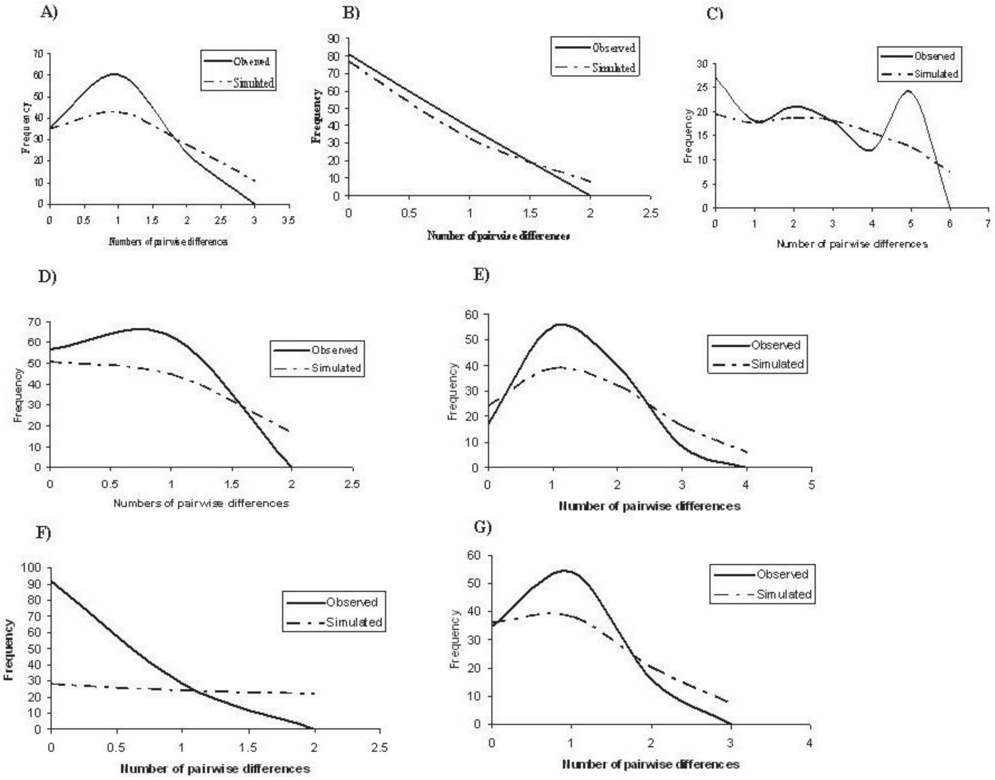


Figure 9. Mismatch distribution of the 7 populations of *H. taichui* studied.

Neighbor-joining and haplotype trees

The neighbor-joining (NJ) tree of 127 sequences of the COI gene showed 3 groups of populations. The major populations in group 1 were NA, CR, PY, and CH, which were populations collected in Thailand (Thai group). The major populations in group 2 were SV, BK, and CP. Populations in this group were obtained from Lao PDR (Lao group). The last group belonged to the population collected from Vietnam (Vietnam group). However, the BK population was related to the Thai group (haplotype no. 007). The NJ tree that was constructed showed long internal branches (Fig. 10).

