TITLE: Phylogenetic relationships within the Opisthorchis viverrini species complex with specific analysis of O. viverrini sensu lato from Sakon Nakhon, Thailand by mitochondrial and nuclear DNA sequencing

AUTHORS: Pitaksakulrat, O; Webster, B L; Webster, J P; Laha, T; Saijuntha, W; Lamberton, P H L; Kiatsopit, N; Andrews, R H; Petney, T N; Sithithaworn, P

JOURNAL: Infection, Genetics and Evolution

PUBLISHER: Elsevier

PUBLICATION DATE: 19 April 2018 (online)

DOI: 10.1016/j.meegid.2018.04.022

Phylogenetic relationships within the *Opisthorchis viverrini* species complex with specific analysis of *O. viverrini* sensu lato from Sakon Nakhon, Thailand by mitochondrial and nuclear DNA sequencing

Opal Pitaksakulrat[a,b,c], Bonnie L. Webster[d,e], Joanne P. Webster[f,g], Thewarach Laha[a], Weerachai Saijuntha[h], Poppy H.L. Lamberton[i], Nadda Kiatsopit[a,b,j], Ross H. Andrews[b,f,j], Trevor N. Petney[j,k], Paiboon Sithithaworn[a,b,j,*]

[a] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[b] Liver Fluke and Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[c] Faculty of Veterinary Science, Prince of Songkla University, Songkhla 90110, Thailand

[d] Department of Life Sciences, Parasites and Vectors Division, Natural History Museum, London SW7 5BD, UK

[e] The London Centre for Neglected Tropical Disease Research (LCNTDR), Imperial College, Faculty of Medicine (St Mary's Campus), Norfolk Place, London W2 1PG, UK

[f] Department of Infectious Disease Epidemiology, Imperial College, Faculty of Medicine (St Mary's Campus), Norfolk Place, London W2 1PG, UK

[g] Department of Pathology and Pathogen Biology, Centre for Emerging, Endemic and Exotic Diseases (CEEED), Royal Veterinary College, University of London, Hertfordshire, London AL9 7TA, UK

[h] Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150

[i] Institute of Biodiversity, Animal Health and Comparative Medicine, and Wellcome Centre for Molecular Parasitology, Graham Kerr Building, The University of Glasgow, Glasgow G12 8QQ, UK

[j] Cholangiocarcinoma Screening and Care Program, (CASCAP,) Khon Kaen University, 40002 Thailand

[k] Institute of Zoology 1: Ecology and Parasitology, Karlsruhe Institute of Technology, Kornblumen Strasse 13, Karlsruhe, Germany

***** Corresponding author at: Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Tel.: +66 43348387; fax: +66 43202475.

E-mail addresses: paibsit@gmail.com, paib_sit@kku.ac.th (P. Sithithaworn).

Abstract

The liver fluke *Opisthorchis viverrini* sensu lato causes serious public-health problems in northeast Thailand and Southeast Asian countries. A hypothesis has been proposed that *O. viverrini* represents a species complex with varying levels of genetic differentiation in Thailand and Lao PDR. This study aimed to clarify whether *O. viverrini* populations can be genetically divided into separate taxa. We collected *O. viverrini* s.l. from eight different locations in Lao PDR and Thailand. The results of *nad1, cox1*, CF-int6, Pm-int9, ITS2 and 28S rDNA sequence analysis revealed that sub-structuring occurred between the eight populations. We found that *O. viverrini* s.l. from Sakon Nakhon (SK), Thailand, shows significant genetic differentiation ($P < 0.05$) from all other isolates from different localities in Thailand and Lao PDR. This was supported by haplotype and phylogenetic tree analyses in which the SK isolate was separated from all other isolates. This suggests that *O. viverrini* s.l. from SK is a cryptic species. The data, however, also confirm the association between genetic groups of *O. viverrini* s.l. and specific wetland systems, and raise important questions regarding the epidemiological significance of these genetic differences.

Keywords: *Opisthorchis viverrini* sensu lato, mitochondrial gene, nuclear DNA gene, phylogenetics

1. Introduction

Opisthorchis viverrini is the most significant food-borne trematodes endemic in southeast Asian countries bordering the Mekong River: Thailand, Lao People's Democratic Republic (Lao PDR), Cambodia, and Vietnam (Andrews et al., 2008). An estimated 10 million people are infected with the majority living in Thailand, although data from the other affected countries are very limited (Andrews et al., 2008; Sithithaworn et al., 2012a; WHO, 1995). *O. viverrini* has a three-host life-cycle which includes freshwater snails (*Bithynia* species) and cyprinid fish as first and second intermediate hosts, respectively. Fish eating carnivores (animals and humans) become definitive hosts when they are infected by ingestion of viable cysts (metacercariae) which occur in the flesh of raw, inadequately cooked, or processed fish (Andrews et al., 2008; Kaewkes, 2003). Infection with *O. viverrini* causes opisthorchiasis which can result in bile duct cancer (cholangiocarcinoma, CCA), a major medical problem in endemic areas especially in northeast Thailand (IARC, 2011; Sithithaworn et al., 2014). There is considerable geographical, local and regional variation in the disease epidemiology of opisthorchiasis and of CCA. Several genetic studies have shown that *O. viverrini* represents a species complex with distinct geographical genetic differentiation appearing between certain populations in Thailand and Lao (PDR) (Ando et al., 2001; Saijuntha et al., 2007; Sithithaworn et al., 2007), which could correspond to differences in the epidemiology *O. viverrini* infection. It has been hypothesized that this could be related to host factors and/or to biological and genetic variation in the parasite populations which comprises currently morphologically similar but genetically very distinct 'cryptic' species (Sithithaworn et al., 2012b). Here we use a multi-locus genetic analysis, involving both mitochondrial and nuclear markers, with the aim to further resolve the *O. viverrini* species complex in Thailand and Lao (PDR).

2. Materials and Methods

2.1 Sample collection and generation

Metacercariae of *O. viverrini* were obtained from naturally infected cyprinid fish caught in eight different geographical sampling areas in Thailand and Lao PDR with details of sampling localities and fish samples (Table 1, Fig. 1). Fish samples were processed for *O. viverrini* using the pepsin A digestion technique (Sithithaworn et al., 1997). Three hamsters were infected by intragastric intubation with 50 metacercariae for each of the eight geographical areas. Four months after infection, the hamsters were euthanized and adult *O. viverrini* worms were recovered from their bile ducts, washed extensively in physiological saline, placed into micro centrifuge tubes containing small amount of PBS and stored frozen at –20 °C for molecular analyses. All animal experimentation was approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 74/2555).

2.2 Molecular characterisation

Nine individual worms (three from each of the three hamsters) from each of the eight sampling localities were molecularly characterised. Genomic DNA (gDNA) was extracted from each individual worm using the DNeasy blood and tissue kit (QIAGEN Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions, and gDNA was eluted in a total of 100 µl. Six different DNA regions (four nuclear, the ITS2 and 28S rDNA regions, and Pm-int9 and CF-int6 coding regions and two mitochondrial gene regions, *cox1* and *nad1*) were amplified by PCR from each individual worm using the primers detailed in Table 2.

For each DNA region PCR's were performed in a total reaction volume of 25 µl using illustraTM puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK), 10 pmols of each primer and 2 µl (~ 50 ng) of genomic DNA. Thermal cycling was performed in a Perkin Elmer 9600 Thermal Cycler and the thermal cycling conditions were 95 °C for 5 min,

40 cycles of 95 °C for 30 sec, Tm °C (ref. to Table 2) for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Four µl of each PCR product was run and visualized on a 1.5% gel-red agarose gel. Positive PCRs were purified using the QIAquick PCR purification Kit (Qiagen Ltd, UK) and then sequenced on a 3730XL 96 capillary automated sequencer (Applied Biosystems, UK) using dilutions of the original PCR primers.

