

## Genetic Differentiation of *Opisthorchis*-Like Eggs in Northern Thailand Using Stool Specimens Under National Strategic Plan to Control Liver Fluke Infection and Cholangiocarcinoma

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**Abstract.** Liver fluke infection caused by *Opisthorchis viverrini* is recognized as a potential risk factor for cholangiocarcinoma (CCA). The National Strategic Plan to Control Liver Fluke Infection and Cholangiocarcinoma has implemented microscopic-based stool examination screening. However, eggs of *O. viverrini* and minute intestinal flukes (MIFs) are nearly morphologically similar and could result in inaccurate *O. viverrini* diagnosis. Stool specimens were collected from eight districts of Chiang Mai Province in northern Thailand. *Opisthorchis*-like eggs were identified with the Kato–Katz technique and differentiated for *O. viverrini* and MIFs using molecular study by PCR and PCR–restriction fragment length polymorphism targeting the internal transcribed spacer 2 (ITS2) gene. Prevalence of *Opisthorchis*-like eggs was 5.9% from a total of 9,570 specimens. From PCR assays, all liver flukes were *O. viverrini* and all MIFs were *Haplorchis taichui*. The distribution of species was *H. taichui* (38.2%), *O. viverrini* (10.5%), coinfection of *H. taichui* and *O. viverrini* (37.2%), and 14.1% were negative from PCR. Totally, *H. taichui* was found in 75.4% of infections from *Opisthorchis*-like specimens. ITS2 nucleotide sequencing analysis showed a single variant of *O. viverrini* with no variation and two variants of *H. taichui*. This study first revealed the genetic background of *Opisthorchis*-like eggs in northern Thailand. Minute intestinal flukes are occasionally misdiagnosed as *O. viverrini* leading to misinterpretation and overestimation of the burden of *O. viverrini* infection. Molecular diagnosis such as PCR could effectively discriminate species of *Opisthorchis*-like eggs and help shape the robustness of epidemiological data to control liver fluke infection and raise awareness of other risk factors for CCA.

### INTRODUCTION

Human liver fluke infection caused by *Opisthorchis viverrini* remains a major health problem in Thailand<sup>1,2</sup> where prevalence is high in the northern and northeastern regions,<sup>3,4</sup> which is associated with the consumption of uncooked freshwater fish containing infective-stage metacercariae.<sup>5</sup> The nationwide prevalence of *O. viverrini* infection in 1980, 2000, and 2009 were 14.0%, 9.4%, and 8.7%, respectively, where the prevalence was greater than 15.0% in the northern and northeastern provinces. In some part of northeastern region, prevalence of *O. viverrini* infection was higher than 50.0%.<sup>6,7</sup>

Infection is typically asymptomatic resulting in low awareness and chronic infection leading to a lethal bile duct cancer, cholangiocarcinoma (CCA). A report from the Office of Disease Prevention and Control revealed that CCA has high mortality associated with high medical expense and poor quality of life. The prevalence and mortality are also expected to increase over time. *Opisthorchis viverrini* is commonly found in Thailand, whereas the other two species, *Opisthorchis felineus* and *Clonorchis sinensis*, are prevalent in Eastern Europe and China, respectively. The flukes need Bithynia snails serving as a first intermediate host and white-scale freshwater fish (cyprinoid) as second intermediate host. Humans are infected by consuming uncooked cyprinoid fish containing infective stage metacercariae. Adult flukes reside in the bile duct within the hepatobiliary system and produce eggs which when excreted through feces into freshwater body, the eggs hatch and infect snails to complete their life cycle.<sup>8–10</sup> Minute intestinal flukes (MIFs) share the same life cycle, including mode of infection. However, the first

intermediate host is usually Thiarid snails. Therefore, the distribution of human liver flukes and MIFs depend on the abundance of their intermediate host in the environment.<sup>11–13</sup> A national health survey conducted in 2015 by the Department of Disease Control reported that uncooked fish consumption was observed in populations in upper northern provinces. In some areas, the consumption was 80% in populations older than 15 years. Local dishes such as *koi pla* and *larb pa*, instantly prepared uncooked fish mixed with spicy herbs, are popular.<sup>14</sup>

Clinical manifestations of *O. viverrini* infection are mostly asymptomatic.<sup>15</sup> When the infection remains chronic, the symptoms could be the result of subsequent CCA including jaundice and an enlarged liver. The International Agency for Research on Cancer declared *O. viverrini* is carcinogenic to human. *Opisthorchis viverrini* infection and CCA were widely studied in northeastern Thailand.<sup>16–19</sup> The carcinogenic process is initiated from excretory/secretory products from flukes and the human host immune response and promoted by chemical compounds such as nitrosamine.<sup>20,21</sup> Incidence of CCA is approximately 100–200 cases/100,000 population in Khon Kaen Province, northeastern Thailand, whereas the incidence in the west is one to two cases/100,000 population.

