Epidemiology of *Opisthorchis* spp. in Central Vietnam

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Dedicated to:

My parents, my husband and my son as a promise!

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With all my best wishes!

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ABBREVIATIONS

CCA	Cholangiocarcinoma
MDA	Mass drug administration
COI (*co*1)	Cytochrome c oxidase subunit I
ITS2	Internal transcribed spacer subunit II
DNA	Deoxyribonucleic acid
Bps	Base pairs
mtDNA	Mitochondrial DNA
rDNA	Ribosomal DNA
rTU	Ribosomal transcriptome unit
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RT-PCR	Real-time-PCR
^{0}C	Celsius grade
pH	Acidity
rpm	Round per minute
ml	Milliliter
mm	Millimeter
mM	Millimol
µl	Microliter
µm	Micrometer
UV	Ultraviolet
spp.	Species (plural)
sp.	Species (singular)
AFA	Alcohol-formaldehyde- acetic acid-glacial solution
WHO	World Health Organization
FAO	Food and Agriculture Organization

GENERAL INTRODUCTION

The liver flukes, *Opisthorchis viverrini*, *Opisthorchis felineus* and *Clonorchis sinensis* (*Opisthorchis* genus, Opisthorchiidae) cause opisthorchiasis and clonorchiasis, which are major public health problems in many parts of East Asia and Eastern Europe. The number of people infected with these flukes was estimated at 45 million worldwide and the number of people at risk at over 600 million (Bouvard et al., 2009; Keiser and Utzinger, 2005; Wagner and Salman, 2004). These liver flukes are fish-borne trematodes with a complicated life cycle involving two intermediate hosts, Bithynia fresh water snails and Cyprinid fish, respectively. The final human and animal hosts get infected by eating raw or undercooked fish infected with metacercariae (Kaewkes, 2003; Nguyen and Le, 2011). The infection is associated with several hepatobiliary abnormalities and associated pathologies and clinical signs. Most important, both experimental and epidemiological evidences strongly show *O. viverrini* infection in the etiology of cholangiocarcinoma (CCA) or bile duct cancer, which has one of the highest mortality rates of any cancer (Fedorova et al., 2017; Kim et al., 2016; Sripa et al., 2012).

Opisthorchiasis and clonorchiasis occur in areas where aquaculture is practiced, where there is a habit of eating raw or undercooked fish and/or where low sanitary conditions prevail. Endemic areas are mainly in developing countries where inhabitants living near streams, ponds and irrigations canals are particularly affected. Man is the natural final host of these parasites but dogs and cats and other fish-eating mammals and birds can act as reservoir hosts (Aunpromma et al., 2012; Bouvard et al., 2009; Chai et al., 2005a; De et al., 2003; Grundy-Warr et al., 2012; Kaewkes, 2003; Nguyen and Le, 2011; Rim, 2005; Sithithaworn et al., 2012; Sripa et al., 2007).

Despite all efforts to prevent and control opisthorchiasis caused by *O. viverrini* in the lower Mekong region (including Thailand, Lao PDR, Cambodia and Vietnam) by the governments and international organizations such as, WHO and FAO (Bouvard et al., 2009), this fatal human fluke is still highly prevalent in some areas where it causes serious morbidity and mortality. In addition, the epidemiological situation has become even more complicated, with an increasing number of new *Opisthorchis* species being reported in a short period of time. Indeed, metacercariae of *Opisthorchis lobatus* were found in fish in Lao PDR in 2011 (Thaenkham et al.,

2011b) and our research group identified an adult *Opisthorchis* sp. in ducks in Central Vietnam (Dao, 2012) that was temporary called *Opisthorchis viverrini*-like, due to its sympatric distribution and morphological and molecular characteristics that it shares with *O. viverrini*. However, the taxonomic position as well as the life cycle and the epidemiology of this *Opisthorchis* sp. in ducks are not known yet. Also, it is not known whether this duck isolate can cause human infections.

Vietnam has been identified as an endemic country of both opisthorchiasis and clonorchiasis, of which *O. viverrini* is endemic in the Central and Southern parts of the country, while *C. sinensis* is present in the North (Nguyen and Le, 2011). The new finding of a *O. viverrini*-like species in the Central endemic part suggests a local complex epidemiological situation. Therefore, the current study was conducted to unravel the epidemiology and taxonomy of *Opisthorchis* spp. in Central Vietnam. Starting with clarification of the current *O. viverrini* infection status in humans in Binh Dinh Province; we performed morphological and molecular identification of a new *Opisthorchis* sp./genotype isolated in ducks; then we pointed out the phylogenetic position of the *O. viverrini*-like duck genotype in the Opisthorchiidae family; followed by estimating its prevalence in different populations of ducks in the same region; finally, we identified the local snail and fish intermediate host species for both *O. viverrini* and *O. viverrini*-like. The results of these studies clarify part of the epidemiology of *Opisthorchis* spp. in Central Vietnam, yet at the same time highlight the complexity of the epidemiology and call for more studies on possible hybridization/introgression of *Opisthorchis* spp. in Central Vietnam.

CHAPTER 1

A LITERATURE REVIEW OF THE GENUS OF *OPISTHORCHIS* (OPISTHORCHIIDAE)

1.1 Taxonomy

Kingdom:	Animalia (Linnaeus, 1758)	
Phylum:	Platyhelminthes (Claus, 1887)	
Class:	Trematoda (Rudolphi, 1808)	
Order:	Opisthorchiida (La Rue, 1957)	
Family:	Opisthorchiidae (Looss, 1899)	
Genus:	*Opisthorchis* (Blanchard, 1895)	*Clonorchis* (Looss,1907)
Species:	*viverrini* (Poirior, 1886)	*sinensis* (Looss,1907)
	felineus (Rivolta, 1884)	
	lobatus (Scholz, 2008)	

1.2 Life cycle

The life cycle of *Opisthorchis* species is similar (Kaewkes, 2003) (Fig 1.1). The adult flukes deposit fully developed eggs that are excreted with the feces into the environment by which they may reach freshwater sources such as ponds, canals, lakes or rivers. After ingestion by a suitable freshwater mollusk species (the first intermediate host), the parasite eggs hatch and release miracidia in the intestinal tract of the snail where the next three developing stages develop, i.e. sporocysts, redia and cercariae by asexual multiplication.

Cercariae, after being released from the snail, attach and penetrate susceptible species of freshwater fish (the second intermediate host), in which they encyst as metacercariae in the muscles or under the scales. The definitive hosts including, various mammals, fish-eating birds and humans become infected by eating raw or undercooked fish containing metacercariae (Kaewkes, 2003; Rim, 2005; WHO, 1995; Young et al., 2010).

After ingestion, the metacercariae excyst and develop in the duodenum as juvenile flukes. These flukes use their oral and ventral suckers to migrate to the common bile ducts, then go to intra-hepatic bile ducts where they mature, fertilize and lay eggs. Sometimes, adult flukes may be found in the common bile ducts, gall bladder or pancreatic ducts of the final hosts (Kaewkes, 2003).