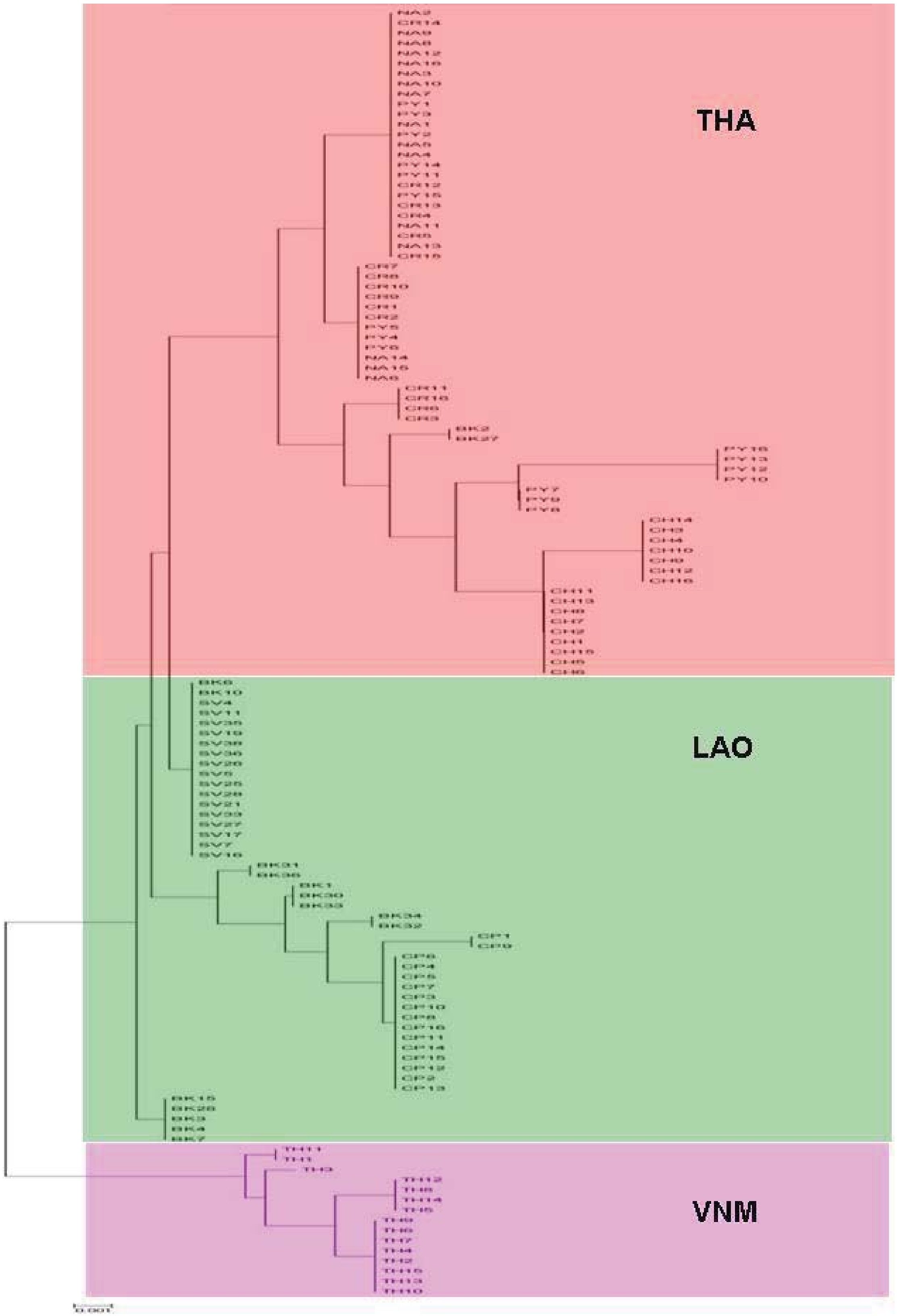
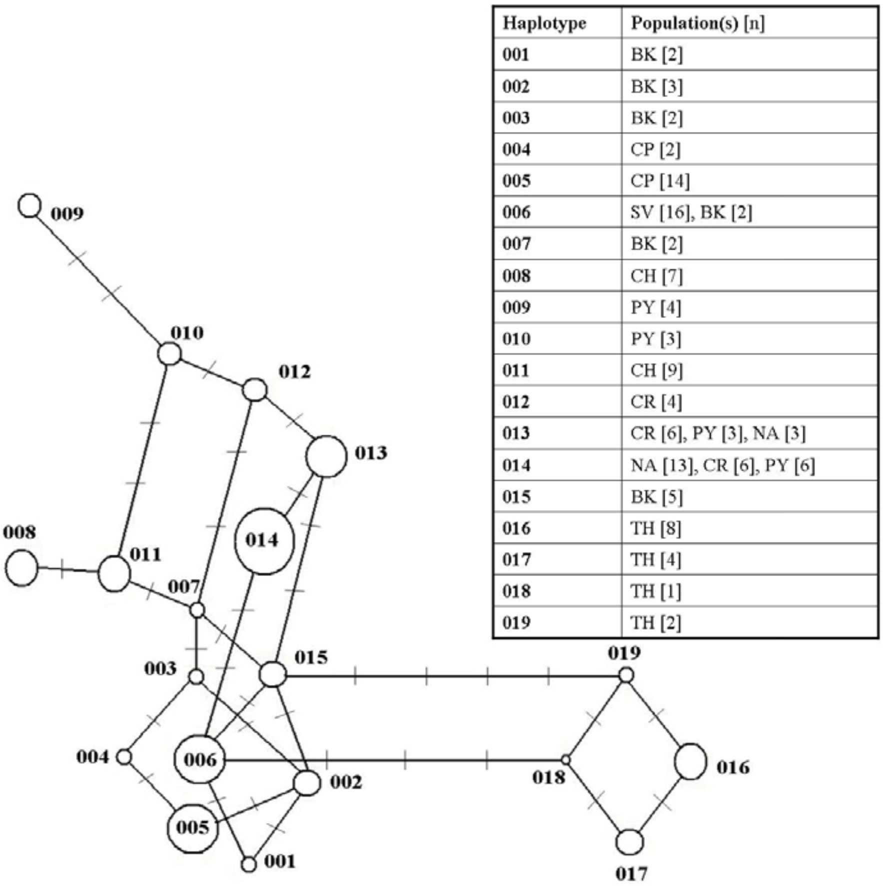


Figure 10. Clustering dendrogram among 8 populations of *H. taichui* from 3 countries (Thailand, Lao PDR, and Vietnam) using the *cox*1 gene as genetic marker.

The relationships between 19 haplotypes were revealed by the haplotype network (Fig. 11). The network was not star-like in shape, but the haplotypes seemed closely related. The highest numbers of individuals appeared in haplotype 014 (comprising populations CR, PY, and NA), with a singleton in haplotype 018 (TH population).



Haplotype	Population(s) [n]
001	BK [2]
002	BK [3]
003	BK [2]
004	CP [2]
005	CP [14]
006	SV [16], BK [2]
007	BK [2]
008	CH [7]
009	PY [4]
010	PY [3]
011	CH [9]
012	CR [4]
013	CR [6], PY [3], NA [3]
014	NA [13], CR [6], PY [6]
015	BK [5]
016	TH [8]
017	TH [4]
018	TH [1]
019	TH [2]

Figure 11. Haplotype network of 8 populations of *H. taichui* suggesting the haplotype diversity of the *cox*1 gene among the parasite populations studied.

Genetic differentiation of populations

The AMOVA test was used to estimate genetic differentiation using the F*st* value from the global AMOVA test. The F*st* value indicated significant difference in genetic variation among the *H. taichui* populations (F*st* = 0.810, *P* < 0.001). According to the NJ tree of the *H. taichui* populations, they could be divided into 3 groups (Thai, Lao, and Vietnam groups). It was possible that the 3 populations differed genetically. The hypothesis was tested by calculating the F_{ct} value. The result indicated a significant difference between those groups of *H. taichui* ($F_{ct} = 0.45$, $P < 0.001$) (Table 11).

Table 11. AMOVA indicated genetic differentiation among 8 *H. taichui* populations from different geographic areas.