In 2013, a screening program led by the Faculty of Public Health, Khon Kaen University, comprised screening and confirmation tests with patient management focusing on populations in the northeastern region where *O. viverrini* infection is prevalent (CCA Screening and Care Program).<sup>22</sup> In 2015, the Ministry of Public Health and collaborating health agencies initiated the National Strategic Plan for Controlling Liver Fluke Infection and Cholangiocarcinoma emphasizing controlling *O. viverrini* infection and CCA screening from 2015 to 2025 and extending the screening area to national coverage, including the upper northern provinces of Thailand, that is, Chiang Rai, Chiang Mai, Phrae, Nan, Mae Hong Son,

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Lamphun, Lampang, and Phayao. Local health authority reported the prevalence and mortality rate from CCA are as high as found in the northeastern regions and higher than other regions of the country. The main strategy of the program aims to prevention and control *O. viverrini* infection to reduce subsequent CCA incidence.<sup>19,20,23</sup> Unlike the northeastern region, data of *O. viverrini* infection as a risk factor for CCA in northern region were still limited.<sup>24</sup>

The screening test for *O. viverrini* infection is made by direct stool examination using the Kato–Katz technique and examining stool samples for *O. viverrini* eggs under a microscope. The method provides good accuracy and remains practical for local ambulatory settings with limited resources.<sup>25–27</sup> However, *O. viverrini* eggs are almost morphologically similar to MIF eggs under a microscope and difficult to differentiate in routine practice,<sup>28,29</sup> which could be called *Opisthorchis*-like eggs. Moreover, *O. viverrini* and MIFs can be distinguished by identifying adult worms,<sup>29,30</sup> but collecting the parasite is impractical because adult liver flukes are rarely expelled from the bile duct to stool.<sup>8,12,31,32</sup>

National Strategic Plan to Control Liver Fluke Infection and Cholangiocarcinoma emphasized on targeting population from high-risk areas of CCA.<sup>33</sup> Therefore, *Opisthorchis*-like eggs identified with the Kato–Katz technique were presumably diagnosed as *O. viverrini* based on previous baseline epidemiological data, which were mainly obtained from endemic northeastern region.<sup>20,34,35</sup> However, *O. viverrini* were not uniformly distributed outside endemic area, and the increased prevalence in northern Thailand could be a result from misdiagnosis of MIFs due to limitation of the Kato–Katz technique.<sup>36</sup> In northern Thailand, the studies of *Opisthorchis*-like eggs have revealed that MIFs are found in both cyprinoid fish and residents.<sup>37,38</sup> A previous PCR study from Chiang Mai Province, northern Thailand, revealed that *Opisthorchis*-like eggs obtained from stool specimen were coinfection of *O. viverrini* and *Haplorchis taichui*.<sup>39</sup> Therefore, additional epidemiological data are needed to determine the prevalence of *Opisthorchis*-like eggs based on geographical distribution. In this study, screening tests were performed using stool examination to determine the prevalence of *Opisthorchis*-like eggs following the National Strategic Plan to Control Liver Fluke Infection and Cholangiocarcinoma. We assumed that *Opisthorchis*-like eggs may not be *O. viverrini* eggs exclusively from microscopy-based stool examination. Consequently, eggs from specimens were further tested using molecular techniques to discriminate *O. viverrini* and MIF infection. We also showed genetic information of *Opisthorchis*-like eggs in the designated study areas. The data will help understanding the distribution of *O. viverrini* and MIF infections within the areas under the screening program. Up-to-date data are needed for policy makers, especially when CCA could also be the result from other causes despite *O. viverrini* infection.<sup>40,41</sup>

## MATERIALS AND METHODS

**Ethics.** The research protocol was reviewed and approved by the Ethics Committee of The Royal Thai Army Medical Department (Ref. S025h/51 and S045h/54).