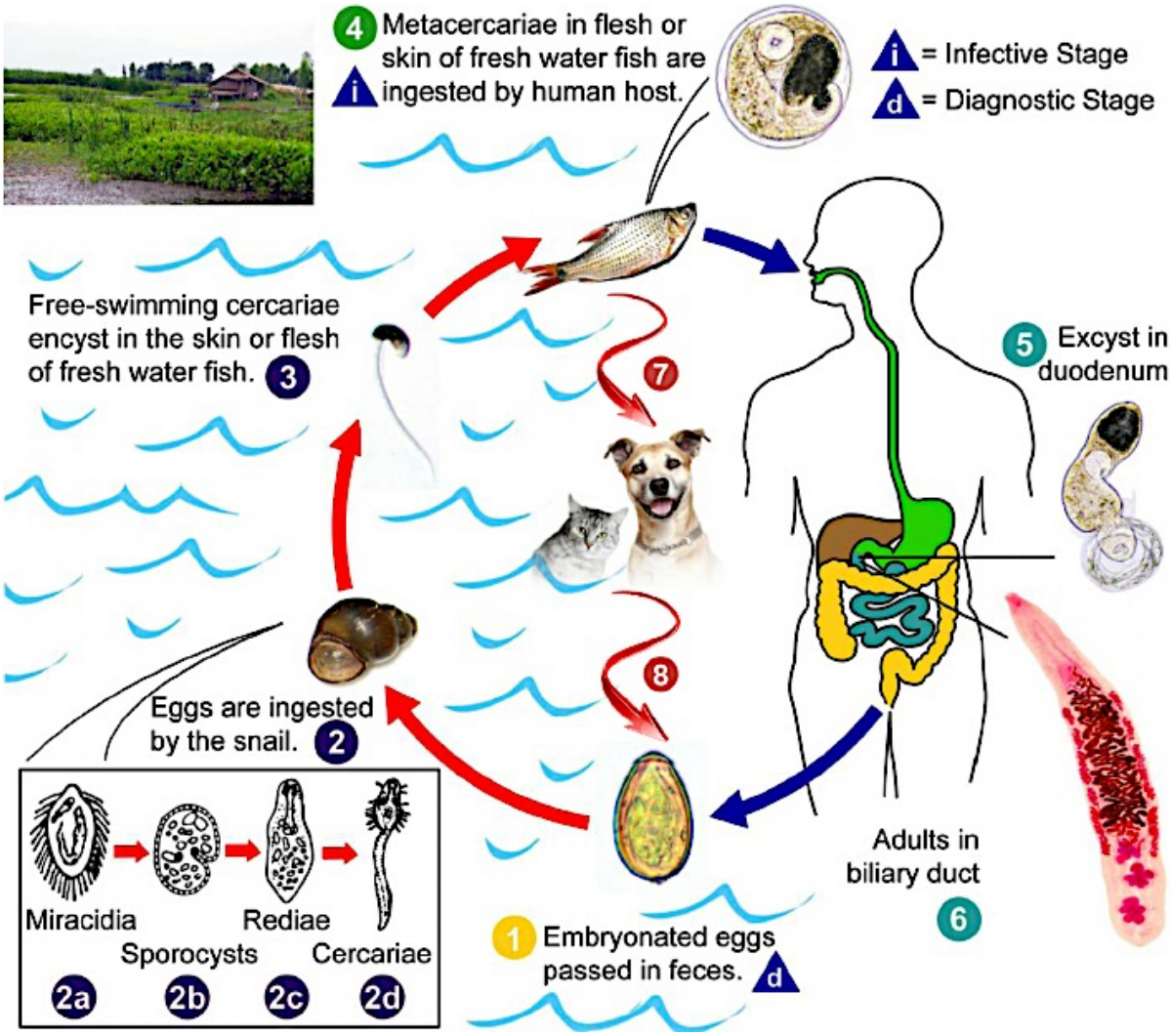


Figure 1.1 Life cycle of *Opisthorchis viverrini* (Sripa et al., 2015).

1.3 Morphology of *Opisthorchis* species

1.3.1 Adult worms

(Bray et al., 2008; Kaewkes, 2003) Adult worms are monoecious[1], dorsal-ventral flattened, transparent, lancet-shaped or leaf-shaped (Fig 1.2). The tegument is unspined. The body length is three to five times as long as the width. Internal organs mainly include:

The oral sucker: terminal or sub-terminal anterior in the body;

The ventral sucker: in the mid-body of the anterior third;

[1] having both male and female reproductive organs in an individual

The pre-pharynx is absent; the pharynx small, round and oval;

The esophagus is short;

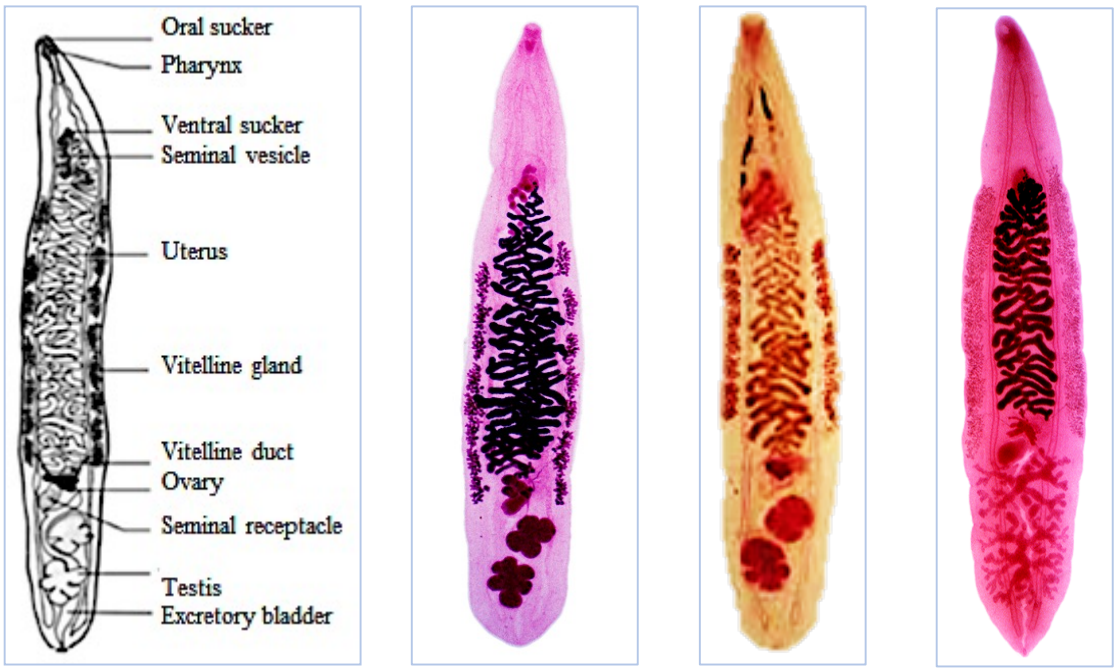
The ovary: lobate, in the posterior third of the body and anterior to the testes;

The intercecal uterus is densely packed with eggs, and loops in the space between the ovary and the ventral sucker;

The vitelline glands form two extracecal chains, span in the middle third of the body, between the ventral sucker and the ovary or the testes;

Two testes: in the last third of the posterior body, dendritic in *C. sinensis* but lobate in *Opisthorchis* species, and in tandem or diagonal range;

Excretory bladder: sinuous stem, passes around the dorsal bodies of testes, bifurcates between the anterior testis and seminal receptacle, the pore terminal.