2.3 Data analysis

All sequences for the different DNA regions were assembled and manually edited using Sequencher v4.6 (http://genecodes.com) to remove any ambiguities between forward and reverse strands. For each sample consensus sequences were aligned in Sequencher and polymorphic positions observed between individuals were checked and confirmed by visualisation of the original sequence chromatograms. The identity (species and gene) of the sequence was also confirmed using the Basic Local Alignment Search Tool (NCBI-Blast). For each DNA region consensus sequences from each individual sample were grouped and aligned in MacClade v4.05 and then collapsed together using Collapse v1.2 (http://darwin.uvigo.es/software/collapse.html) to identify individual samples with identical sequences. Each group of identical sequences and also any unique sequences became unique haplotypes and consensus sequences were created and given a unique haplotype number. The numbers of individuals that presented each haplotype in each locality were also recorded.

2.4 Sequence analysis of cox1, nad1, Pm-int9 *and* CF-int6 *DNA regions*

2.4.1 Haplotype analysis

To estimate genealogical relationships between haplotypes, the individual haplotype sequences were aligned in MacClade v4.05 and then a minimum spanning network was created in the programme TCS (http://darwin.uvigo.es/software/tcs.html).

2.4.2 Phylogenetic analysis

For each DNA region all haplotype sequences were aligned in MacClade v4.05 and exported into Mega v5 (Tamura et al., 2011). Evolutionary relationships between the haplotypes were inferred using the Neighbour-Joining methods. The relative support for clades was determined by using 1,000 bootstrap replicates. The numbers of variable and parsimony informative sites of each DNA sequence were also analyzed. The topologies were rooted by out-group sequences available from Genbank. For *nad1* gene; *Opisthorchis felineus* (EU921260.2), *Clonorchis sinensis* (FJ381664.2), *Taenia solium* (AF338826.1), *T. asiatica* (NC004826.2) and *Ascaris suum* (NC001327.1). For *cox1*; *O. felineus* (DQ469316.1 and DQ469317.1), *C. sinensis* (AF181889.3), *T. solium* (AB271234.1), *T. asiatica* (NC004826.2), *T. saginata* (AB107245.1), *Schistosoma japonicum* (EU340353.1) and *A. suum* (NC001327.1). For Pm-int9; *O. felineus* (AF311774.1), *C. sinensis* (EF071860.1), *T. solium* (AY034087.1) and *A. suum* (AEUI02001283.1). No out-group was available for CF-int6. Genetic structure and fixation indices were calculated using Arlequin v3.5 (Excoffier et al., 1992).

3. Results

3.1 Mitochondrial DNA

3.1.1 Cox1 *analysis*

A total of 23 *cox1* sequences from the eight geographical localities were analyzed for genetic variation. The *cox1* sequence (705-bp) showed 11 haplotypes and contained 23 variable sites (14 parsimony informative). Haplotype 3 had the highest frequency, while haplotypes 1, 2, 4 and 7 were unique (Table 3).

The haplotypes separated into two distinct groups (Fig. 2). One generated from haplotype 9 and haplotype 11 which are only found in SK (Thailand), and the other

group with all the *O. viverrini* samples from all the sampling localities (Thailand and Lao) and excluding SK.

3.1.2 Nad1 *analysis*

A total of 24 *nad1* sequences were analyzed together with six *O. viverrini* sequences from different geographical localities from Thailand and Lao PDR available from Genbank: Thailand ; Sakaeo-SK5 (GQ401100), Cambodia (Kandal-KD12 (GQ401093) and Lao PDR; Vientiane-VT13 (GQ401037), Khammoune-KM1 (GQ401040), Savannakhet-SV14 (GQ401062), Champasak-CP18 (GQ401081) (Thaenkam et al., 2010). Within the 713 DNA region, 31 variable sites (18 parsimony informative) and 18 haplotypes were observed. Haplotypes 4 and 5 had the highest frequencies, while haplotypes 1, 2, 3, 7, 8, 10, 11, 13, 16, 17 and 18 were unique (Table 4).

Again the 18 haplotypes were separated into two distinct groups. One group contained haplotypes 12 and 13 from SK (Thailand) and the other group contain the rest haplotypes included in the analysis (Fig. 3). The AMOVA revealed that the main genetic variation of the fluke (58.6%) were among the population within the group (country) although genetic differentiation were significant at population and country (F_{ST} = 0.49, F_{SC} = 0.53, $P < 0.001$) (Table 5).

3.2 Nuclear DNA

3.2.1 CF-int6 analysis

A total of 23 CF-int6 sequences from eight geographical localities were analyzed. The CF-int6 sequence (709-bp) showed 4 haplotypes and contained 11 variable sites. Haplotype 2 had the highest frequency, while haplotype 4 was unique (Table 6).

The four haplotypes separated into two distinct groups. One clade was generated from haplotype 3 (SK, Thailand) and another group from all the other isolates (Fig. 4).

3.2.2 Pm-int9 analysis

The 24 Pm-int9 sequence (174-bp) contained only 4 variable sites, which resolved in 2 haplotypes. Haplotype 1 had the highest frequency with 87.5% and was found all sampling localities except SK which again formed a separate group (Table 7, Fig. 5).

3.3 ITS2 and 28S rDNA analysis

All ITS2 and 28S rDNA sequences from all the *O. viverrini* samples were identical.

4. Discussion

By multi sequencing analyses of 6 DNA regions, the nucleotide sequences of 4 regions, *nad1*, *cox1*, Pm-int9 and CF-int6, revealed highly significant genetic variation between the eight isolates of *O. viverrini* s.l. from different geographic origins in Thailand and Lao PDR. In this study 3 isolates from RE, SK and SSK from Thailand included in the analysis for the first time. Data from phylogenetic tree and haplotype network analyses all showed consistent and distinct separation of SK from all other *O. viverrini* in Thailand and Lao PDR. This finding confirmed the existence of a new and second cryptic species within the *O. viverrini* species complex for Sakon Nakhon, as proposed previously (Kiatsopit et al., 2011; Laoprom et al., 2009; Saijuntha et al., 2007). It is interesting to note that Saijuntha et al. (2007) reported that *O. viverrini* from Sakon Nakhon (SK) and Nakhon Phanom (NP) from Thailand were grouped in the same cluster with those from Lao PDR (Nam Ngum River wetland and the Sae Bang Heang River wetland, as well as TL analyzed in this study) and did not share the same cluster with the other populations from Thailand. Our study confirms that the SK isolate from the Songkram River wetland in Thailand differs from other specific wetlands along the Chi (KBp, KLp) and Mun (SSK) River wetlands in Thailand and the Nam Ngum River (TL, NK) wetlands in Lao PDR.

While both *nad1* and *cox1* sequences separated SK from the rest of the 7 populations from Thailand and Lao PDR, two previous reports, based on a similar mitochondrial DNA region,

failed to detect a separation between different geographical population of *O. viverrini* from Thailand, Lao PDR (including *O. viverrini* from Savannakhet) and Cambodia (Saijuntha et al., 2008; Thaenkham et al., 2010). The observed discrepancy may stem from a different set of parasite was isolated from specific geographical locality, temporal as well as seasonal factors (Sithithaworn et al., 2012b). Indeed, the recent discoveries of *Opisthorchis*-like worms, which have similar morphology to *O. viverrini*, have made genetic as well as phenotypic analysis necessary in order to understand its systematic and phylogenetic relationship (Dao et al, 2013; Nawa et al, 2015; Thaenkham et al., 2010).

For nuclear DNA, a Pm-int9 sequence analyses indicated a similar pattern of haplotype network and phylogenetic relationship of SK to other *O. viverrini* isolates by mitochondrial DNA. It also provided evidence that *O. felineus* and *C. sinensis* are in the same cluster and separated from *O. viverrini*. A previous report by Shekhovtsov et al. (2009) showed low intraspecific variation in *O. felineus* and *C. sinensis*, as well as in *O. viverrini*, and suggested that *C. sinensis* should be considered the sister species of *O. viverrini*. The reason for the different results between the current and previous report (Shekhovtsov et al., 2009) is not known but it may be due to several possibilities. For example, the sample population, localities as well as the target DNA region analyzed differ. By geographical distribution, the boundary distribution between species, i.e. *C. sinensis* and *O. felineus* in part of China and Russia (Mordvinov et al., 2012), may facilitate the gene flow between species. Thus future analysis of worm samples from the species boundary between the two human liver fluke species would help to clarify their phylogenetic relationship.