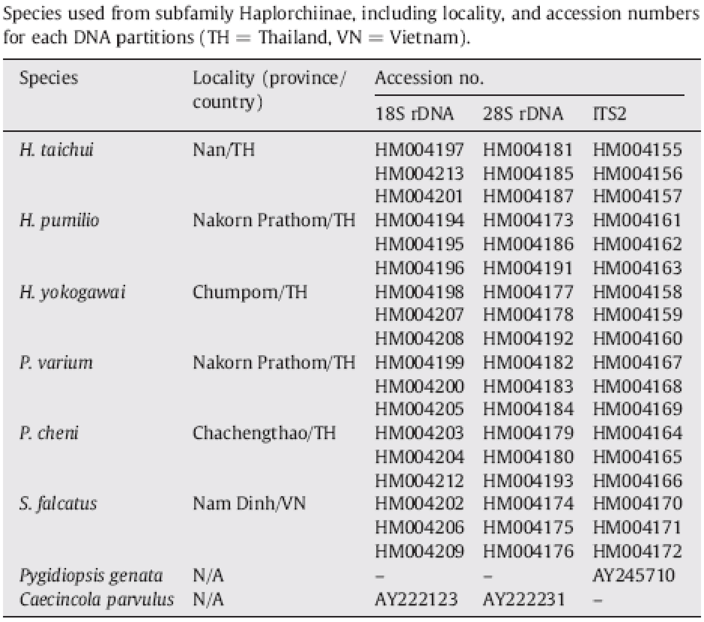
Population tested	AMOVA	Source of variance	Degree of freedom	% of variation	Fixation index
8 populations	Global AMOVA	Among populations	7	81.08	Fst=0.810*
		Within population	119	18.91	
	3 groups	Among groups	2	45.29	Fst=0.83*
		Among population /within group	5	38.59	Fct = 0.45*
		Within population	119	16.12	

Research #6

Molecular systematics of heterophyid minute intestinal flukes in the subfamily Haplorchiinae (Trematoda: Heterophyidae), based on nuclear ribosomal DNA genes and ITS2 region

Metacercariae of *H. taichui, H. pumilio, H. yokogawai, P. varium,* and *P. cheni* were collected from fish at selected localities in Thailand by pepsin digestion technique (Srisawangwong *et al*., 1997) and identified under a light microscope. Around 1000 metacercariae of each species were inoculated into Syrian golden hamsters (*Mesocricetus auratus*). The hamsters were sacrificed 3 days' post-infection to obtain adult worms from the small intestine; the worms were preserved in 70% alcohol at -20 °C until used. Adult *S. falcatus* worms were collected from human feces after deworming intestinal flukes with praziquantel + MgSO4, in Nam Dinh Province, Vietnam, and were stored in the same manner as above (Table 12).

Table 12.

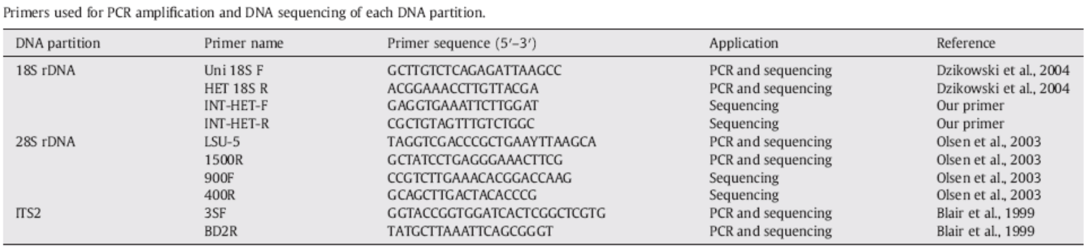


Species used from subfamily Haplorchiinae, including locality, and accession numbers for each DNA partitions (TH = Thailand, VN = Vietnam).

Species	Locality (province/ country)	Accession no.		
		18S rDNA	28S rDNA	ITS2
H. taichui	Nan/TH	HM004197	HM004181	HM004155
		HM004213	HM004185	HM004156
		HM004201	HM004187	HM004157
H. pumilio	Nakorn Prathom/TH	HM004194	HM004173	HM004161
		HM004195	HM004186	HM004162
		HM004196	HM004191	HM004163
H. yokogawai	Chumporn/TH	HM004198	HM004177	HM004158
		HM004207	HM004178	HM004159
		HM004208	HM004192	HM004160
P. varium	Nakorn Prathom/TH	HM004199	HM004182	HM004167
		HM004200	HM004183	HM004168
		HM004205	HM004184	HM004169
P. cheni	Chachengthao/TH	HM004203	HM004179	HM004164
		HM004204	HM004180	HM004165
		HM004212	HM004193	HM004166
S. falcatus	Nam Dinh/VN	HM004202	HM004174	HM004170
		HM004206	HM004175	HM004171
		HM004209	HM004176	HM004172
Pygidiopsis genata	N/A	–	–	AY245710
Caecincola parvulus	N/A	AY222123	AY222231	–

DNA was extracted separately from at least 3 individuals of each species of Haplorchiinae, using a Genomic DNA mini kit (Tissue) (Geneaid, Taiwan) according to the manufacturer's protocol. The PCR amplicons were amplified from different partitions, 18S rDNA, 28S rDNA, and ITS2 region (Table 13), of the parasite genome, using the appropriate PCR conditions.

Table 13. PCR primers for this study

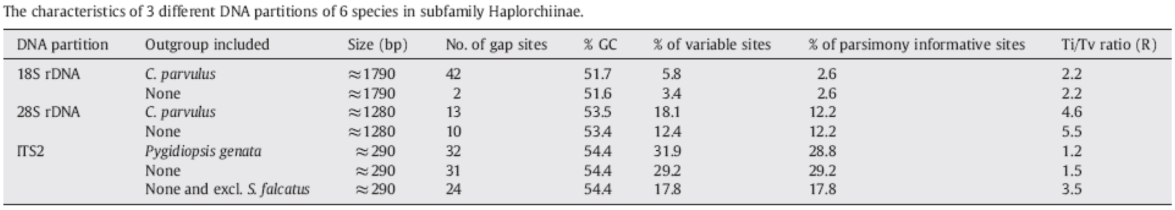


Primers used for PCR amplification and DNA sequencing of each DNA partition.