O. lobatus [1] *O. viverrini* [2] *O. felineus* [2] *C. sinensis* [2]

Figure 1.2 Adult *Opisthorchis* species and *Clonorchis sinensis* (Opisthorchiidae).

(1) (Thaenkham et al., 2011b),

(2) http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/MR/Opisthorchiasis/body_Opisthorchiasis_il4.htm

Opisthorchis viverrini: reddish-bile colored, 5.4 - 10.2 mm length x 0.8 - 1.9 mm width. The oral sucker is sub-terminal. The ventral sucker locates at the approximately anterior one-fifth length of the body. Two testes are deeply lobate, diagonal, sited near the posterior extremity. The seminal vesicle is long-slightly coiled, terminates in the ejaculatory duct, which opens through the genital pore immediately in front of the ventral sucker. The vitelline glands form 7 – 8 groups on each lateral side of the body, between testis and ventral sucker. The multilobate ovary is in front of the anterior testis. The seminal receptacle and the Laurer's canal are nearby the ovary. The uterus packed with eggs loops irregularly in the middle part of the body, runs from the ovary to the ventral sucker. The excretory bladder is long, S-shaped and runs between the two testes (Bray et al., 2008; Kaewkes, 2003).

Opisthorchis felineus: the body size is 7 – 12 mm length x 2 – 3 mm width. The morphology of adult flukes resembles that of *O. viverrini*: the oral sucker is terminal. The ventral sucker is sited at approximately the anterior one-fifth length of the body; the uterus loops irregularly in the mid-body, between the ovary and the ventral sucker. The ovary is disposed in front of the anterior testis; the vitelline follicles form 7 – 8 groups on each lateral side of the body. Two testes are lobed and diagonal. However, the adult worm of *O. felineus* can be differentiated from that of *O. viverrini* by having the ovary and the testes not as deeply lobate as those of *O. viverrini*; also, the vitelline glands on each lateral side of the body span between the ovary and the ventral sucker, not between the testes and the ventral sucker as in *O. viverrini* (Chai et al., 2005a; Chai et al., 2005b; Mordvinov et al., 2012).

Opisthorchis lobatus: the adult measures 4 – 6 mm length x 0.7 – 1.1 mm width. The oral sucker is terminal and very small. The ventral sucker is small, located in the mid-body of the anterior third. The pre-pharynx is absent. The pharynx is small and long. The esophagus is short. The caeca are at both sides of the body and almost reach to the posterior extremity. Two testes are tandem and irregularly lobate. Vitelline follicles are relatively large and form 7 – 8 groups on each lateral side of the body, between the ovary and the ventral sucker. The ovary is lobate, looks like 3 – 4 fingers. The uterus loops and forms transverse irregular folds in the

middle of the body, between caeca, with some folds overlapping caeca. The excretory bladder is tubular, winding between testes and seminal receptacle (Thaenkham et al., 2011b).

Clonorchis sinensis: the body size is much larger than that of *Opisthorchis* spp., reaches 10 – 25 mm in length. The oral sucker is sub-terminal and is bigger than the ventral sucker; the pre-pharynx is absent; the esophagus is short. The caeca extend almost to the posterior extremity. The tandem and dendritic testes overlap the caeca laterally and site in the posterior third of the body. The vitelline follicles form two extra-caeca, uninterrupted bands between the level of the ventral sucker and the anterior testis. The seminal receptacle is voluminous, posteromedial to the ovary. The ovary is lobate, slightly sub-median, pretesticular. The Laurer's canal is present; the uterus loops almost entirely between the two caeca, runs from the ovary to the ventral sucker. The excretory vesicle with sinuous stem, passes around the dorsal bodies of the testes, bifurcates between the anterior testis and the seminal receptacle; the pore is terminal (Bray et al., 2008).

1.3.2 Metacercariae

Metacercariae encyst in the muscles of fish. Most of the metacercariae are oval shape; a few are round and have a two-layered wall. The body of metacercaria folding inside the cyst bears some internal organs (Fig 1.3).

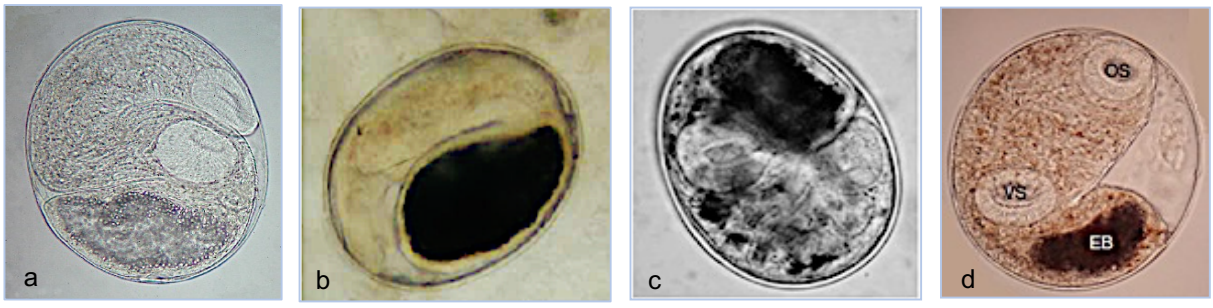


Figure 1.3 Metacercariae of *Opisthorchis* species, (a) *O. viverrini* (Touch et al., 2009); (b) *O. felineus* (Armignacco et al., 2008); (c) *O. lobatus* (Thaenkham et al., 2011b); (d) *C. sinensis* (Sohn et al., 2009). OS: oral sucker; VS: ventral sucker; EB: excretory bladder.

O. viverrini metacercariae (Fig 1.3a): the average size of encysted metacercariae is 201 μm x 167 μm. The cyst walls are thin. The outer wall is 1 – 3 μm thick. The

body of the metacercariae inside the cyst bears oral and ventral suckers, and a tubular excretory bladder at the posterior end of the worm. Under the microscope, the excretory bladder appears as an oval area containing a dense mass of granules and the brownish-yellow pigments are distributed over the whole body (Kaewkes, 2003; Touch et al., 2009).

O. felineus metacercariae (Fig 1.3b): the shape of the encysted metacercariae is oval. They are big in size of 225 – 320 μm length x 165 – 225 μm width (Kaewkes, 2003).

O. lobatus metacercariae (Fig 1.3c): the encysted metacercariae are oval, transparent, a single-layered cyst wall; the size is 100 – 200 μm length x 70 – 90 μm width. The oral sucker is terminal. The ventral sucker is spherical. Pigment granules scatter densely over the whole body. The excretory bladder is large, oval and sited in the posterior third of the body (Thaenkham et al., 2011b).

C. sinensis metacercariae (Fig 1.3d): the encysted metacercariae are elliptical; the size is 158 – 193 μm length x 153 – 183 μm width. The oral and ventral suckers have nearly equal sizes. The O-shaped excretory bladder is a dense brown mass, posteriorly located. The brownish pigment granules are scattered over the entire body of the metacercariae (Sohn et al., 2009).

1.3.3 Cercariae

The cercariae of the *Opisthorchis* genus are oculate, pleurolophocercous, pipe-formed (Fig 1.4b) and cannot be determined to species level. The cercaria has two pigmented eye spots and lateral fin folds. The body averages 154 μm x 75 μm and is covered with minute spines; the tail measures 392 μm x 26 μm (Kaewkes, 2003).