A novel nuclear DNA sequence used in the current study is the cathepsin F (CF-int6) region which plays an essential role in the physiology of helminth parasites, such as in the production of excretory-secretary molecules which are implicated in pathogenesis within the host, i.e. inflammation surrounding the parasite. The CF-int6 product has important

biological functions such as the digestion of ingested material (host cells or tissue) (Pinlaor et al., 2009; Sripa et al., 2010). In *C. sinensis*, this gene is more conserved when compare with other trematodes (Kang et al., 2004). Whether the CF-in6 sequence variations have implications in parasite biology, morphology and require further study.

Habitat heterogeneity may influence parasite transmission between different host species, particularly the *Bithynia* snail intermediate hosts (Wang et al., 2015). The habitat type for SK in the previous report was Nong Harn Reservoir, the largest water reservoir located in the Meung district, Sakon Nakhon Province northeast Thailand, while the SK locality in the current study is a rice paddy field in Phang Khon district, Sakon Nakhon province. These sites are 54 km apart. SK was separated from other populations examined in this study by varying distances ranging from 116 to 310 km. The largest distance from the current SK locality is SSK in Srisaket province, in the southern part of northeast Thailand. As a rice paddy wetland habitat in SK, it systematically received irrigated water supplied through a canal network from the Nam Un Dam. This dam receives water from the Nam Un river originating in the Phu Phan Mountain in Sakon Nakhon province. This water head also serves as the origin of the Songkram River. Due to the irrigation system from Nam Un dam, these cultivation wetlands are able to produce two crops a year compared to one crop per year for rain fed paddy fields. The irrigated water from Nam Un dam finally joins with Songkram River before it drains into the Mekong River in Nakorn Phanon province. The rice paddy environment in SK is known to allow active transmission from human and animal reservoir hosts to *Bithynia goniomphalos siamensis* (Kiatsopit et al., 2012; Kopolrat et al., 2016; Namsanor et al., 2015). Whether different habitat types and/or other environmental attributes such as snail (Kiatsopit et al., 2013), fish and human hosts are responsible to the occurrence of this cryptic species in Sakon Nakhon is not clear. Our previous study suggested that distant separation, especially macro-scale separation (up to

770 km), is an important factor for population structure (Laoprom et al., 2012). But population structure can be observed in spatially closer populations of *O. viverrini* (10–60 km separation), which is comparable to the *O. viverrini* sampling localities in Sakon Nakhon in this study. The genetic diversity may be a consequence of selection against specific genotype of parasite by different host species in the parasite's life cycle. The snail intermediate host, which shows high levels of genetic diversity (Kiatsopit et al., 2013), and the possibility of co-evolution between *O. viverrini* and *Bithynia* may play role in the observed genetic and biological variation in of the parasite.

In this study, a novel sequence marker for the sixth intron of the CF gene (CF-int6) was developed. The CF-int6 sequence analysis revealed the separation of the SK population from other populations from Thailand and Lao PDR, similar to mitochondrial and other nuclear DNA sequencings.

Further study is needed to reaffirm the genetic variation and the occurrence of the new cryptic species observed in this study using a population genetics approach such that population structure and gene flow can be examined. In addition, the biological importance of the distinct SK isolate should be delineated, as reported previously by Laoprom et al. (2009, 2010). The potential weakness of the current study is that we relied on analyses of adult *O. viverrini* harvested from laboratory animals (hamsters), which may be subjected to host selection bias. To avoid such an effect, direct analysis of parasite life stages such as metacercaria is desirable and is now being done using a more comprehensive set of microsatellite DNA as genetic markers.

In conclusion, by using a multi-gene sequence approach (phylogenetic and haplotyping network), based on sequences of mitochondrial DNA (*nad1* and *cox1*) and nuclear DNA (Pm-int9 and CF-int6), we provide clear evidence of the new *O. viverrini* cryptic species from SK locality in Sakon Nakhon compared with other

isolates from northeast Thailand and Lao PDR. The detection of this new cryptic species confirmed that there are at least two cryptic species within the *O. viverrini* species complex in Sakon Nakhon and Nakon Phanom belonging to the Songkram River Wetland in northeast Thailand. The new SK population came from an irrigated rice paddy wetland where active transmission of *O. viverrni* in the snail intermediate host has been reported (Kiatsopit et al., 2012; Namsanor et al., 2015). Currently it is not clear whether habitat ecology, connectivity and/or land use that encourages snail host population growth or a history of chemotherapeutic control by praziquantel has affected the genetic difference of the new SK isolate. Although more evidence is still needed, previous population genetic data for inbreeding or self-fertilisation in *O. viverrini* (Laoprom et al., 2012) suggested that the principle of "allopatric speciation" is a plausible explanation.

The findings in this study provide further support for the previous studies (Laoprom et al., 2012; Saijuntha et al., 2007) which hypothesize that SK contains a cryptic species of *O. viverrini*. The observed data in this study have led to a better understanding not only of the genetic variation present in this pathogenic liver fluke, but they may also shed light on the cause of the observed heterogeneity in prevalence, intensity and disease presentation (opisthorchiasis), as well as the varying incidences of cholangiocarcinoma within and between different geographical areas and specific wetlands in the Mekong Sub-region.

Acknowledgements

This research was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (grant PHD/0210/2550 to O.P.), and Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through health cluster (SHeP-GMS). We acknowledge the support of the Faculty of Medicine, Khon Kaen University Visiting

International Professor Program. We also wish to thank the Office of Research Affairs and the Invitation Research fund from faculty of Medicine, Khon Kaen University, Thailand, and the Deutsche Forschungsgemeinschaft (grant PE1611/1–3) for the assistance. We acknowledge the support of the Cholangiocarcinoma Screening and Care Program, (CASCAP) Khon Kaen University, 40002, Thailand.

Reference

Ando, K., Sithithaworn, P., Nuchjungreed, C., Tesana, S., Srisawangwong, T., Limviroj, W., Chinzei, Y., 2001. Nucleotide sequence of mitochondrial CO I and ribosomal ITS II genes of *Opisthorchis viverrini* in northeast Thailand. Southeast Asian J. Trop. Med. Public Health 32 Suppl 2, 17–22.

Andrews, R.H., Sithithaworn, P., Petney, T.N., 2008. *Opisthorchis viverrini*: an underestimated parasite in world health. Trends Parasitol. 24, 497–501.

Barker, S.C., Blair, D., 1996. Molecular phylogeny of *Schistosoma* species supports traditional groupings within the genus. J. Parasitol. 82, 292–298.

Bowles, J., Blair, D., McManus, D.P., 1995. A molecular phylogeny of the human schistosomes. Mol. Phylogenet. Evol. 4, 103–109.

Dao, T.H., Nguyen, T.G., Victor, B., Gabriël, S., Dorny, P., 2014. *Opisthorchis viverrini*-like liver fluke in birds from Vietnam: morphological variability and rDNA/mtDNA sequence confirmation. J. Helminthol. 88, 441–446.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

IARC, 2011. IARC monographs on the evaluation of carcinogenic risks to humans. World Health Organization, International Agency for Research on Cancer, Lyon.

Kaewkes, S., 2003. Taxonomy and biology of liver flukes. Acta Trop. 88, 177-186.

Kang, T.H., Yun, D.H., Lee, E.H., Chung, Y.B., Bae, Y.A., Chung, J.Y., Kang, I., Kim, J., Cho, S.Y., Kong, Y., 2004. A cathepsin F of adult *Clonorchis sinensis* and its phylogenetic conservation in trematodes. Parasitology 128, 195–207.