**Study area.** The research employed a cross-sectional design using a laboratory study. Stool specimens were examined using the molecular technique from a stool bank collected from the Stool Screening Program under the National

Strategic Plan to Control Liver Fluke Infection and Cholangiocarcinoma. Study areas comprised eight districts in Chiang Mai Province, upper northern Thailand. The areas are under the supervision of the Chiang Mai Provincial Health Office.

**Stool preparation.** According to the screening program, the Office of Public Health in each district performed the Kato–Katz stool examination for *Opisthorchis*-like egg detection. Specimens presenting *Opisthorchis*-like eggs were collected by the Provincial Health Office to the stool bank and later sent to the laboratory at the Phramongkutklao College of Medicine. Stool specimens were then prepared for molecular study. DNA was extracted from the sediment of phosphate buffer saline (PBS) ethyl acetate stool preparation. PCR and PCR–restriction fragment length polymorphism (PCR–RFLP) were performed to discriminate opisthorchiid flukes (*O. viverrini* and *C. sinensis*) and heterophyid flukes using RTFluke primers<sup>42</sup> targeting the ITS2 gene. The protocol was developed by *Buathong*<sup>28,43</sup> and published elsewhere. From the protocol, MIFs detected from this study would be heterophyid flukes accordingly.

**DNA extraction of *Opisthorchis*-like eggs.** *Opisthorchis*-like eggs, collected from PBS ethyl acetate concentration technique, were used for DNA extraction. Each positive sample of 200  $\mu$ L of sediment was incubated at 4°C for 2 days,<sup>44</sup> added with 1.4 mL ATL tissue lysis buffer (Qiagen, Hilden, Germany), and mixed continuously for 1 minute or until the stool samples were thoroughly homogenized. The suspension was subjected for six cycles of freezing in liquid nitrogen followed by thawing at 98–100°C. Subsequently, the suspension was heated at 70°C for 20 minutes before continuously mixing and centrifuging at 20,000 g for 1 minute to solidify sediment fecal pellets. The amount of 1.2 mL supernatant was transferred to a new 2-mL microcentrifuge tube. The DNA was extracted from the supernatant using QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol by modifying the standard protocol by occasional vortex using a thermomixer applied every 5 minutes for 0.5 hours during incubation at 70°C to improve DNA quality.<sup>45</sup> At the final step, DNA was eluted with 50  $\mu$ L of elution buffer.

**PCR assay for amplifying of *Opisthorchis*-like eggs in stool samples.** The positive samples of *Opisthorchis*-like eggs were subjected to triplicate PCR amplification. Two primer sets, ITS2<sup>46</sup> and RTFluke,<sup>42</sup> designed for ITS2 of opisthorchiid and heterophyid flukes, were used in this study. All positive samples were first validated using ITS2 primer, and negative samples of ITS2 primer were subjected to RTFluke PCR assay to confirm the true-negative results of PCR amplification. PCR amplifications were performed in a final volume of 50  $\mu$ L, consisting of DNA template, 10 pmole of each primer, 200  $\mu$ M dNTP, 2 mM of MgCl<sub>2</sub>, 1 $\times$  buffer PCR, and 1 unit of *Taq* polymerase (5U/ $\mu$ L) (Promega, Madison, WI). The PCR products were amplified in the Mastercycle<sup>®</sup> Personal Cycler (Bio-Rad, Hercules, CA). For the PCR assay of ITS2 primer, the DNA samples were initially denatured at 94°C for 5 minutes followed by amplification of 40 cycles consisting of desaturating at 94°C for 1 minute, annealing at 60°C for 30 seconds, and elongating at 72°C for 2 minutes for ITS2 primer. The PCR products of *O. viverrini*, *C. sinensis*, *Haplorchis pumilio*, and *H. taichui* comprised 380, 381, 380, and 530 bp, respectively.