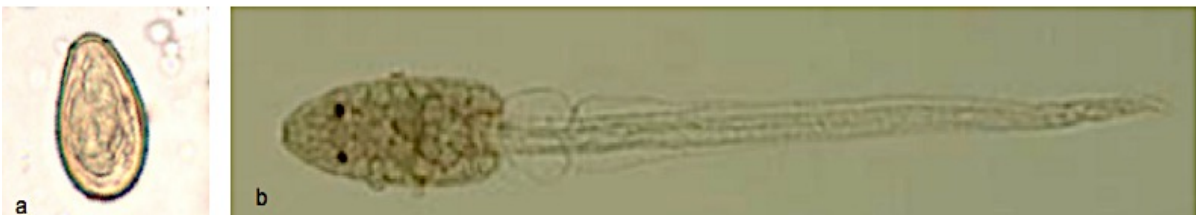


Figure 1.4 (a) Embryonated egg of *Opisthorchis* spp. (https://www.cdc.gov/dpdx/opisthorchiasis/); (b) Pleurolophocercous cercaria of *Opisthorchis* spp. (Apiporn, 2011).

1.3.4 Eggs

The eggs of *Opisthorchis* spp. and *C. sinensis* (Fig 1.4a) are similar, in oval or electric-bulb shaped, measuring 27 μm x 15 μm on average. The egg has a yellowish-brown color and a well-developed operculum with the prominent margins of the operculum seen as “shoulders”. The surface structure consists of filaments, approximately 0.2 μm thick, predominantly found in the lengthwise direction of the egg (Kaewkes et al., 1991).

1.4 *Opisthorchis* spp. and *Clonorchis sinensis* genetics and its genetic variations

1.4.1 Mitochondrial genome (mtDNA)

Nearly complete mtDNA genomes of *O. viverrini* and *C. sinensis* and complete mtDNA genome of *O. felineus* were sequenced revealing the length of around 14000 base pairs (bps). As other eukaryotes, the mitochondrial genome of *Opisthorchis* genus sequences have been extensively used as genetic markers for studying the taxonomy, systematics and population genetics of animals due to their maternal inheritance, fast rate of evolutionary change, lack of recombination, and relatively conserved genome structure. The sequences of the COI and *nad*1 markers are the most frequently used for this genus (Cai et al., 2012a; Le et al., 2017; Mordvinov et al., 2009).

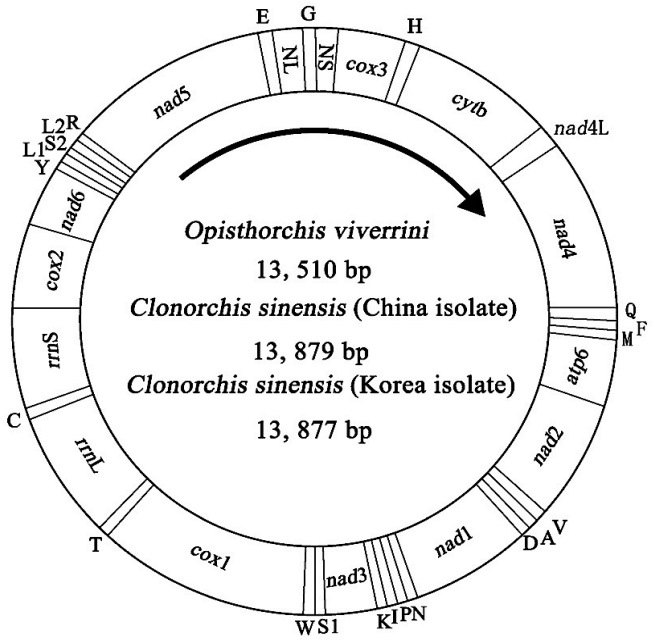


Figure 1.5 Mitochondrial genome (mtDNA) of the *Opisthorchis* genus (Cai et al., 2012a).

1.4.2 Ribosomal nucleus genome (rDNA)

Together with mtDNA, ribosomal transcription unit (rTU) of rDNA is commonly used for molecular diagnosis and systematic/ phylogenetic studies. A single rTU consists of three coding regions including 18S, 5.8S and 28S rRNA genes separated by two internal transcribed spacers (ITS1 and ITS2) (Le et al., 2017; Torres-Machorro et al., 2009). The ITS1 and ITS2 are mostly used for diagnosis in the *Opisthorchis* genus (Huang et al., 2012).

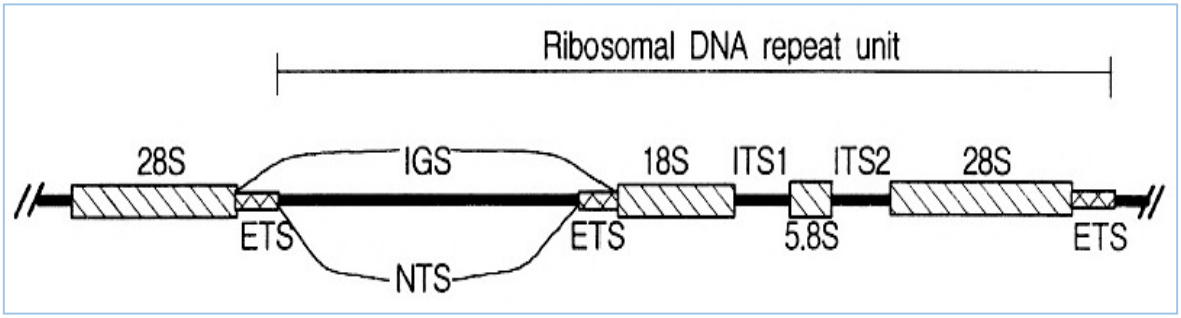


Figure 1.6 An illustrated figure of a eukaryote rTU: The gene segment of eukaryotic rDNA contains 18S, 5.8S, and 28S tracts and forms a tandem repetitive cluster. NTS: non-transcribed spacer, ETS: external transcribed spacer, ITS: internal transcribed spacers 1 and 2, numbered from 5' end (Hwang and Kim, 1999).

1.4.3 Genetic variations

Several studies on genetic characteristics of small liver flukes have shown that there is variation in the genome within the different species. In *C. sinensis*, the genome of worms isolated from distinct areas of China differed from each other from 0 – 1.6 % in mtDNA; however, very little variation in rDNA was found in isolates of Korean origin (Lee and Huh, 2004; Liu et al., 2012). However, *O. viverrini* in Laos is distinct from that of isolates from Thailand with "fixed differences at 33 – 44% of loci" by random amplified polymorphic DNA and "the magnitude of the genetic differences among *O. viverrini* specimens ranges from 3 to 73%". This species tends to split into at least two sub-species, possibly six according to the geographical location, although morphological traits are not different between those genetically different isolates (Andrews et al., 2008; Pitaksakulrat et al., 2017; Saijuntha et al., 2007; Sithithaworn et al., 2007).

1.5 Clinic and symptoms

Infection with adult flukes in human causes opisthorchiasis and clonorchiasis. Most infections are sub-clinical; heavy infection or chronic infections may cause nonspecific signs and symptoms such as, pain in the right upper abdominal quadrant, flatulence, fatigue, anorexia, nausea, diarrhea, loss of weigh or obstructive jaundice. Pathological signs mainly occur in the liver, the bile ducts and the gall bladder with inflammation and enlargement of these organs or stones inside them. These lesions can be detected by abdominal ultrasound examination (Bouvard et al., 2009; WHO, 2008).