Kiatsopit, N., Sithithaworn, P., Saijuntha, W., Boonmars, T., Tesana, S., Sithithaworn, J., Petney, T.N., Andrews, R.H., 2012. Exceptionally high prevalence of infection of *Bithynia siamensis goniomphalos* with *Opisthorchis viverrini* cercariae in different wetlands in Thailand and Lao PDR. Am. J. Trop. Med. Hyg. 86, 464–469.

Kiatsopit, N., Sithithaworn, P., Saijuntha, W., Petney, T.N., Andrews, R.H., 2013. *Opisthorchis viverrini*: Implications of the systematics of first intermediate hosts, *Bithynia* snail species in Thailand and Lao PDR. Infect. Genet. Evol. 14, 313–319.

Kiatsopit, N., Sithithaworn, P., Sithithaworn, J., Boonmars, T., Tesana, S., Pitaksakulrat, O., Saijuntha, W., Petney, T.N., Andrews, R.H., 2011. Genetic relationships within the *Opisthorchis viverrini* species complex with specific analysis of *O. viverrini* from Savannakhet, Lao PDR by multilocus enzyme electrophoresis. Parasitol. Res. 108, 211–217.

Kopolrat, K., Sithithaworn, P., Kiatsopit, N., Pitaksakulrat, O., Tesana, S., Andrews, R.H., Petney, T.N., 2016. Comparison of infectivity, metacercarial burden and host mortality induced by *Opisthorchis viverrini* sensu lato cercariae from Lao PDR compared with Thailand in cyprinid fish, *Barbonymus gonionotus*. Trans. R. Soc. Trop. Med. Hyg. 110, 46–54.

Laoprom, N., Saijuntha, W., Sithithaworn, P., Wongkham, S., Laha, T., Ando, K., Andrews, R.H., Petney, T.N., 2009. Biological variation within *Opisthorchis viverrini* sensu lato in Thailand and Lao PDR. J. Parasitol. 95, 1307–1313.

Laoprom, N., Sithithaworn, P., Ando, K., Sithithaworn, J., Wongkham, S., Laha, T., Klinbunga, S., Webster, J.P., Andrews, R.H., 2010. Microsatellite loci in the

carcinogenic liver fluke, *Opisthorchis viverrini* and their application as population genetic markers. Infect. Genet. Evol. 10, 146–153.

Laoprom, N., Sithithaworn, P., Andrews, R.H., Ando, K., Laha, T., Klinbunga, S., Webster, J.P., Petney, T.N., 2012. Population genetic structuring in *Opisthorchis viverrini* over various spatial scales in Thailand and Lao PDR. PLoS Negl. Trop. Dis. 6, e1906.

Mordvinov, V.A., Yurlova, N.I., Ogorodova, L.M., Katokhin, A.V., 2012. *Opisthorchis felineus* and *Metorchis bilis* are the main agents of liver fluke infection of humans in Russia. Parasitol. Int. 61, 25–31.

Namsanor, J., Sithithaworn, P., Kopolrat, K., Kiatsopit, N., Pitaksakulrat, O., Tesana, S., Andrews, R.H., Petney, T.N., 2015. Seasonal transmission of *Opisthorchis viverrini* sensu lato and a lecithodendriid trematode species in *Bithynia siamensis goniomphalos* snails in northeast Thailand. Am. J. Trop. Med. Hyg. 93, 87–93.

Nawa, Y., Doanh, P.N., Thaenkham, U., 2015. Is *Opisthorchis viverrini* an avian liver fluke? J. Helminthol. 89, 255–256.

Pinlaor, P., Kaewpitoon, N., Laha, T., Sripa, B., Kaewkes, S., Morales, M.E., Mann, V.H., Parriott, S.K., Suttiprapa, S., Robinson, M.W., To, J., Dalton, J.P., Loukas, A., Brindley, P.J., 2009. Cathepsin F cysteine protease of the human liver fluke, *Opisthorchis viverrini*. PLoS Negl. Trop. Dis. 3, e398.

Saijuntha, W., Sithithaworn, P., Wongkham, S., Laha, T., Chilton, N.B., Petney, T.N., Barton, M., Andrews, R.H., 2008. Mitochondrial DNA sequence variation among geographical isolates of *Opisthorchis viverrin*i in Thailand and Lao PDR, and phylogenetic relationships with other trematodes. Parasitology 135, 1479–1486.

Saijuntha, W., Sithithaworn, P., Wongkham, S., Laha, T., Pipitgool, V., Tesana, S., Chilton, N.B., Petney, T.N., Andrews, R.H., 2007. Evidence of a species complex within the food-borne trematode *Opisthorchis viverrini* and possible co-evolution with their first intermediate hosts. Int. J. Parasitol. 37, 695–703.

Shekhovtsov, S.V., Katokhin, A.V., Romanov, K.V., Besprozvannykh, V.V., Fedorov, K.P., Yurlova, N.I., Serbina, E.A., Sithithaworn, P., Kolchanov, N.A., Mordvinov, V.A., 2009. A novel nuclear marker, Pm-int9, for phylogenetic studies of *Opisthorchis felineus*, *Opisthorchis viverrini*, and *Clonorchis sinensis* (Opisthorchiidae, Trematoda). Parasitol. Res. 106, 293–297.

Sithithaworn, P., Andrews, R.H., Nguyen, V.D., Wongsaroj, T., Sinuon, M., Odermatt, P., Nawa, Y., Liang, S., Brindley, P.J., Sripa, B., 2012a. The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol. Int. 61, 10–16.

Sithithaworn, P., Andrews, R.H., Petney, T.N., Saijuntha, W., Laoprom, N., 2012b. The systematics and population genetics of *Opisthorchis viverrini* sensu lato: implications in parasite epidemiology and bile duct cancer. Parasitol. Int. 61, 32–37.

Sithithaworn, P., Nuchjungreed, C., Srisawangwong, T., Ando, K., Petney, T.N., Chilton, N.B., Andrews, R.H., 2007. Genetic variation in *Opisthorchis viverrini* (Trematoda: Opisthorchiidae) from northeast Thailand and Laos PDR based on random amplified polymorphic DNA analyses. Parasitol. Res. 100, 613–617.

Sithithaworn, P., Pipitgool, V., Srisawangwong, T., Elkins, D.B., Haswell-Elkins, M.R., 1997. Seasonal variation of *Opisthorchis viverrini* infection in cyprinoid

fish in north-east Thailand: implications for parasite control and food safety. Bull. World Health Organ. 75, 125–131.

Sithithaworn, P., Yongvanit, P., Duenngai, K., Kiatsopit, N., Pairojkul, C., 2014. Roles of liver fluke infection as risk factor for cholangiocarcinoma. J. Hepatobiliary Pancreat. Sci. 21, 301–308.

Sripa, J., Laha, T., To, J., Brindley, P.J., Sripa, B., Kaewkes, S., Dalton, J.P., Robinson, M.W., 2010. Secreted cysteine proteases of the carcinogenic liver fluke, *Opisthorchis viverrini*: regulation of cathepsin F activation by autocatalysis and trans-processing by cathepsin B. Cell. Microbiol. 12, 781–795.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

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

Wang, Y.C., Ho, R.C., Feng, C.C., Namsanor, J., Sithithaworn, P., 2015. An ecological study of Bithynia snails, the first intermediate host of *Opisthorchis viverrini* in northeast Thailand. Acta Trop. 141, 244–252.

WHO, 1995. Control of food born trematode infections. World Health Organ. Tech. Rep. Ser., 849.

Figure Legends

Fig. 1 Map show the *Opisthorchis viverrini* sampling areas including 8 localities from 4 river wetlands in Thailand and Lao PDR. Abbreviations used for locality are described in Table 1.

Fig. 2 A: Phylogenetic relationships of *O. viverrini* with the other parasitic trematodes based on a neighbor-joining analysis of cox1 nucleotide sequence data. *A. suum* (Nematoda) was used as the outgroup in the analyses. Bootstrap values (> 50%) are indicated above branches. The small table shows the members of each haplotype from Thailand and Lao PDR. **B:** The phylogenetic network of 11 haplotypes (1–11) of cox1 sequence from 8 geographically different populations from Thailand (SSK, KLP, NP, RE, KBP, SK) and Lao PDR (TL, NK). Abbreviations used for locality are described in Table 1.