RTFluke consisted of an initial stage of denaturation at 94°C for 15 minutes, annealing temperature at 60°C for 1 minute,

extension step at 72°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, a final extension at 72°C for 7 minutes, and a holding temperature of 12°C to complete amplifications. The amplicons of *O. viverrini*, *C. sinensis*, and *H. taichui* were 375, 381, and 526 bp, respectively.<sup>42</sup>

**Fragment length polymorphism methods for discriminating eggs of *O. viverrini*, *C. sinensis*, and MIFs.** The PCR products of *O. viverrini*, *C. sinensis*, and *H. pumilio* showed the same size in agarose gel.<sup>46</sup> Therefore, PCR-RFLP was performed to discriminate eggs of *O. viverrini* and *C. sinensis* using a restriction enzyme, *FauI* (New England Biolabs, Ipswich, MA).<sup>28</sup> PCR products were digested with 2 units of *FauI* in a total volume of 20 µL at 55°C for 6–8 hours. Digesting with *FauI* restriction enzyme, PCR-RFLP analysis was used to discriminate *O. viverrini* from *C. sinensis*, which generated the fragments of 129 bp and 247 bp for *O. viverrini* but produced undigested amplicon for *C. sinensis* and *H. pumilio*.<sup>28</sup>

**Agarose gel electrophoresis and DNA sequencing.** The PCR products were detected in 2% agarose gel in 1× Tris/borate/EDTA (TBE) buffer. For DNA staining, 10 mL of agarose gel was mixed with 0.3 µL SYBR® safe DNA Gel Stain (Invitrogen, Waltham, MA). A 100-bp DNA ladder (Vivantis Technologies, Selangor, Malaysia) was used as a marker to estimate the sizes of PCR products. The PCR products were electrophoresed at 100 V at room temperature for 40 minutes. Finally, the agarose gel was visualized by molecular image® Gel Doc™ XR + Imaging System (Bio-Rad). The PCR products were purified before DNA sequencing using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The randomly selected PCR products of *Opisthorchis*-like eggs were sent to U2Bio Co., Ltd. (Songpa-gu, South Korea) for DNA sequencing.

**Data management and analysis.** Data entry and analysis were conducted using STATA® for Windows, Version 14 (StataCorp, College Station, TX). Prevalence of *Opisthorchis*-like eggs, *O. viverrini*, and small intestinal fluke infections were reported as percentage.

**DNA analysis and phylogenetic tree of *Opisthorchis*-like egg.** The randomly selected PCR products of 380 bp and 530 bp were sent to the First BASE laboratories (First BASE Laboratories, The Gemini, Singapore) for DNA sequencing.

The sequences were subjected to an NCBI BLAST search to identify species. The DNA sequences were aligned using the BioEdit Program, version 7.0.9, and MEGA5, version 5.05 (<http://www.megasoftware.net/>), to determine the similarity by generating the phylogenetic relationships of Opisthorchiidae and Heterophyidae families. For this, *Metagonimus guianensis* and *Centrocestus formosanus* were chosen as an out-group for Opisthorchiidae, whereas *C. sinensis* was chosen for Heterophyidae. RAxML analysis was used to construct the ITS2 trees of both Opisthorchiidae and Heterophyidae based on RAxML, version 7.4.2, with a GTR matrix (GTR +  $\Gamma$  model)<sup>47</sup> using raxmlGUI, version 1.<sup>48</sup> The clade stability of the tree topology was evaluated using 1,000 replicates of RAxML bootstrap values.

**Accession numbers.** All data are available through the GenBank database with the following accession numbers. *Haplorchis taichui*: GQ176380.1, AB517576.1, MH991969.1, MK790157.1, KP165440.1, KP165440.1, MH991968.1, MH991968.1, MT006044, MT006045, MT006046, MT006047, MT006048, MT006049, MT006050, MT006051, MT006052, MT006053, MT006054, and MT006055. *Metagonimus guianensis*: KX387524.1. *Centrocestus formosanus*: KY075665.1. *Opisthorchis viverrini*: KT726408.1, HQ328548.1, AY584735.1, MK886660.2, MK886661.2, MK886662.2, MK886663.2, MT002729, MT002730, MT002731, MT002732, MT002733, MT002734, and MT002735. *Opisthorchis labotus*: HQ328545.1. *Opisthorchis parageminus*: KX258657. *C. sinensis*: EF688144.1.