1.6 Opisthorchiasis/ clonorchiasis and induction of cholangiocarcinoma (CCA)

The International Agency for Research on Cancer (IARC) has classified two liver flukes, *O. viverrini* and *C. sinensis* as carcinogenic to humans Group 1 (Bouvard et al., 2009; IARC, 1994).

The epidemiological correlation between the prevalence of *O. viverrini* infection and the incidence of CCA has been demonstrated. The highest CCA incidence in the world is in Khon Kaen Province of Northeast Thailand, at a rate of 96 per 100,000 people, coinciding with a high prevalence of opisthorchiasis (Sripa et al., 2007). The CCA can develop asymptomatically for a long time, especially in intrahepatic locations. Many pathways leading to CCA in *O. viverrini* infected humans were studied using experimental infections in the Syrian golden hamster. Three intricate mechanisms are involved: (i) the direct damage caused by adult worms on the bile duct epithelium, (ii) the immunopathological processes related to chronic inflammation (oxidative stress) and (iii) the mitogenic and anti-apoptotic effects of the proteins secreted by the parasite (Sripa et al., 2012; Sripa et al., 2007).

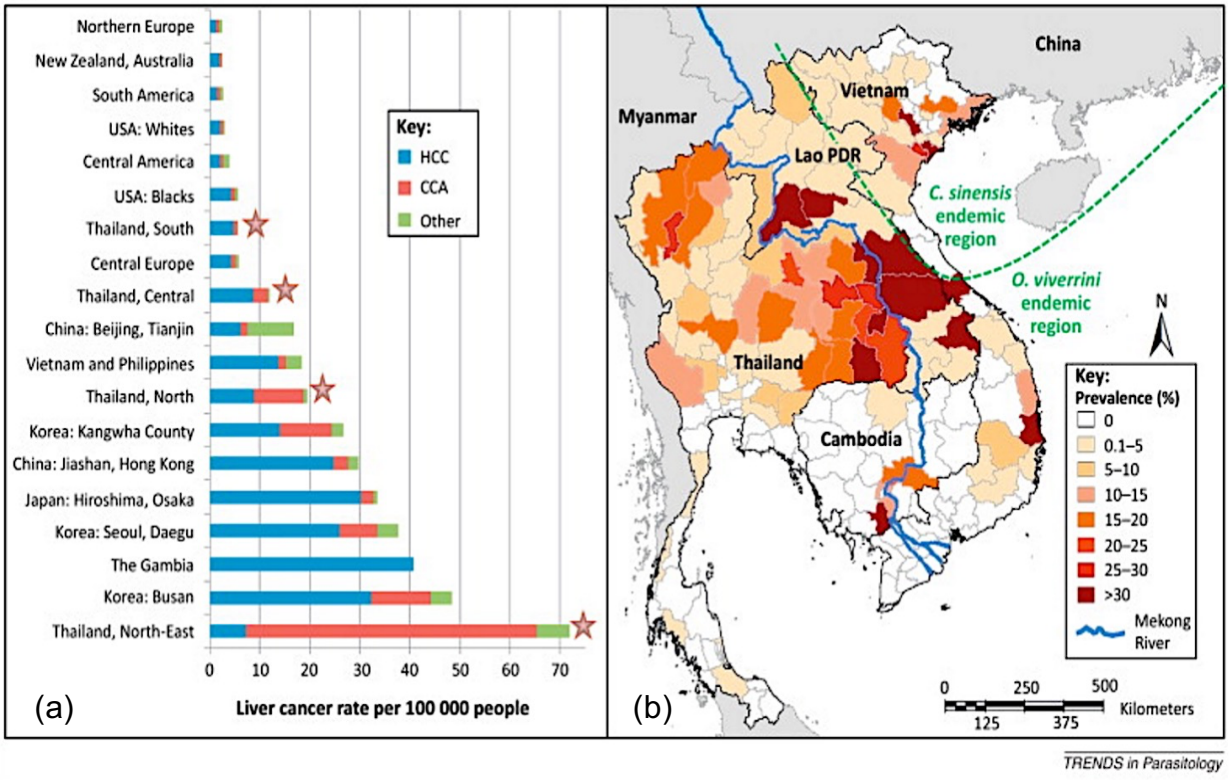


Figure 1.7 Incidence of liver cancer and prevalence of liver fluke infections.

(a) Liver cancer rates are divided into the major subtypes of hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA) and other less common subtypes. Regions within Thailand are highlighted with red stars (Sripa et al., 2012);

(b) The prevalence of *O. viverrini* and *C. sinensis* in the Mekong Basin sub-region. Endemicity level is defined based on prevalence of infections: low, 1-5%; medium, 5.1-15%; high, >15%. Taken from (Sithithaworn et al., 2012).

1.7 Diagnostic methods

Diagnosis of opisthorchiasis and clonorchiasis can be accomplished by several approaches. The three main diagnostic approaches that can be undertaken are (1) direct parasitological diagnosis, being detection of eggs in feces or examination of adult worms; (2) immunological tests; or (3) molecular approaches. An additional test (4) is ultrasonography.

1.7.1 Parasitological diagnosis

Coprological examination: the Kato-Katz technique (WHO, 1991) is the most often used technique to detect the eggs in human fecal samples. However, the

morphological characteristics of eggs of all species of human liver flukes as well as those of minute intestinal flukes are similar; therefore, microscopy has limitations for diagnosis of opisthorchiasis and clonorchiasis in areas where these trematodes and minute intestinal flukes are co-endemic.

Morphological examination of adult flukes: differential diagnosis based on morphological characteristics of adult flukes is possible and widely used (Bray et al., 2008; Kaewkes, 2003). The mature parasites collected from stool following deworming or from necropsy are stained in Semichon's acetic carmine and examined under a light microscope. Morphological criteria on size, shape and location of reproductive organs (ovary, uterus, vitelline glands, testes) are the most important characteristics (Bray et al., 2008; Fried et al., 2004; Kaewkes, 2003).

1.7.2 Immunological tests

Several immunological tests have been developed for clonorchiasis and opisthorchiasis. They are mostly used as supplementary tools for diagnosis. Immunological tests, either using crude antigen, excretory/ secretory antigen or recombinant antigen have shown low specificity (around 70%), due to cross reactions with other parasites, including those between *C. sinensis* and *O. viverrini* (Choi et al., 2003; Nagano et al., 2004; Rim, 2005). However, ELISA using specific purified antigens shows higher values of both specificity and sensitivity than ELISA using crude antigen. ELISA is used as a popular screening tool in endemic areas (Choi et al., 2003; Hong and Fang, 2012; Nagano et al., 2004; Ruangsittichai et al., 2006).