DNA partition	Primer name	Primer sequence (5′–3′)	Application	Reference
18S rDNA	Uni 18S F	GCTTGTCTCAGAGATTAAGCC	PCR and sequencing	Dzikowski et al., 2004
	HET 18S R	ACGGAAACCTTGTTACGA	PCR and sequencing	Dzikowski et al., 2004
	INT-HET-F	GAGGTGAAATTCTTGGAT	Sequencing	Our primer
	INT-HET-R	CGCTGTAGTTTGTCTGGC	Sequencing	Our primer
28S rDNA	LSU-5	TAGGTCGACCCGCTGAAYTTAAGCA	PCR and sequencing	Olsen et al., 2003
	1500R	GCTATCCTGAGGGAAACTTCG	PCR and sequencing	Olsen et al., 2003
	900F	CCGTCTTGAAACACGGACCAAG	Sequencing	Olsen et al., 2003
	400R	GCAGCTTGACTACACCCG	Sequencing	Olsen et al., 2003
ITS2	3SF	GGTACCGGTGGATCACTCGGCTCGTG	PCR and sequencing	Blair et al., 1999
	BD2R	TATGCTTAAATTCAGCGGGT	PCR and sequencing	Blair et al., 1999

Sequence data analyses and preliminary phylogenetic analysis. After sequence alignment of each DNA marker, gap sites were excluded from the sequences. A repeat region (166 bp nucleotide long) in the ITS2 sequences of *H. taichui*, reported by Van *et al.* (2009), was also found in our ITS2 sequences of *H. taichui*. This repeat region was removed from our ITS2 DNA sequences (Van et al., 2009). The final lengths of alignments of the partial 18S rDNA, 28S rDNA and ITS2 regions, after removal of gap sites, were 1752 bp, 1263 bp, and 232 bp, respectively. The GC contents of the three DNA partitions were > 50%. Although the ITS2 region was far shorter (>1/5) than 18S and 28S rDNA in length, the percentage variation (31.9%) and parsimony informative sites (28.8%) within ITS2 region were the highest among them. Moreover, the transition/transversion ratio (Ti/Tv) of the ITS2 region was 1.24, which was 4.4 times smaller than the 28S rDNA genes. However, when the ITS2 sequences of *P. genata* were excluded, variable and parsimony informative sites were 29.2 and 29.2%, respectively, and the Ti/Tv ratio was 1.54. Moreover, when the ITS2 sequence of *S. falcatus* was excluded. together with the outgroup *P. genata*, the percent of variable sites and parsimony informative sites of *Haplorchis* spp. plus *Procerovum* spp. were both 17.8%, and the Ti/Tv ratio was 3.45 (Table 14). These data revealed that the ITS2 sequence of *S. falcatus* is markedly different from other species of the subfamily Haplorchiinae.

Table 14. Characteristics of 3 different DNA partitions of 6 species in the subfamily Haplorchinae



The characteristics of 3 different DNA partitions of 6 species in subfamily Haplorchiinae.

DNA partition	Outgroup included	Size (bp)	No. of gap sites	% GC	% of variable sites	% of parsimony informative sites	Ti/Tv ratio (R)
18S rDNA	*C. parvulus*	≈1790	42	51.7	5.8	2.6	2.2
	None	≈1790	2	51.6	3.4	2.6	2.2
28S rDNA	*C. parvulus*	≈1280	13	53.5	18.1	12.2	4.6
	None	≈1280	10	53.4	12.4	12.2	5.5
ITS2	*Pygidiopsis genata*	≈290	32	54.4	31.9	28.8	1.2
	None	≈290	31	54.4	29.2	29.2	1.5
	None and excl. *S. falcatus*	≈290	24	54.4	17.8	17.8	3.5

As a preliminary study, the phylogenetic tree for each DNA marker was reconstructed using the *p*-distances for the NJ method. For construction of the ITS2 tree, the previously deposited homologous sequences of *Procerovum* sp., *H. pumilio*, and *H. taichui* from the different geographical localities were used, together with our sequences. The results showed that the phylogenetic trees constructed from the 18S to 28S rDNA sequences were congruent, while the tree constructed from the ITS2 region was incongruent with 18S and 28S rDNA. In all 3 DNA partitions, no significant genetic variation was seen in the species tested (data not shown).

To increase the accuracy of the phylogenetic data, the DNA partitions were combined before reconstructing the phylogenetic tree. The partition homogeneities of the combined data sets (18S+28S rDNAs, 18S rDNA+ITS2, 28S rDNA+ITS2, and 18S rDNA+28S rDNA+ITS2) were examined. Significant incongruence, after comparisons of DNA partitions, was found at P >0.02. Only the combination between 18S and 28S rDNA sequences was non-significant (P= 1.00). The combinations of ITS2 with 18S rDNA, or 28S rDNA, or both genes were significant (P= 0.001). The ML and BI methods were used to construct the phylogenetic trees of each DNA marker (the trees constructed from the 18S to 28S rDNA markers separately are not shown) and combined the sequences of the 18S and 28S rDNA genes (Fig. 12). The likelihood scores (¯*ln* L) of the ML trees for 18S rDNA, 28S rDNA, ITS2 region, and the combined data of the two rDNA genes, were 3154.36, 2978.65, 778.15, and 6215.15, respectively. The non-parametric branch support values calculated based on a Shimodaira-Hasegawa-like procedure (SH-like) are shown at each node of the trees. The standard deviation of split frequencies at the end of running Bayesian analysis of 18S rDNA, 28S rDNA, ITS2 region, and combined data of two rDNA markers were 0.0047, 0.0046, 0.0045, and 0.0038, respectively. The ML and BI trees showed good agreement among 18S rDNA, 28S rDNA and the combined data of these two genes. Phylogenetic trees constructed from the combined 18S and 28S rDNA sequences showed *Procerovum* spp. and *H. taichui* grouped as closely related species, while *H. pumilio* and *H. yokogawai* were separated from them into another clade. *S. falcatus* was separated from the genera *Procerovum* and *Haplorchis* (Fig. 12). When the relationships of the topology of those 6 species to several morphological diagnostic characters were examined, discrimination by the number of testes is congruent with the topology of the species, in that *Procerovum* spp. and 3 *Haplorchis* spp., having a single testis, form a single clade separated from *S. falcatus*, which has two testes (Fig.