## RESULTS

In 2019, a total 9,570 stool specimens were collected. The prevalence of *Opisthorchis*-like eggs was 5.9% (563/9,570). Then 304 samples (54.0%) were randomly selected from 563 specimens with *Opisthorchis*-like eggs for molecular study.

**Molecular study.** PCR assay using ITS2 plus RTFLuke primers was 85.9% (261/304) positive in specimens with *Opisthorchis*-like eggs from the Kato–Katz stool examination. All liver flukes detected were *O. viverrini*, and all MIFs detected by PCR were *H. taichui*, as shown in Figure 1.

From Table 1, PCR-RFLP shows that 48.7% (148/304) were single infection and 37.2% (113/304) were coinfection. Combining single and coinfection, 75% of positive results included

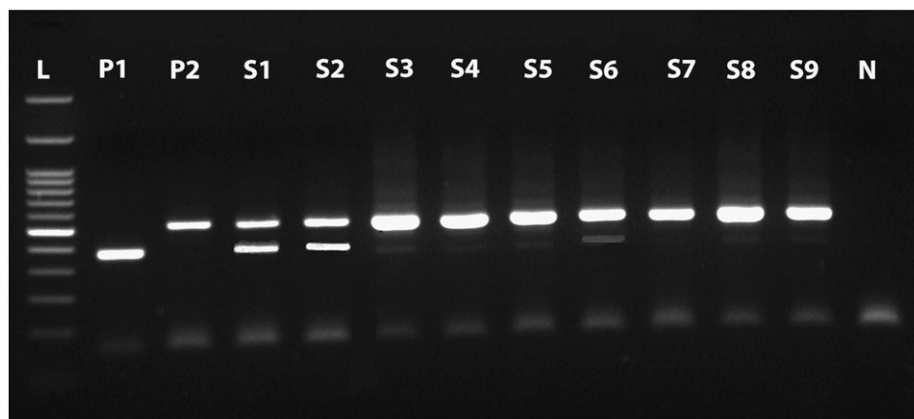


FIGURE 1. PCR products with *Opisthorchis*-like eggs using ITS2 primer. P1 was *Opisthorchis viverrini* positive, P2 was *Haplorchis taichui* positive, and N was negative. S1 to S9 samples were tested showing single and coinfection, accordingly. Lanes S1, S2, and S6 were coinfection of *O. viverrini* and *H. taichui*. Lanes S3 to S5 and S7 to S9 were *H. taichui*.

TABLE 1  
Proportion of *O. viverrini* and MIF infection detected by PCR-RFLP

PCR-RFLP result	Total specimen (n = 304)	Excluded for negative PCR (n = 261)
	n (%)	n (%)
Negative for PCR	43 (14.1)	–
<i>H. taichui</i>	116 (38.2)	116 (44.4)
<i>O. viverrini</i>	32 (10.5)	32 (12.3)
Coinfection of <i>H. taichui</i> and <i>O. viverrini</i>	113 (37.2)	113 (43.3)

PCR-RFLP = PCR–restriction fragment length polymorphism; *H. taichui* = *Haplorchis taichui*; *O. viverrini* = *Opisthorchis viverrini*.

*H. taichui* and 47.7% included *O. viverrini* infection. When excluded for negative results, highest proportion of infection was *H. taichui* (44.4%) followed by coinfection (43.3%). Single *O. viverrini* infection was 12.3% in positive results.

From Table 2, the highest numbers of *Opisthorchis*-like specimens were collected from Doi Tao/Chom Thong districts followed by Mae Taeng District. The highest proportion of *H. taichui* and *O. viverrini* infection was 56.4% in Mae Taeng District and 27.3% in Doi Tao/Chom Thong districts, respectively. Coinfection was highest in Samoeng District (66.7%). Chai Prakan District had the highest proportion of negative PCR results (30.8%). Among 304 specimens from eight districts, four specimens were from unknown areas. However, they were collected from within the studied districts for which three specimens (75%) were *H. taichui* and one specimen (25%) indicated coinfection.