1.7.3 Molecular approaches

Some specific molecular methods (targeting on rDNA and mtDNA) have been developed for identification and differential diagnosis, such as: a mtDNA-based multiplex PCR for discrimination of adult worms of *C. sinensis* and *O. viverrini* (Le et al., 2006); a PCR-RFLP using the mtDNA COI marker to distinguish *O. viverrini* from *Haplorchis taichui* in mixed infection (Thaenkham et al., 2007); a rDNA PCR targeted on the ITS1 and ITS2 regions to discriminate *O. viverrini*, *C. sinensis*, *Haplorchis pumilio* and *H. taichui* on the egg stage; a TaqMan RT-PCR for detection of *C. sinensis* in stool and fish (Cai et al., 2012b; Sato et al., 2009); a

specific PCR for diagnosis of *C. sinensis* infection in final, reservoir and intermediate hosts using the ITS1 and ITS2 of the rDNA (Huang et al., 2012), etc. Although these techniques may reach high values of sensitivity and specificity, they are not widely used in diagnosis, but rather in epidemiological studies.

1.7.4 Sonographical diagnosis

Due to pathological changes in the liver and gall bladder and cholelithiasis in patients with liver fluke infections, ultrasound (US) scans can be used as complementary diagnostic tools in clinical practice, especially in the diagnosis of active liver flukes in endemic areas and in heavy or chronic infections in individuals. Because of the low cost and the ease to practice, US scans are widely used in screening of *O. viverrini* infections in Thailand. Several researches on using US scans in the diagnosis of *O. viverrini* infections showed that infection with *O. viverrini* can lead to changes in ultra-sonographic features in the liver that are different from other parenchymal liver diseases; and the grades of fibrosis can exactly indicate the burden and chronic status of the *O. viverrini* infection (Choi et al., 2004; Mairiang, 2017; Mairiang et al., 2012).

1.8 Epidemiology

1.8.1 Geographical distribution, prevalence and species composition

O. viverrini is highly endemic in the Mekong Basin, including Lao PDR, Thailand, Cambodia, South and Central Vietnam and Myanmar. The prevalence is as high as 60% in Nakhon Panom Province (Thailand) and the total number of infected cases in Thailand is estimated at 6 million (Aung et al., 2017; Bouvard et al., 2009; Sithithaworn et al., 2012). In Lao PDR, 98% of the population is at risk of *O. viverrini* infection (WHO, 2008).

O. felineus is endemic in Siberia, Russia and parts of Europe, where the number of infected people is estimated at 1.2 million (Armignacco et al., 2008; Keiser and Utzinger, 2009).

C. sinensis is most prevalent in China, South Korea, the Amur River Basin, Japan and North Vietnam. The number of infected cases is estimated at 12 million and 1.3 million in China and Korea respectively.

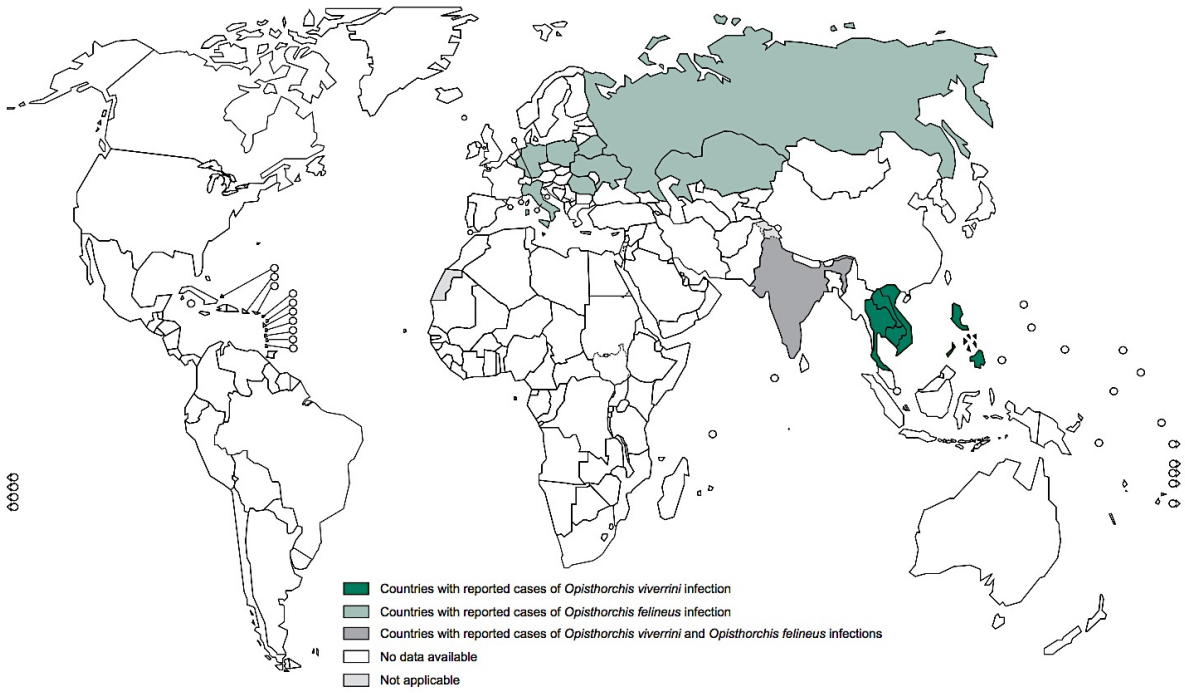


Figure 1.8 Worldwide distribution of opisthorchiasis, 2017.
(http://www.who.int/foodborne_trematode_infections/Distribution_of_opisthorchiasis_worldwide_1280x876px.png?ua=1)

1.8.2 Risk factors

Opisthorchiasis and clonorchiasis mainly occur in areas where aquaculture is practiced, where there is a habit of eating raw or undercooked fish and where low sanitation conditions prevail. Endemic areas are mainly in developing countries at the level of commune or district; inhabitants living closely to streams, ponds and irrigations connected to nearby rivers are particularly affected. Humans, dogs, cats and other fish eating mammals are the final hosts of these liver flukes. Dogs and cats may act as important reservoir hosts. Humans of any age and any gender are infected, but humans between 40 to 50 years old, and men are most infected (Aunpromma et al., 2012; Chai et al., 2005a; De et al., 2003; Grundy-Warr et al., 2012; Nguyen and Le, 2011; Sithithaworn et al., 2012; Sripa et al., 2007). Recently,

raw fish sharing and level of education were recognized as additional risk factors of *O. viverrini* and *C. sinensis* infections (Hoang et al., 2017; Saenna et al., 2017).

1.9 Opisthorchiasis in Vietnam

1.9.1 An overview on O. viverrini *and* C. sinensis *in Vietnam*

In Vietnam, both *O. viverrini* and *C. sinensis* have been reported as co-existing with an estimation of two million infections. However, *O. viverrini* is known as occurring typically in the South and Central parts, but not in the North where *C. sinensis* is endemic (see Fig 1.7b) (Nguyen and Le, 2011; Sithithaworn et al., 2012; WHO, 2008).

The key risk factor of the infection with *O. viverrini* is the habit of consuming a raw fish dish called 'goi ca' by the local population. There are some differences in the way of eating raw fish between Central and North Vietnam. In the North raw fish is sliced and mixed with rice powder, while in the Centre, residents consume whole live small fish. Unfortunately, the silver carp, the most favorite fish species for raw fish preparation in the North was identified as having the highest occurrence and prevalence of *C. sinensis* metacercariae; while 'diec' fish (crucian carp - *Carasius auratus*), a small wild freshwater fish that is served alive in Central Vietnam has the highest prevalence of *O. viverrini* metacercariae (Dao et al., 2016b; Phan et al., 2010a).