Fig. 3 A: Phylogenetic relationships of *O. viverrini* with the other parasitic trematodes based on a neighbor-joining analysis of nad1 nucleotide sequence data. *A. suum* (Nematoda) was used as the outgroup in the analyses. Bootstrap values (> 50%) are indicated above branches. The table shows the members of each haplotype from Thailand and Lao PDR. **B:** The phylogenetic network of 18 haplotypes (1–18) of nad1 sequence from 14 geographically different populations from Thailand (SSK, KLP, NP, RE, KBP, SK) and Lao PDR (TL, NK) and retrieved sequences (Thaenkam et al., 2010) from NCBI database (KM, CP, KD, VT, SV). VT13-Vientiane (Accession number GQ401037), KM1-Khammoune (Accession number GQ401040), SK5-Sakaeo (Accession number GQ401100), KD12-Kandal, Cambodia (Accession number GQ401093), SV14-Savannakhet (Accession number GQ401062), CP18-Champasak (Accession number GQ401081).

Fig. 4 A: Phylogenetic relationships of *O. viverrini* with the other parasitic trematodes based on a neighbor-joininganalysis of CF-int6 nucleotide sequence data (No out groups). The small table shows the members of each haplotype from Thailand and Lao PDR. **B:** The phylogenetic network of 4 haplotypes (1–4) of CF-int6 nucleotide sequence from 8 geographically different populations from (SSK, KLP, NP, RE, KBP, SK) and Lao PDR (TL, NK) Thailand and Lao PDR. Abbreviations used for locality are described in Table 1.

Fig. 5 A: Phylogenetic relationships of *O. viverrini* with the other parasitic trematodes based on a neighbor-joining analysis of Pm-int9 nucleotide sequence data. *A. suum* (Nematoda) was used as the outgroup in the analyses. Bootstrap values (> 50%) areindicated above branches. The small table shows the members of each haplotype from Thailand and Lao PDR. **B:** The phylogenetic network of 3 haplotypes (1–3) of Pm-int9 nucleotide sequence from 8 geographical different populations from Thailand and Lao PDR and *O. felineus*. Abbreviations used for locality are described in Table 1.