12). On the other hand, species discrimination with two other markers, modification of the ventral sucker and the presence of an expulsor, did not fit with the topology of the species in the combined 18S and 28S rDNA phylogenetic tree.

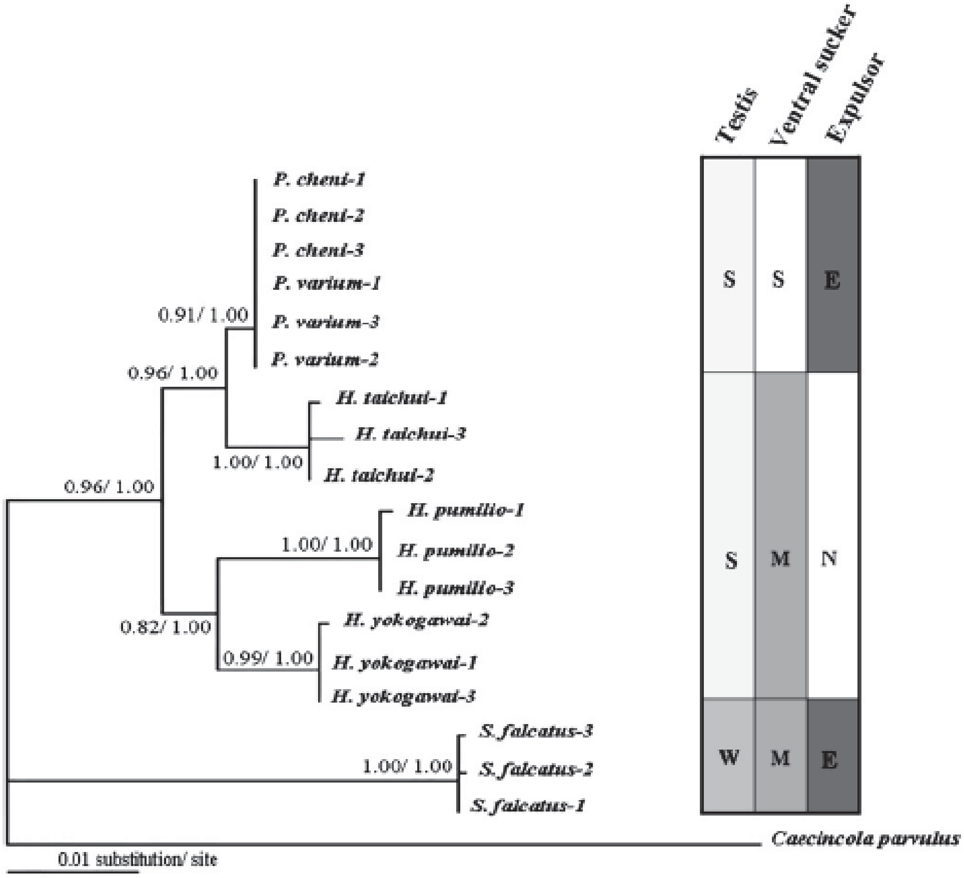


Figure 12. Phylogenetic relationships among 6 species in the subfamily Haplorchiinae, reconstructed from combined DNA partitions between 18S and 28S rDNA sequences. The ML tree was constructed by simultaneous NNI moves, using *Caecincola parvulus* as an outgroup. *P*-values of the approximate likelihood ratios (aLTR) of the SH-test and the Bayesian posterior probability values (BPP) are placed on each node (aLTR/BPP). Three morphological characters related to the phylogenetic relationships are shown beside the topology. Testis: single [S] or two testes [W], Ventral sucker: simple [S] or modified [M], Expulsor: present [E] or absent [N].

The ML and BI topology of the ITS2 region revealed that, like the analyses of the combined 18S and 28S rDNA markers, the genera *Procerovum* and *Haplorchis* were grouped together, to the exclusion of *S. falcatus* (Fig. 13). However, the positions of the *Haplorchis* species were incongruent with those inferred from the 18S and 28S rDNA markers in Fig. 12. In the ITS2 tree (Fig. 13), *H. pumilio* was more closely related to *Procerovum* spp. than to *H. taichui*. When the topology of 6 species of the subfamily Haplorchiinae in the ITS2 tree was compared with 3 morphological characters, the groups having a single testis (genera *Procerovum* and *Haplorchis*) formed a single group, far removed from *S. falcatus*, which has two testes. The characters of the modified ventral sucker and expulsor were not related to the topology of the species in the molecular phylogenetic tree (Fig. 13).