Even though documents show that 21 northern provinces and four central provinces are endemic for *C. sinensis* and *O. viverrini,* respectively, at prevalences up to 40% (Nguyen and Le, 2011; WHO, 2008), until recently few studies have been conducted on clinical implications and on the transmission of those cancerogenic liver flukes in Vietnam. Recently, several reports on the prevalence of *C. sinensis* infection in humans, the range of fish host species serving as the second intermediate host of *C. sinensis*, and reservoir host species were published, mainly in the framework of the FIBOZOPA project (A Danish Project on zoonotic trematodes transmitted to human from fish) (Do et al., 2007; Nguyen et al., 2015; Nguyen et al., 2010; Nguyen, 2005; Phan et al., 2010a; Phan et al., 2010b). There are almost no studies conducted on *O. viverrini* in Vietnam.

1.9.2 Opisthorchiasis in Central Vietnam: status and transmission conditions

O. viverrini was first discovered in Central Vietnam in 1991, after which it was confirmed to be an endemic parasite in the region by its occurrence in several central provinces. Nevertheless, only sporadic local researches on fish hosts, focusing only on 'diec' fish were conducted. No studies confirmed *O. viverrini* infection in the first intermediate Bithynia hosts (Nguyen, 2005; Pham and Nawa, 2016; WHO, 2008).

A typical village in Central Vietnam (Fig 1.9) is organized in a different way than in the North: in northern villages, most households own a private fish pond near the house (Phan et al., 2010a); in the South, household farms locate along a 'bau' that is shared by the commune. 'Bau' or lake is a complex of irrigation canals, rice fields and small ponds. Flooding occurs all over the 'bau' in the rainy season that spans from September to December, turning the area into a big lake during this period. Freshwater activities of the commune, such as rice and vegetable production, and duck farming, typically occur in the 'bau'. A wide diversity of freshwater snails and wild fish species susceptible to *O. viverrini* as intermediate hosts inhabit the 'bau'. Domestic dogs and cats usually roam around the lake and feed on fish and kitchen leftovers containing fish. Toilets in the houses generally have no sedimentation tank but the feces is disposed of directly into the 'bau'. The population living around the 'bau' has fish on the daily menu; 'diec' fish is eaten mainly on special occasions and mainly by men (Dao et al., 2016b). Those conditions create an ideal setting for the *O. viverrin*i life cycle and a dynamic transmission.

1.9.3 New finding of an Opisthorchis *sp. in duck in Central Vietnam*

In 2012, we reported on the occurrence of a new *Opisthorchis* species in ducks in the South-Central Coast region of Vietnam (Dao, 2012) (Fig 1.10 & Fig 1.11). This finding not only increases the number of *Opisthorchis* species described so far, but it is also the first record of an *Opisthorchis* sp. naturally infecting birds in Central Vietnam.

In November 2009, important mortality of ducks in Central Vietnam was reported by the local veterinary service. All flocks of ducks were vaccinated with avian influenza vaccine according to the Campaign of National Vaccination. At necropsy, numerous

small flukes were recovered in the bile ducts of these ducks. These flukes were obtained by Dr. Nguyen Thi Giang Thanh – National Institute of Veterinary Research Vietnam, and were carmine stained at the Institute of Tropical Medicine Antwerp, Belgium for morphological analysis. These preliminary morphological analyses revealed some morphological features of *O. viverrini* that occurs in the region, but also displayed some differences. It was temporary called *Opisthorchis viverrini* – like.

Figure 1.9 A picture of a typical rural villages in dry season in Central Vietnam.

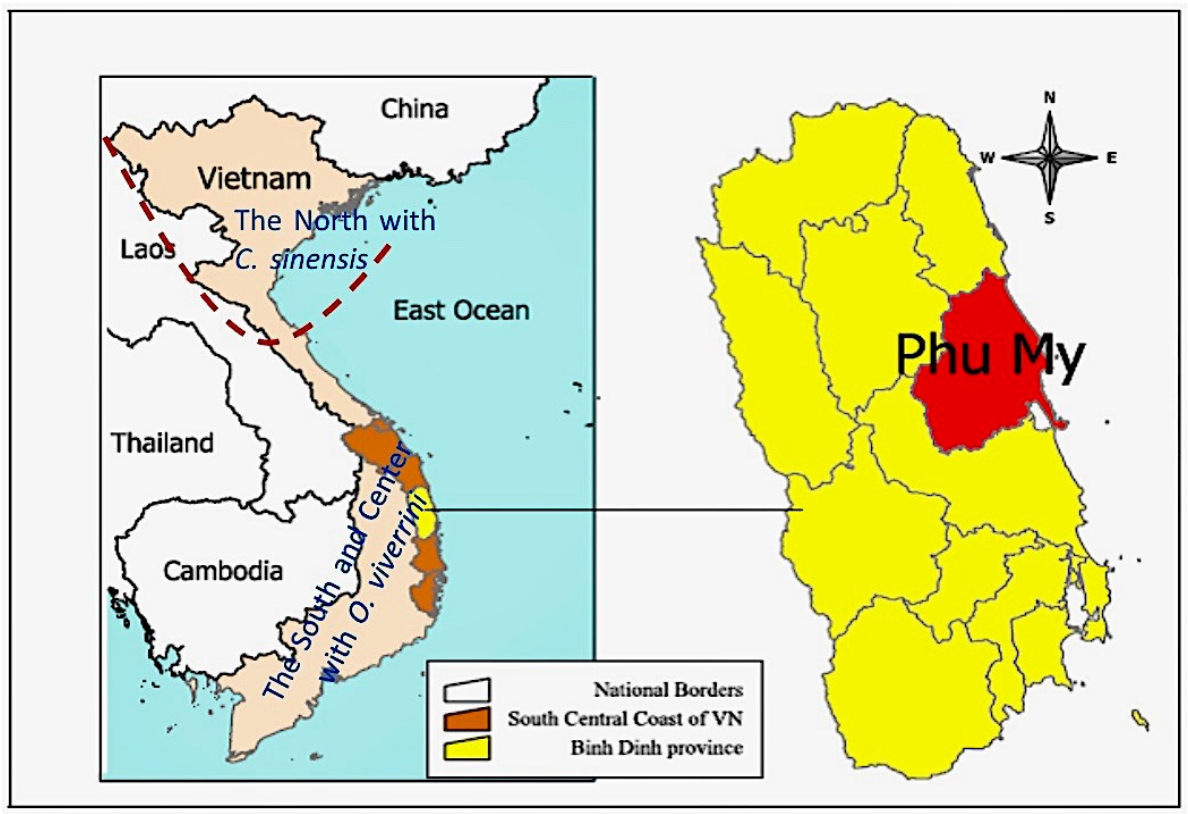


Figure 1.10 Localization of the place where the *O. viverrini*-like was identified in Binh Dinh Province, Central Vietnam (14°05.09' to 14°24.23' N, 108°57.50' to 109°17.44' E).