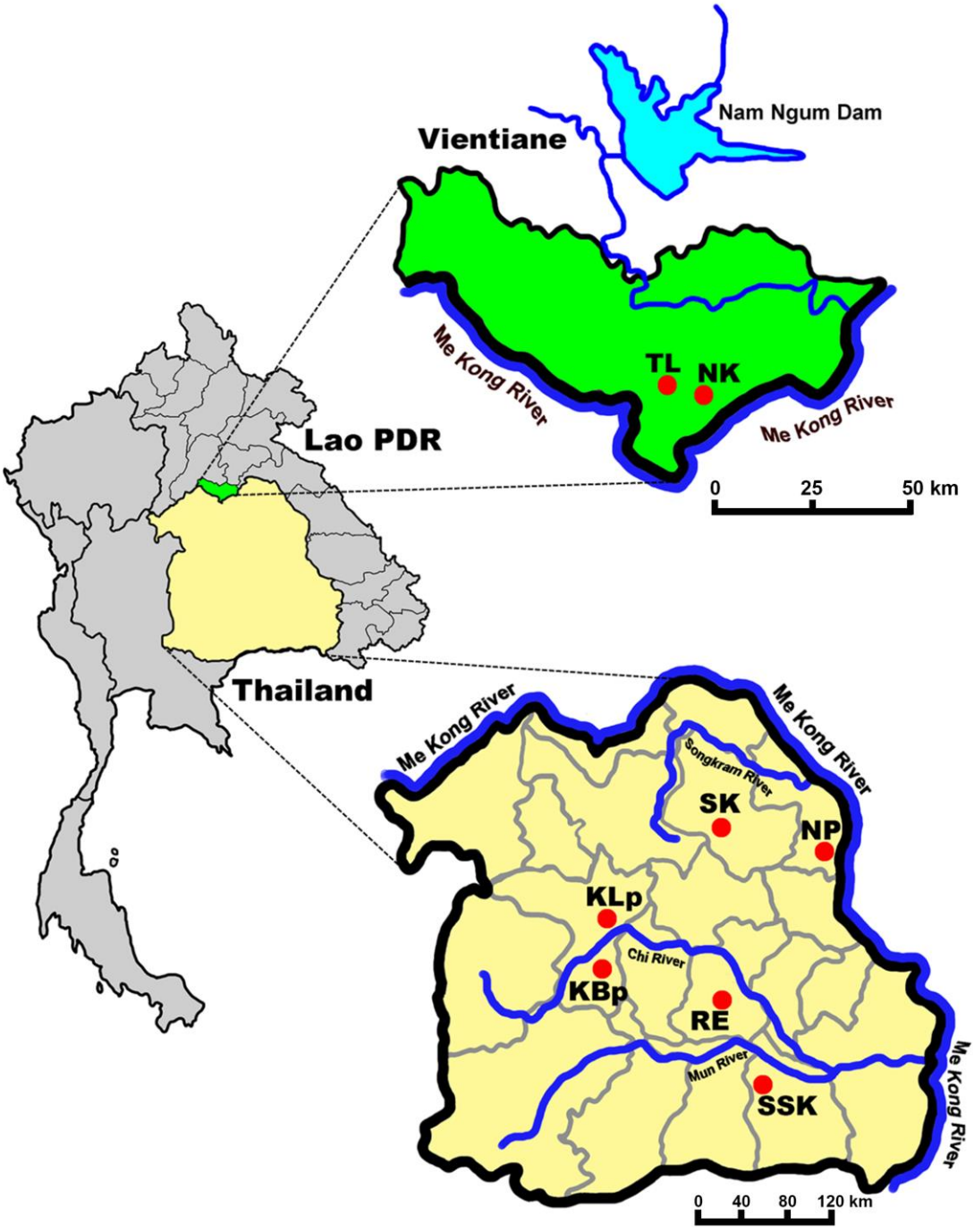


Fig. 1

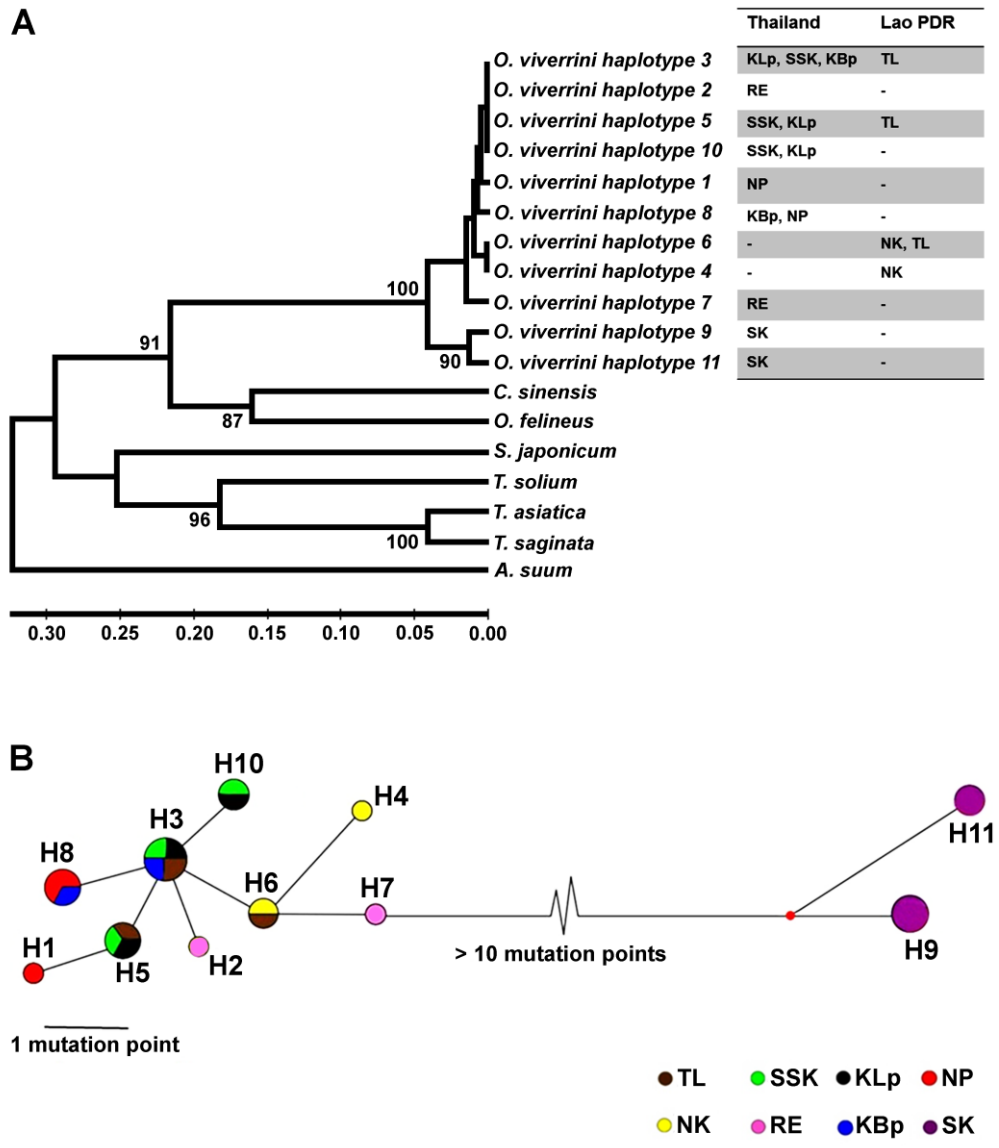


Fig. 2

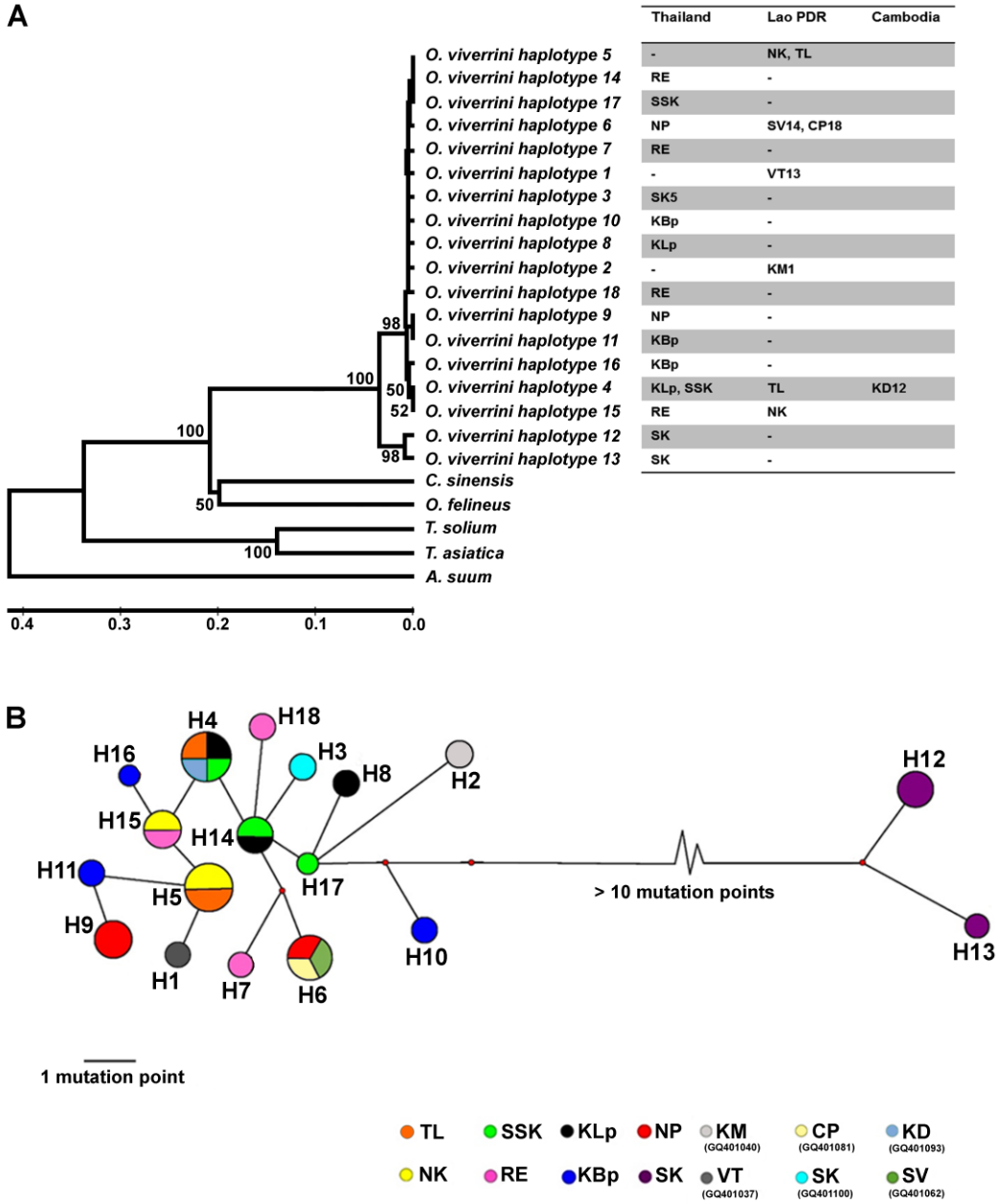


Fig. 3

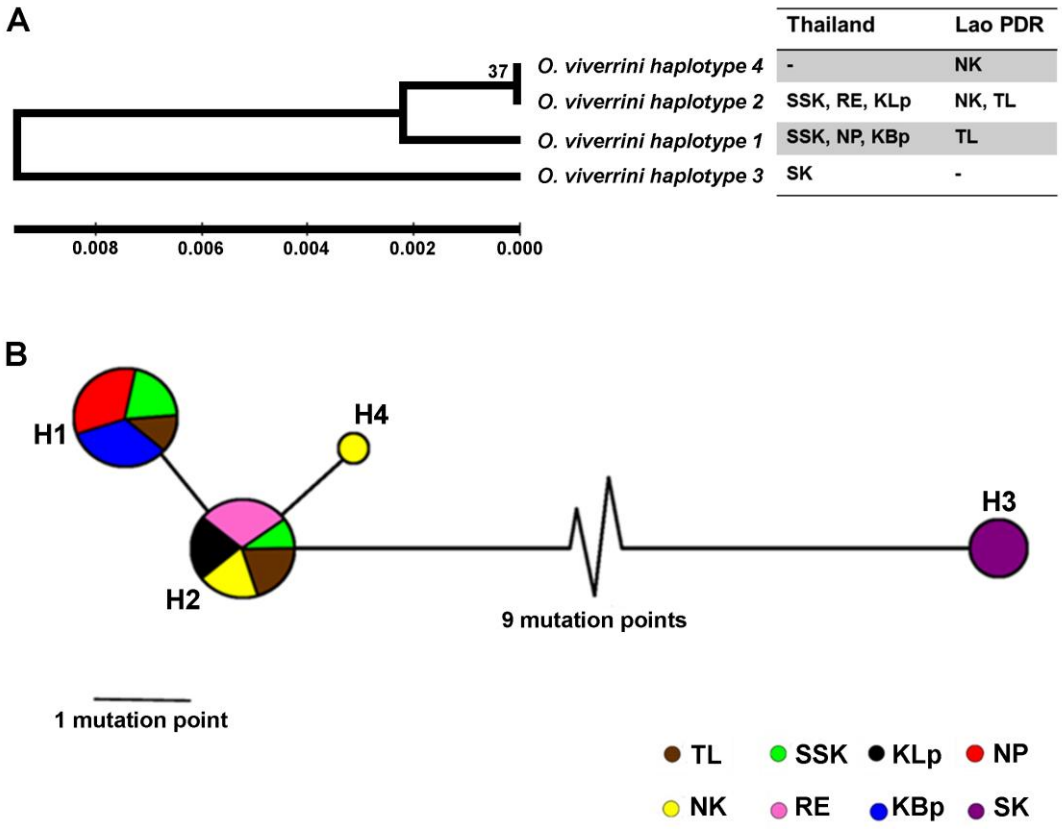


Fig. 4

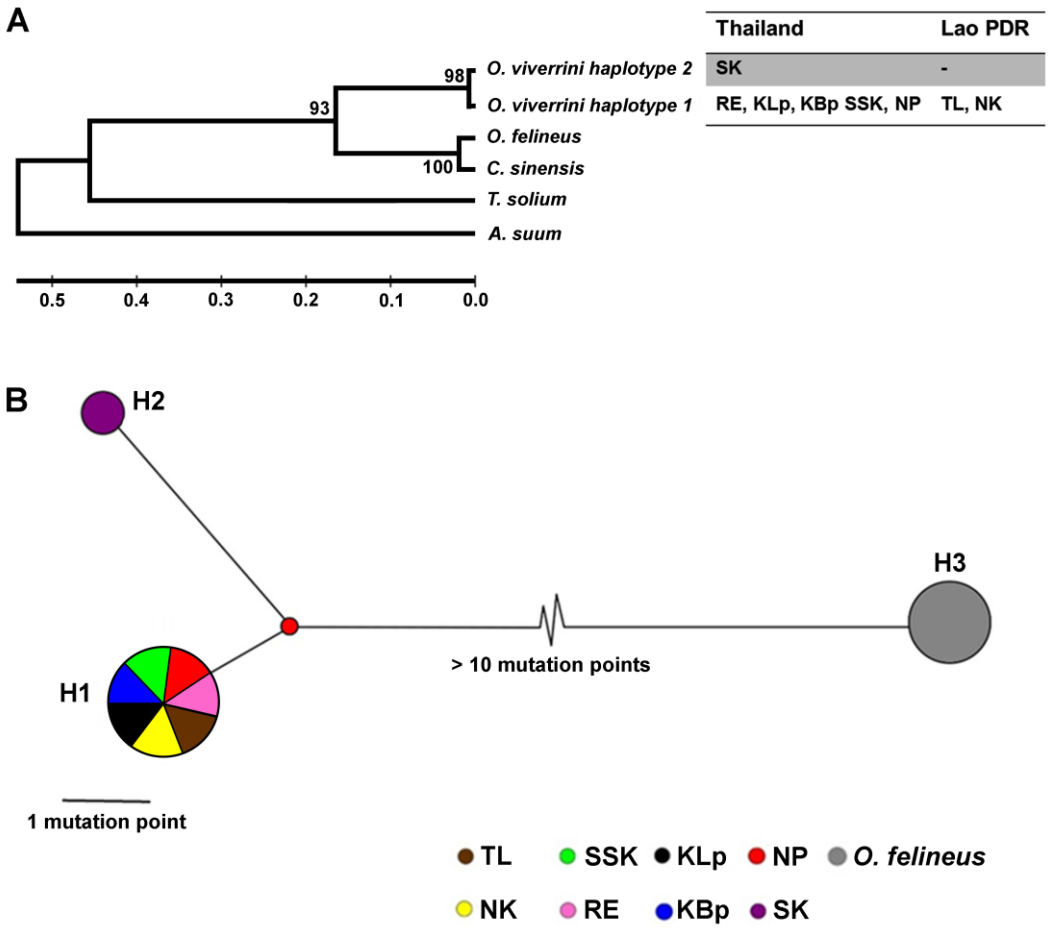


Fig. 5

Table 1 Details of the *Opisthorchis viverrini* and fish sampling areas including 8 localities from 4 river wetlands in Thailand and Lao PDR. Data shown were details of countries, wetlands, province, district, coordinate, distance from Khon Kaen, fish species, number and weight of fish.

Country	Wetland	Province	District	Code	Coordinate	Distance from Mueang District, Khon Kaen Province (km)	Fish species	No. of fish	Weight (kg)
Lao PDR	Num Ngum (NG)	Vientiane	That Luang	TL	17°58.526' N, 102°38.908' E	200	*Cyclocheilichthys armatus*	255	1.05
	Num Ngum (NG)	Vientiane	Ban Na Kluay	NK	17°57.502' N, 102°42.306' E	194	*C. armatus*	122	0.7
Thailand	Chi (CR)	Khon Kaen	Ban Phai	KBp	16°06.533' N, 102°40.567' E	47.6	*C. armatus*	64	1.2
	Chi (CR)	Khon Kaen	Ban Lerngpluey	KLp	16°25.808' N, 102°52.782' E	7	*C. armatus*	156	1.6
	Chi (CR)	Roi Et	Mueang	RE	16°01.745' N, 103°4	128	*Barbonymus gonionotu*	55	2

				0.223' E		*s*		
Songkram (SR)	Nakhon Phanom	Renu Nakhon	NP	17°02.603' N, 104°36.155' E	322	*C. armatus*	17 5	2