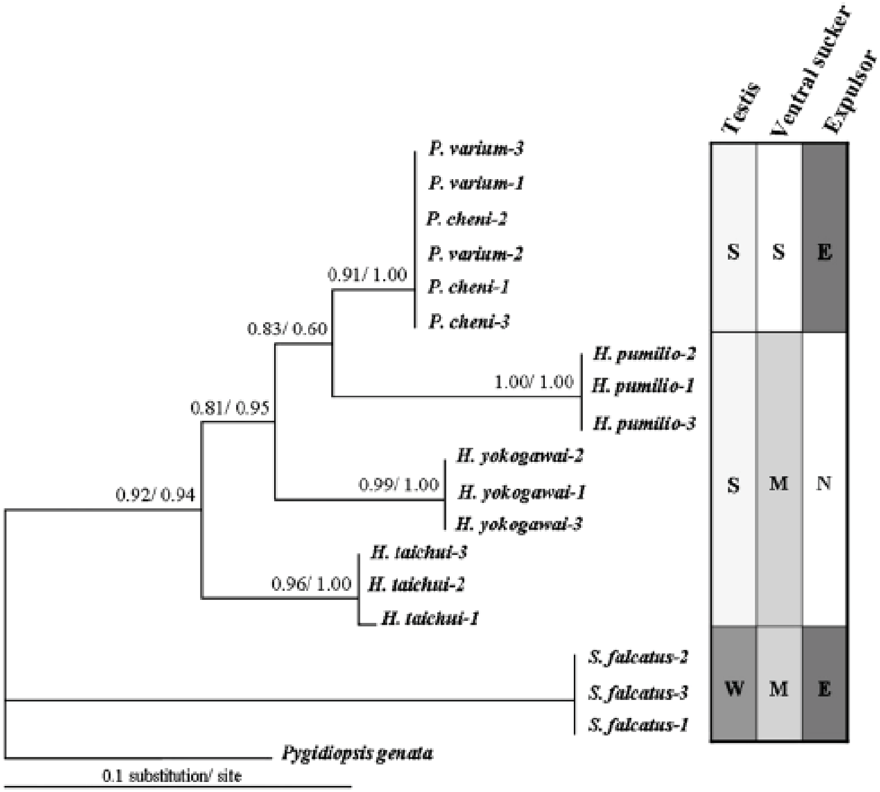


Figure 13. Phylogenetic relationships among 6 species in the subfamily Haplorchiinae, reconstructed from ITS2 sequences. The ML tree was constructed by simultaneous NNI moves using *Pygidiopsis genata* as an outgroup. *P*-values of the approximate likelihood ratios (aLTR) of SH-test and the Bayesian posterior probability values (BPP) are supported on each node (aLTR/BPP). Three morphological characters related to phylogenetic relationships are shown beside the topology. Testis: single [S] or two testes [W], Ventral sucker: simple [S] or modified [M], Expulsor: present [E] or absent [N].

Research #7

Molecular diagnosis of OLFs and HIFs based on ribosomal ITS regions

Species discrimination

The positive PCR bands were confirmed in 1.0% agarose gel. No intraspecies variation was observed in 10 samples per species and region; then, amplicons of 4 species were electrophoresed together. For the ITS1 region, the sizes of the PCR amplicons from *O. viverrini* and *C. sinensis* were approximately 800 and 820 bp, respectively. The PCR amplicon of *H. pumilio* was about 1250 bp, and that of *H. taichui* 930 bp (Fig. 14A). However, *O. viverrini* and *C. sinensis* showed a small difference when separated with 2.0% agarose gel. The ITS2- region amplicon sizes of *O. viverrini*, *C. sinensis*, *H. pumilio,* and *H. taichui* were approximately 380, 390, 380, and 530 bp, respectively (Fig. 14B). The amplicon size of *H. taichui* was clearly different from the other 3 species.

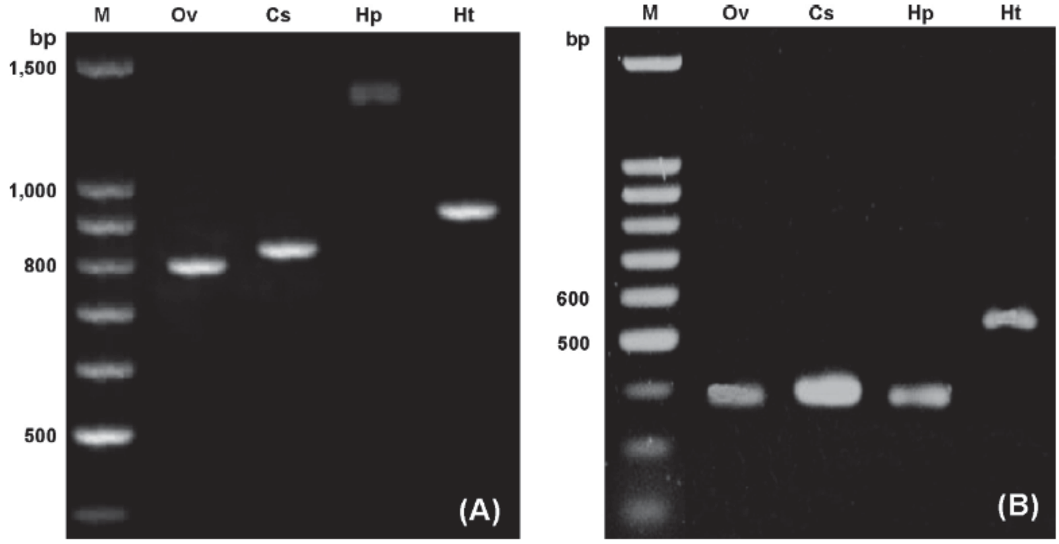


Fig. 14. Species-specific amplicons. Lane 1: 100 bp DNA marker (Promega). Lane 2: *O. viverrini* (Ov), Lane 3: *C. sinensis* (Cs), Lane 4: *H. pumilio* (Hp) and Lane 5: *H. taichui* (Ht). (A) Using primer set ITS1-F and ITS1-R targeting rDNA ITS1 region in 2.0% agarose gel electrophoresed for 2.5 h. (B) Using primer set ITS2-F and ITS2-R targeting rDNA ITS2 region in 1.0% agarose gel electrophoresed for 1.5 h.