**DNA sequencing of *Opisthorchis*-like eggs.** Seven randomly selected *O. viverrini* ITS2 sequences from eight districts in Chiang Mai were analyzed and subjected to BLAST NCBI. The result of BLAST showed that ITS2 sequences of *O. viverrini* obtained from this study were genetically identical to *O. viverrini*, accession number MK886660.2, MK886663.2, MK886662.2, and MK886661.2, with 100% identity. DNA sequence alignment presented single variant and no genetic variation of ITS2 sequences. Moreover, 12 sequences of *H. taichui* PCR products were genetically identical to *H. taichui*, accession number MK790157.1, GQ176380.1, MH991969.1, and AB517576.1, isolate with 100% identity. In addition, DNA sequencing alignment of 12 sequences of *H. taichui* showed two variants. Seven sequences of *O. viverrini* and 12 sequences of *H. taichui* obtained from this study were submitted to GenBank to obtain accession number.

**Phylogenetic tree analysis.** A phylogenetic tree of MIFs based on 492 bp of the ITS2 gene was constructed using 12 *H. taichui* DNA sequences isolated in this study and eight referent *H. taichui* (Figure 2) sequences retrieved from GenBank. *Metagonimus guianensis* and *C. formosanus* were used

as an out-group. The tree topologies of MIFs revealed that *H. taichui* formed paraphyletic relationships with other two species of MIFs (bootstrap value = 100%). Isolates of *H. taichui* (n = 12) (MT006045–MT006054) collected in this study were clustered within the *H. taichui* group (bootstrap value = 100%) forming strictly monophyletic relationships with others from Thailand (MH991969.1 and AB517576.1) and Vietnam (MK790157.1 and GQ176380.1) representing shared ancestor. However, two referent isolates (HM004155.1 and MH991968.1) were separated from the others. Regarding strong support of the bootstrap value (bootstrap value = 96%), the exchanges between branches were less likely to occur, representing shared ancestor in *H. taichui* isolates of this study.

The ITS2 tree of *O. viverrini* based on 377 bp, constructed using seven isolate sequences in this study and nine reference *Opisthorchis* genera sequences with *C. sinensis* as an out group, demonstrated paraphyletic relationships among *O. viverrini* with bootstrap value less than 50% causing uncertain diversity in the *O. viverrini* group (Figure 3). Interestingly, the clade of our seven isolates (MT002729–MT002735) strictly formed a monophyletic group with that of a related report from eastern Thailand (MK886660.2, MK886661.2, MK886662.2, and MK886663.2) (bootstrap value = 78%) and clearly separated from five reference isolates (HQ328545, HQ328548, KT726408, AY584735, and KX258657).

## DISCUSSION

This is the first report to discriminate *O. viverrini* and MIF infection from *Opisthorchis*-like eggs using PCR assays in northern Thailand. According to the National Strategic Plan for Controlling Liver Fluke Infection and CCA, the program emphasizes controlling *O. viverrini* infection, and reports show that the incidence of CCA is high in upper northern provinces where the practice of consuming uncooked fish is popular. However, the program primarily examined stool samples to

TABLE 2  
Distribution of *O. viverrini* and minute intestinal fluke infection as detected by district

District	Prevalence of <i>Opisthorchis</i> -like eggs (%)	Number of <i>Opisthorchis</i> -like eggs	<i>H. taichui</i>	<i>O. viverrini</i>	Coinfection of <i>H. taichui</i> and <i>O. viverrini</i>	Negative for PCR
			n (%)			
Doi Tao/Chom Thong	8.1	99	12 (12.1)	27 (27.3)	54 (54.5)	6 (6.1)
Mae Taeng	13.3	94	53 (56.4)	2 (2.1)	27 (28.7)	12 (12.8)
Chiang Dao	6.6	67	31 (46.3)	0	18 (26.9)	18 (26.9)
Fang	2.0	16	7 (43.8)	3 (18.8)	5 (31.3)	1 (6.3)
Chai Prakan	2.8	13	7 (53.8)	0	2 (15.4)	4 (30.8)
Samoeng	2.7	9	3 (33.3)	0	6 (66.7)	0
Mae Ai	1.5	2	0	0	0	2 (100.0)

*H. taichui* = *Haplorchis taichui*; *O. viverrini* = *Opisthorchis viverrini*.

n = 300, excluding four samples from unknown areas within eight studied districts.