Songkram (SR)	Sakon Nakhon	Phang Khon	SK	17°22.360' N, 103°47.267' E	207	*C. armatus*	82	1.4
Mun (MR)	Sri Sa Ket	Mueang	SS K	15°05.935' N, 104°19.898' E	301	*B. gonionotus*	30	1

Table 2 PCR primers from the different DNA regions.

Gene	Primers	Sequence 5'–3'	Tm (°C)	Reference*
Cytochrome c oxidase (*cox1*)	COXI_F	TTT TGA TCC GTC TGG GGG TG	60	This study
	COXI_R	TTA CGC ACC GCC ATA GAC TC		
NADH dehydrogenase (*nad1*)	NDI_F	TAC GCA GGT GGT TTG GTT GG	52	This study
	NDI_R	CCC AAA GCT CAC ACC TTG T		
Cathepsin F cysteine protease (CF)	CF_F	AGC TGA ACT GAA CCA CGC TGT TC	64	This study
	CF_R	TGC GCG GTT GAT TCC GCA AG		
Paramyosine (Pm-int9)	PME_F	GCA GAG AAT ATG CGA CTC AAG	60	Shekhovtsov et al. (2009)
	PMI_R	AAA TTA TCC CGT TCC GCT TC		
Internal transcribed spacers 2 (ITS2)	ITS2_3S	GGT ACC GGT GGA TCA CTC GGC TCG TG	55	Bowles et al. (1995)
	ITS2_BD2	TAT GCT TAA ATT CAG CGG GT		
28S ribosomal RNA (28S rRNA)	JB10_Fw	GAT TAC CCG CTG AAC TTA AGC ATA T	55	Barker and Blair (1996)
	JB9_Rv	GCT GCA TTC ACA AAC ACC CCG ACT C		

*New primers were designed for certain DNA regions within this study to improve PCR specificity. New primers were designed using Primer3 and reference DNA sequences obtained from GenBank.