Stool examination

With the ITS1 PCR, an 800 bp band, the same size as *O. viverrini*, was found in 16 of 21 samples (76.2%), and the 930 bp band of *H.taichui* appeared in 1 sample (Fig. 15A). For the ITS2 PCR, 16 of 21 showed the 380 bp band, the same size as *O. viverrini* and/or *H. pumilio*, and 4 samples had bands of 530 bp, the same as *H. taichui*; both band sizes appeared in 3 samples (Fig. 15B). In all, the samples showed positive results in 20 of 21 samples (95.2%). *C. sinensis* was excluded, since the parasite was not prevalent in the study area. Eggs per gram (EPG) ranged between 0 and 4400 (Fig. 15). The kappa test was used to study agreement between the two methods. The kappa value was 0.75 for ITS1 PCR, and 0.95 for ITS2, indicating a medium and a high association with the modified thick smear method. The PCR targeting ITS1 was less sensitive than the standard fecal-smear method, while ITS2 exhibited a high capacity to detect flukes in the Superfamily Opisthorchoidea.

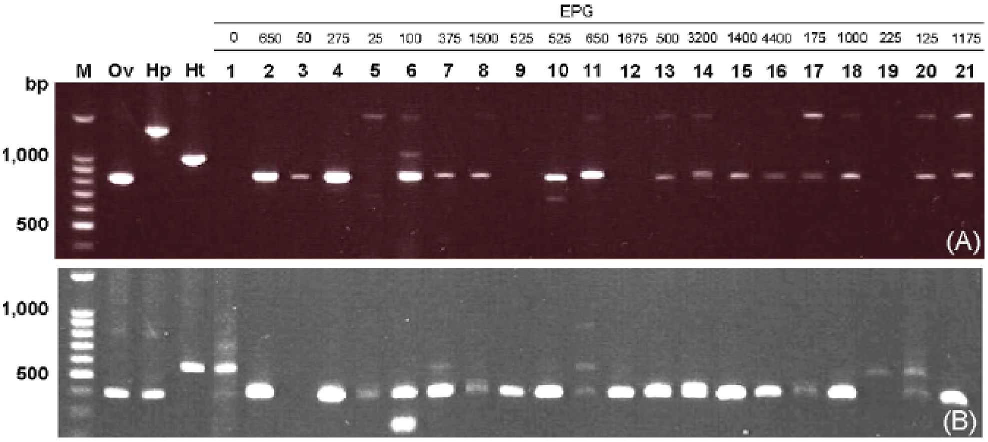


Fig. 15. PCR results for fecal samples targeting the ITS region. The PCR products were electrophoresed in 1.0% agarose gel for 1.5 h. Lane M: 100 bp DNA marker (Promega), Lane Ov: *O. viverrini*, Lane Hp: *H. pumilio*, Lane Ht: *H. taichui* and Lanes 1-21: fecal samples. (A) ITS1 region and (B) ITS2 region.

DISCUSSION

This research study was performed according to the objectives and permissions prescribed by the Thailand Research Fund (TRF). Our results are reported in seven papers, published in international journals. This research was delayed by unpredictable variations in the seasons during the study period. We were unable to collect fish from the field in Thailand several times, especially in 2010-2011. Therefore, the results for the prevalence of metacercariae in 2010 may not represent the true prevalence of OLFs and HIFs (Research #1). However, from the available data it may be concluded that OLF and HIF metacercariae were found mostly in

Puntioplites falcifer and *Henicorhynchus lineatus* (Cyprinids), and *Trichogaster trichopterus* (non-Cyprinid).

In Lao PDR, the sample fish were collected in 2008-2009. OLF-HIF co-infection was found frequently (39.4% in *Barbonymus brevis*) (Research #1). In Lao PDR, we discovered one new member of the genus Opisthorchis. Previous reports have not recognized any other species of Opisthorchis, and only *O. viverrini* was reported in the lower Mekong Basin. In this research study, we reported the presence of an additional species in the lower Mekong Basin, *Opisthorchis lobatus,* which was first reported in Pakistan (Bilqees et al., 2003) (Research #2). The metacercarial stage of this parasite was found in *Channa limbata* in Vientiane Province, Lao PDR. By morphology, both their metacercariae and adult worms are quite difficult to distinguish from *O. viverrini*. This discovery will inform further studies of the true prevalence of *O. viverrini* in the region. Although this species has so far only been found in Laos, it may be distributed into neighboring areas and countries, such as Thailand, Cambodia, and Vietnam. Moreover, whether this parasite is capable of infecting humans remains a research question for further work.

In Central Vietnam, we collected freshwater fish only once, during the period 17-20 August 2009, in Danang and Hue. Almost 100% of the fish were infected with *H. taichui*, especially the climbing perch, *Anabas testudineus*. Although 259 fish were examined, no fish was found to be infected with *O. viverrini*. In Vietnam, *O. viverrini*-endemic areas are around the central and southern parts of the country. We were unable to obtain *O. viverrini* metacercariae at this time. This may be due to: 1) the sampling period not being a period of *O. viverrini* infection in fish, 2) too few samples of fish and fish species, and 3) the sampled area being non-endemic for *O. viverrini*. To clarify the reasons for this finding, further studies of *O. viverrini* in Vietnam will be needed, with longer sampling periods and broader sampling areas.

In Cambodia (Research #1), fish were collected from two provinces, Kandal and Prey Veng, and examined from 2010. Cyprinids were the major intermediate hosts of *O. viverrini* in both provinces. However, no information was found on human *O. viverrini* infection in Cambodia (Touch *et al*., 2009). The prevalence of opisthorchiasis in Cambodia may be under-estimated. Therefore, more extensive investigations of opisthorchiasis are recommended in Cambodia. In addition, surprisingly, we were unable to detect any HIF in the fish sampled.

Research #3: we collected fish from Kandal Province in southern Cambodia during 2008-2009, to study the seasonal variation of *O. viverrini* metacercarial infection in fish. The month of March had the highest prevalence of *O. viverrini* infections, while July and August had none. These findings may be useful for predicting periods of *O. viverrini*-infected fish, so as to avoid eating fish in high infection periods. However, these data were derived from normal seasons in Cambodia, and this information may not represent the results of fish infection from abnormal seasonal periods. To know and predict *O. viverrini* infection during periods of fluctuating seasons, more data on fish infections should be collected continuously for at least 5 years, together with the level of water and all conditions related to fish infection by *O. viverrini*.