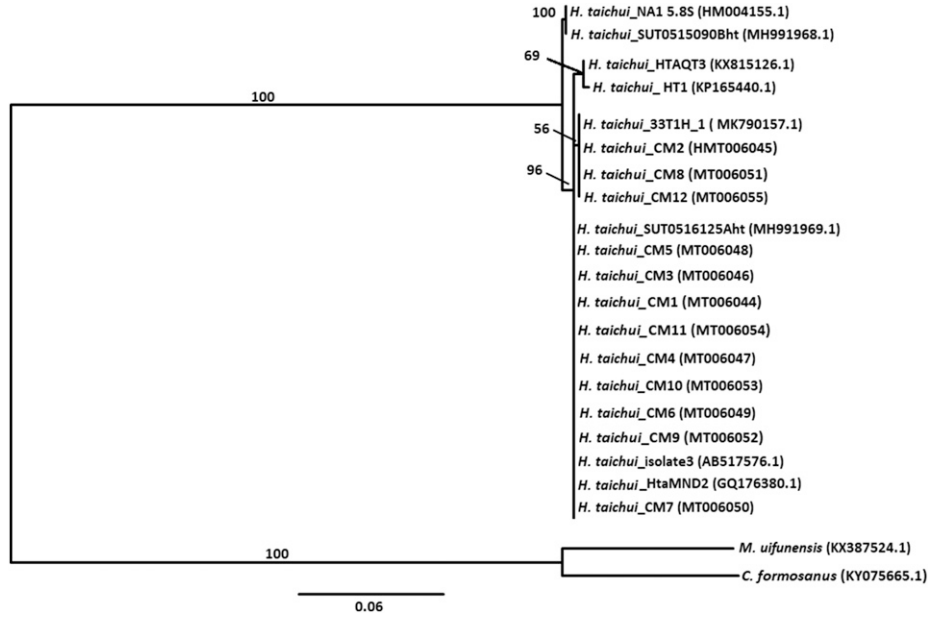


FIGURE 2. Randomized Axelerated Maximum Likelihood tree based on the ITS2 sequences of minute intestinal flukes. The alignment of 429 nucleotide sequences without gaps and 12 isolates from eight villages (isolates CM1–CM12) were analyzed. *Haplorchis taichui* isolates NA1 5.8 S, SUT0515090Bht, HTAQT3, HT1, 33T1H\_1, SUT0516125Aht, isolate3, and HtaMND2 were used as the reference isolates. The percentages of 1,000 replications (bootstraps) of more than 50% are shown at the nodes.

determine *O. viverrini* infection, which could not effectively discriminate *O. viverrini* and MIF infection from specimens. Distribution data of *Opisthorchis*-like eggs could play an important role for program planning. Some areas with high incidences of CCA might not have high prevalence of *O. viverrini* infection.

Diagnosis of *O. viverrini* and MIF infection is practically made by microscopy-based stool examination. Direct simple smear can identify various parasitic infections, including helminth eggs and protozoa. Preparation technique to increase fecal material concentration such as the Kato–Katz thick smear or formalin–ethyl concentration technique (FECT) could increase sensitivity of the test.<sup>26,27,29,49</sup> However, diagnosis depends on identifying eggs under light microscope. Studies of *Opisthorchis*-like eggs conducted by Radomyos et al.<sup>37,50</sup> and Wijit et al.<sup>32</sup> in upper northern provinces revealed that most adult flukes collected from participants were MIFs, which is not a risk factor for CCA. In central and northeastern Thailand, *Opisthorchis*-like eggs were mostly identified as *O. viverrini* infection<sup>5,45</sup> and are significantly related to CCA occurrence.<sup>18,40</sup> Several works used PCR to study rDNA ITS1 and the ITS2 region to differentiate *O. viverrini* and MIFs.<sup>46,51</sup> PCR showed better performance than concentration techniques such as FECT, especially in low infection intensity areas. Many studies of *Opisthorchis*-like eggs in northern provinces of Thailand revealed MIFs, particularly *H. taichui* was a dominant fish-borne trematode in these regions. From 2015 to 16, one study in upper north provinces using purgative magnesium sulfate revealed 70% of *Opisthorchis*-like eggs were positive for *H. taichui*, but *O. viverrini* was undetected by PCR.<sup>32</sup> The PCR assay-based rDNA ITS2 region was used to detect *Opisthorchis*-like eggs from stool specimens in this study. ITS2 primer was mainly applied to detect *Opisthorchis*-like eggs with a high sensitivity of 95.2% to detect, whereas

RTFluke primer indicated only 71.0%.<sup>42</sup> ITS2 primer revealed a higher proportion of infection of *H. taichui* and coinfection of *H. taichui* and *O. viverrini* than single *O. viverrini* infection.