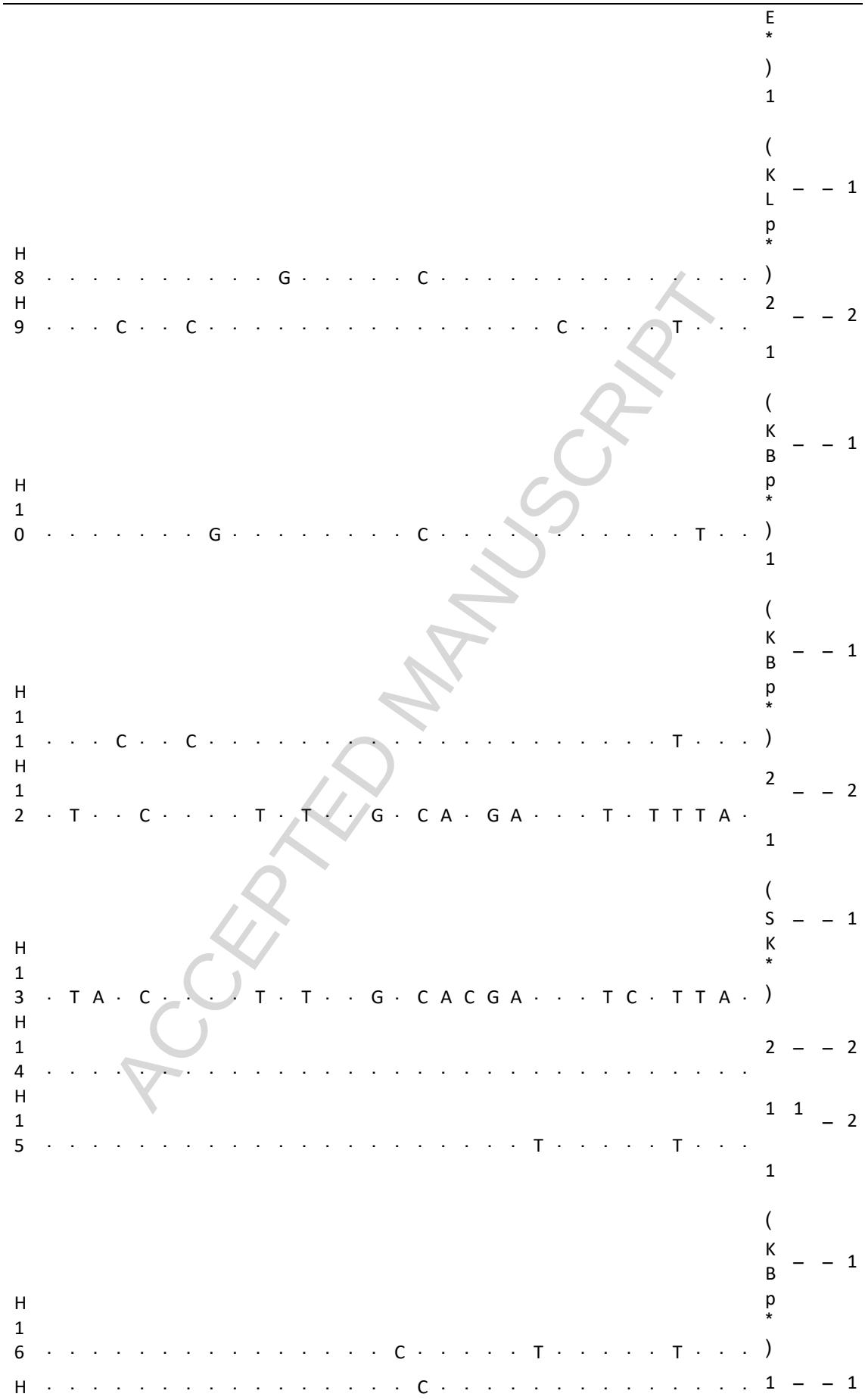
Table 3 Variation sites of 705-bp of *cox1* gene from sequence study. Haplotypes 1 to 11 belonged to *O. viverrini* from 8 geographical different populations. The unique haplotypes (singletons) are shown with their populations. TH = Thai population; LA = Lao population.

Haplotype	040	007	709	097	173	196	199	253	257	274	298	316	340	364	391	430	541	561	592	646	667	679	694	No. of individual per population		
																								TH	LA	Total
H1	C	G	C	C	T	T	T	C	A	T	G	T	T	A	T	T	T	T	C	C	T	G	A	1 (NP*)	–	1
H2	·	·	·	·	·	·	·	T	·	·	·	·	·	G	C	·	·	·	·	·	C	·	·	1 (RE*)	–	1
H3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	3	1	4
H4	·	·	T	·	·	·	·	·	·	·	·	C	·	·	C	·	·	·	·	·	·	·	·	–	1 (NK*)	1
H5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	2	1	3
H6	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	2	2
H7	·	·	T	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	1 (RE*)	–	1
H8	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	3	–	3
H9	T	A	T	T	·	C	C	·	G	C	·	·	C	·	·	·	C	·	T	·	C	A	G	3	–	3
H10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	2	–	2
H11	T	A	T	T	C	C	C	·	G	·	·	·	C	·	·	·	C	C	T	·	C	·	G	2	–	2

*Abbreviations used for locality are described in Table 1.

Table 4 Variation sites of 713-bp of *nad1* gene from sequence study. Haplotypes 1 to 18 belonged to *O. viverrini* from 14 geographical different populations. The unique haplotypes (singletons) are shown with their populations. TH = Thai population; LA = Lao population; CB = Cambodian population.

Haplotype	0 0 0 1 1 1 1 2 2 2 2 2 3 3 3 3 4 4 4 4 4 4 4 4 5 5 5 5 5 5 6 1 2 6 1 3 4 8 9 0 2 6 8 8 4 7 9 5 4 5 6 2 5 1 7 8 8 0 8 2 4 3 4 6 4	No. of individual per population			
		TH	LA	CB	Total
H1	G C T T A G T A T C A C C T A T T G T A G C T C C A C T C G T	–	1 (VT13[a])	–	1
H2	A . . . . . C . . . T . . . C . . . . . C . . .	–	1 (KM1[b])	–	1
H3	 C	1	(SK5[c])	–	–	1
H4	. . . . . T	2	1	1	4
H5	. . . . . T . . .	–	4	–	4
H6	. . . . . C . . . . . T	1	2	–	3
H7	. . . . . A . . . . . T	1 (R)	–	–	1



17		(SSK*) 1			
H18	. . . . C	(RE*)	–	–	1

[a] VT13-Vientiane (Accession number GQ401037), [b] KM1-Khammoune (Accession number GQ401040), [c] SK5-Sakaeo (Accession number GQ401100). *Abbreviations used for locality are described in Table 1.

Table 5 Analysis of molecular variance based on *nad1* sequence of 8 populations of *O. viverrini* classified into two groups defined by country (Thailand and Lao PDR).

Significance: $P < 0.05$.

Source of variation	df	Sum of squares	Variance component	% of variation	Fixation index
Among groups	2	4.595	−0.223	−8.66	$F_{ST} = 0.49^*$
Among population/within group	11	50.239	1.507	58.6	$F_{SC} = 0.53^*$
Within population	16	20.667	1.291	50.14	(* = $P <$ 0.001)

F_{ST} : the variance among groups (country) relative to the total variance

F_{SC} : the variance among populations within group (country)

Table 6 Variation sites of 709-bp of CF-int6 DNA sequence study. Haplotypes 1 to 4 belonged to *O. viverrini* from 8 geographical different populations. The unique haplotypes (singletons) are shown with their populations. TH = Thai population; LA = Lao population.

Haplotype	98	103	110	164	255	261	344	418	480	673	701	No. of individual per population		
												TH	LA	Total
H1	C	A	C	A	C	T	A	T	T	T	G	8	1	9
H2	.	.	G	.	.	.	.	.	.	.	.	6	4	10
H3	T	G	.	G	T	C	C	C	A	.	T	3	–	3
H4	.	.	.	.	.	.	.	.	.	C	.	–	1 (NK*)	1

*Abbreviations used for locality are described in Table 1.

Table 7 Variation sites of 174-bp of Pm-int9 DNA sequence study. Haplotypes 1 to 3 belonged to *O. viverrini* from 8 geographical different populations and *O. felineus*. TH = Thai population; LA = Lao population.

Haplotype	2	3	4	5	8	14	20	23	27	29	30	35	37	44	49	50	56	82	108	No. of individual per population		
																				TH	LA	Total
H1	A	C	C	C	C	T	T	G	T	A	G	A	C	G	A	C	A	C	G	153	60	213
H2	A	T	C	C	T	T	T	G	T	A	G	G	C	G	A	T	A	C	G		—	

Highlights

- Genetic sub-structuring exists in *Opisthorchis viverrini* populations in Thailand and Lao PDR.
- *Opisthorchis viverrini* from Sakon Nakhon was distinct from other isolates.
- Occurrence of a cryptic species of *O. viverrini* from Sakon Nakhon, Thailand.