Research #4 studied *O. viverrini* population structure in the lower Mekong Basin. The study was based on the hypothesis that *O. viverrini* has sufficiently high genetic variation for separation into different types or complex species, which might be related to the severity of opisthorchiasis and CCA (Sripa, 2003). We collected *O. viverrini* metacercariae from 6 geographical localities and infected hamsters to obtain adult worms. The adult worms were confirmed morphologically as being *O. viverrini* before use. The results of the study using the nad1 gene as a DNA marker revealed that *O. viverrini* sensu lato, in the lower Mekong Basin, was not a species complex; their genetic background had a monophyletic relationship. No geographical barrier or other factor appeared to limit gene flow among these populations of *O. viverrini* (Thaenkham *et al*., 2010a). If no species complex within *O. viverrini* was present in this region, the variability of patients' symptoms may be due to other factors, such as traditional-fermented foods (Pla La and Pla Som), and the numbers of treatments, which also cause CCA to develop (Sripa, 2003). However, this hypothesis might require another marker to confirm these suspicions, and this will require further work.

Research #5 studied the population structure of *H. taichui* among 8 populations--Thai (4 populations), Lao (3 populations), and Vietnamese (1 population). The results showed high degrees of genetic differentiation among the *H. taichui* populations in these three countries. Within populations, genetic variation was low, while between/among populations, it was high. This means that the *H. taichui* populations might not be influenced by gene flow, like the *O. viverrini* population (Manuscript in preparation). However, the genetics of the three *H. taichui* populations in Vietnam showed high variation, compared with populations in Thailand and Lao

PDR. This might because of the local geography, which is mountainous, and where the rivers have few tributaries, and branches do not interconnect (Dung *et al*., 2012). In contrast, Thailand and Laos share the Mekong River border, with many interconnecting channels.

Research #6: morphologically, HIFs are quite similar, and the diagnostic characteristics used to classify them are not clear. Therefore, we sought to clarify the classification characteristics for use in classical taxonomy, by analyzing and combining morphological and molecular characteristics. The results revealed that the number of testes should be the main characteristic for classifying HIF species (Thaenkham *et al*., 2010b). This knowledge may help develop the systematics of the Family Heterophyidae.

Research #7: molecular diagnosis was developed to discriminate among OLFs and HIFs, *O. viverrini, Clonorchis sinensis, H. pumilio*, and *H. taichui*, using the ribosomal DNA ITS regions as a DNA marker. This study was a pilot project, to improve the sensitivity and specificity of OLF-HIF molecular diagnosis. The results showed that the method developed might be applicable for use with stool samples in the field (Sato *et al*., 2009, 2010).

All of the research projects undertaken were completed. Research #2, 4, 6, and 7, were published in international journals. Research #5 was divided into 2 parts; the part studying the *H. taichui* population in Vietnam has been published (Dung *et al*., 2012). For the other part, the population structure of *H. taichui* in the GMS (overview), we are preparing the results for a manuscript for publication. For Research #1 and 2, we have already published a paper on the discovery of *O. viverrini* in Cambodia. The information on the other countries has already been compiled, and we will submit a manuscript to an international journal in the near future, for publication.

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OUTPUT

1. Publications submitted to international journals

1.1. Sato M, Pongvonsa T, Sanguakiat S, Yoonuan T, Dekumyoy P, Kalambaheti T, Keomoungkhoun M, Phimmayoi I, Boupha B, Moji K, Waikagul J. 2010. Copro-DNA diagnosis of *Opisthorchis viverrini* and *Haplorchis taichui* infection in endemic area of Lao PDR. Southeast Asian J Trop Med Public Health 41: 28-35.

1.2. Thaenkham U, Nuamtanong S, Sa-nguankiat S, Yoonuan T, Touch S, Manivong K, Vonghachack Y, Sato M, Waikagul J. 2010a. Monophyly of *Opisthorchis viverrini* populations in the lower Mekong Basin, using mitochondrial DNA nad1 gene as the marker. Prasitol Int 59: 242-247.

1.3. Thaenkham U, Dekumyoy P, Komalamisra, Sato M, Dung DT, Waikagul J. 2010. Systematics of subfamily Haplorchiinae (Trematoda: Heterophyidae), based on nuclear ribosomal DNA genes and ITS region. Parasitol Int 59: 460-465.

1.4. Thaenkham U, Nuamtanong S, Vonghachack Y, Yoonuan T, Sanguankiat S, Dekumyoy P, Prommasack B, Kobayashi J, and Waikagul J. 2012. Discovery of *Opisthorchis lobatus* (Trematoda: Opisthorchiidae): New recorded species of small liver flukes in the Greater Mekong Sub-region. J Parasitol 2012 (In press).

1.5. Dung DT, Hop NT, Thaenkham U, Waikagul J. 2012. Genetic differences among Vietnamese *Haplorchis taichui* in Vietnam. J Helminthol (In press).

1.6. Population structure and dynamics of *Haplorchis taichui* in the GMS (preparing manuscript)

1.7. Variations in *Opisthorchis viverrini* metacercarial infection in Cyprinid fish (preparing manuscript)

2. The utility of basic knowledge

The information of prevalence of HIFs and OLFs in endemic areas, biodiversity of *Opisthorchis* spp., and population structure of both group of parasites will be use as information to effective control program. The knowledge of seasonal variation of *O. viverrini* infection will be useful for surveillance of *O. viverrini* infection. The knowledge in population structure and genetic variation of *O. viverrini* will be useful to plan the further work about the risk factor of CCA development.

Appendices

Attached hard copies of reprint or accepted manuscript