This study first showed a high coinfection of *O. viverrini* and *H. taichui* of 37.2% using PCR assay. Only *H. taichui* and *O. viverrini* were found in this study, but *H. pumilio* and *C. sinensis* were not observed, suggesting the distribution pattern of fish-borne trematode in northern Thailand. Using two primer sets could increase the sensitivity of detecting *Opisthorchis*-like eggs in stool specimens. RTFluke primer could amplify *Opisthorchis*-like eggs in 9.5% (29/304) of negative samples from ITS2 primers. DNA extraction might

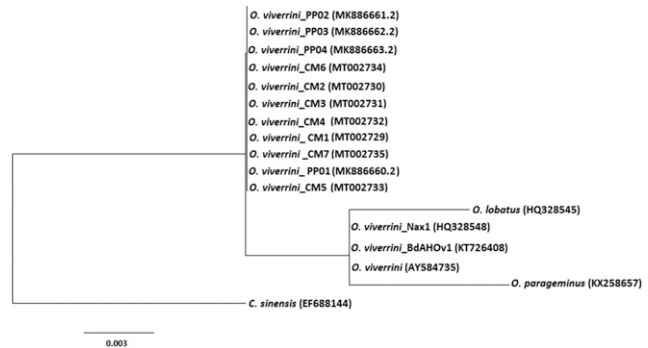


FIGURE 3. Randomized Axelerated Maximum Likelihood tree based on the ITS2 sequences of liver flukes. The alignment of 377 nucleotide sequences without gaps and seven isolates from eight districts (isolate; CM1–CM17) were analyzed. *Opisthorchis viverrini* isolates PP01, PP02, PP03, PP04, Nax1, BdAHov1, and AY584735.1; *Opisthorchis lobatus*; and *O. parageminus* were used as the reference isolates. The percentages of 1,000 replications (bootstraps) of more than 50% are shown at the nodes.

have been affected by inadequate amounts of stool specimens. Therefore, the quality of DNA extraction, sensitivity and specificity of primers, and optimal PCR conditions are crucial to detect and discriminate *Opisthorchis*-like eggs in stool specimens. However, this work emphasized on molecular study from the collected specimen. Demographic, risk behavior, and spatial data could be incorporated in further studies to determine the risk for acquiring *O. viverrini* and MIF infection.

DNA sequencing analysis of ITS2 sequences of *O. viverrini* showed only single variants without variation. Moreover, ITS2 sequences of *O. viverrini* in this study were genetically identical to submitted sequences in the GenBank database and had no variation of rDNA in the ITS2 region. Therefore, other genetic markers, that is, cytochrome c oxidase subunit I and reduced nicotinamide adenine dinucleotide dehydrogenase subunit 1 should be used to study the genetic population of *O. viverrini* in northern Thailand.<sup>28,52,53</sup> Minute intestinal flukes in this study were predominantly identified as *H. taichui* and clustered into two variant groups. Moreover, *H. taichui* obtained from this study was genetically similar to *H. taichui* found in Vietnam (accession number MK790157.1 and GQ176380.1) and Thailand (accession number MH991969.1 and AB517576.1) with 100% identity. *Opisthorchis viverrini* obtained from this study was genetically similar to *O. viverrini* isolated from eastern Thailand with 100% identity.<sup>45</sup>

Epidemiological data of *O. viverrini* and MIF infection and biological diversity will be valuable for public health purposes and helpful in shaping healthcare policy. *Opisthorchis*-like eggs, diagnosed from microscopic-based examination, could be either *O. viverrini* or MIF eggs. In some area with high prevalence of MIF infections, CCA might be influenced by other factors in addition to parasitic infections which could potentially affect the strategic plans to control *O. viverrini* infection to reduce CCA. More broadly, *O. viverrini* is not the only carcinogenic factor of CCA; other examples could similarly benefit from proactively bridging infectious and noninfectious factors. Using molecular studies could help shape the robustness of epidemiological data to control liver fluke infection and raise awareness for other risk factors for CCA despite *O. viverrini* infection.

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