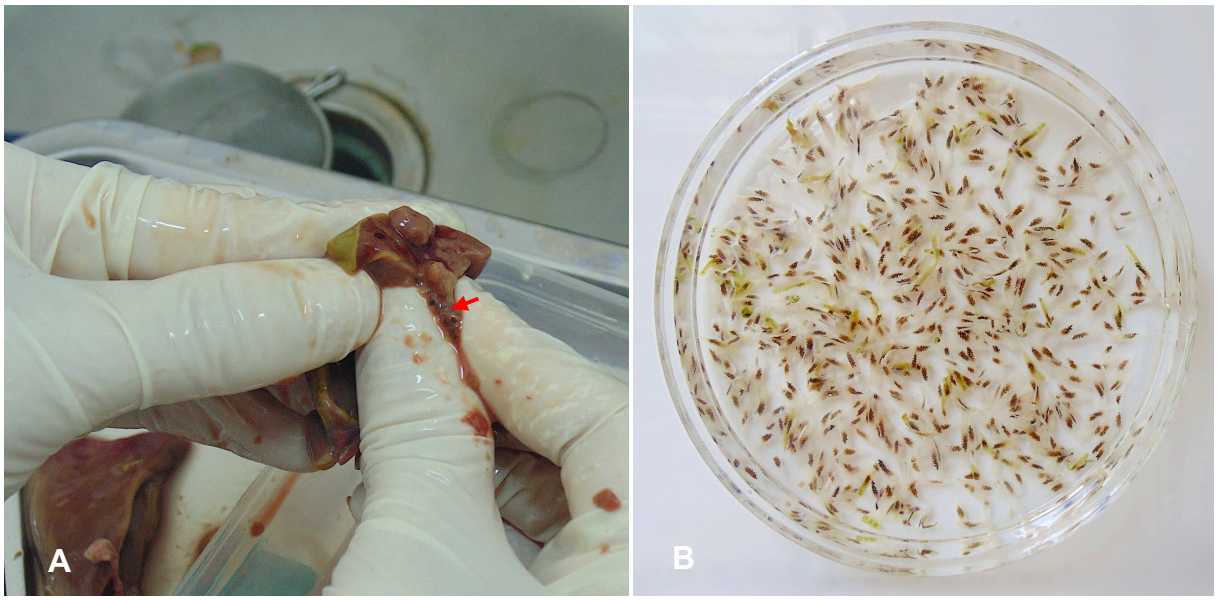


Figure 1.11 *Opisthorchis* species isolated from the bile ducts of a domestic duck (*Anas platyrhynchos)* in Central Vietnam.

1.10 Control and prevention

Avoiding consumption of eating raw or inadequately cooked fish is the most practical way to prevent humans from infection with small liver flukes. On the other hand, constructing hygienic toilets with a sedimentation reservoir and a good management of reservoir domestic animals will avoid the contamination of fishponds with egg-infected feces, contributing to breaking the life cycle. Controlling opisthorchiasis and clonorchiasis in endemic areas only by mass praziquantel administration (MDA) of the human population not only fails but also boosts environmental contamination with the parasite eggs. In addition, reinfection of hosts in a contaminated environment occurs very fast. Only one year after praziquantel-MDA, the incidence in the population of a village in Thailand reached 94%, compared to 97.4% before MDA. The combination of praziquantel-MDA, improvement of the hygienic conditions and health education are significant keys to reduce transmission of human liver fluke infection (Buisson, 2017; Choi et al., 2004; Rim, 2005; Sithithaworn and Haswell-Elkins, 2003; Sripa et al., 2017).

RATIONALE AND OBJECTIVES

Rationale

The discovery of an *Opisthorchis viverrini*-like fluke in domestic ducks in an *O. viverrini* endemic area in Central Vietnam revealed the complex situation of the epidemiology of opisthorchiasis in Vietnam due to the co-existence of two related genotypes. This finding raised many research questions:

1. What is the occurrence of opisthorchiasis in human and birds in Central Vietnam? Does the *O. viverrini*-like genotype occur in humans?

2. What is the position of the *O. viverrini*-like genotype in the phylogenetic tree of the Opisthorchiidae family?

3. Do the two genotypes of *O. viverrini* occurring in the same endemic area share the same intermediate hosts and does this have epidemiological consequences?

Objectives

The general objective of this thesis is to study the occurrence and life cycle of an *O. viverrini-like* fluke, recently identified in domestic ducks in Central Vietnam; thereby assessing the epidemiological complexity of the co-existence of the human and duck *O. viverrini* genotypes in Binh Dinh Province Central Vietnam.

Specific objectives:

1. To estimate the prevalence and associated risk factors of opisthorchiasis in humans in Central Vietnam,

2. To morphologically and molecularly identify the *O. viverrini-like* fluke, a new finding in the liver of domestic ducks in an *O. viverrini* endemic area, and clarify its position in the phylogenetic tree of the Opisthorchiidae family,

3. To estimate the prevalence and intensity of infection of *O. viverrini*-like flukes in domestic ducks in Binh Dinh province,

4. To clarify the range of intermediate hosts by studying the prevalence and intensity of *O. viverrini* and *O. viverrini-like* infections in snails and fish hosts.

CHAPTER 2

OPISTHORCHIASIS IN HUMANS IN AN ENDEMIC AREA OF BINH DINH PROVINCE CENTRAL VIETNAM

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Opisthorchis viverrini infections and associated risk factors in a lowland area of Binh Dinh Province, Central Vietnam

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2.1 Introduction

Opisthorchis viverrini, a trematode belonging to the family Opisthorchiidae causes opisthorchiasis, a potentially fatal fishborne zoonotic infection in humans and carnivores in the Mekong Basin in the South East Asian region (Andrews et al., 2008; Sithithaworn et al., 2012). More than 8 million people are infected with this parasite in Thailand, mainly in the northeastern region; and 98% of Lao PDR's population is at risk of infection because of the habit of eating raw fish dishes (Sripa et al., 2011; Sripa et al., 2007; WHO, 2008). A strong association was found between opisthorchiasis and cholangiocarcinoma (CCA), or bile duct cancer. In Thailand, it was found that the reported number of liver cancer cases in men in the opisthorchiasis endemic areas was 20 times higher than in non-endemic areas (Bouvard et al., 2009; Sripa et al., 2012). In that country, economic losses resulting from this disease account for 120 million USD annually (Andrews et al., 2008).

While *O. viverrini* and opisthorchiasis are well documented in Thailand (Chaiputcha et al., 2015; Chudthaisong et al., 2015; Kaewpitoon et al., 2012), Lao PDR (Forrer et al., 2012; Sripa et al., 2011) and Cambodia (Yong et al., 2012), which are considered *O. viverrini* endemic areas in the international literature, Vietnam is usually not mentioned as an endemic area in the region because "no data are available" (Andrews et al., 2008). More attention in this country has gone to the related *Clonorchis sinensis* that is endemic in the northern part and has a very similar lifecycle and epidemiology, and to fish-borne intestinal flukes (Do et al., 2007; Nguyen et al., 2015; Nguyen and Le, 2011). However, there are some local reports on the presence of *O. viverrini* infections in Central Vietnam (Nguyen et al., 2009). A lack of knowledge on *O. viverrini* infection in Vietnam may hinder prevention and control programs of this fluke. Therefore, a cross-sectional survey was conducted in My Tho commune, in the lowland area of Binh Dinh Province, Central Vietnam where the local population has maintained the culinary habit of eating raw fish dishes. The objective of this study was to investigate the prevalence and associated risk factors of *O. viverrini* infections, aiming at confirming the opisthorchiasis endemic status in this part of Vietnam.

2.2 Materials and methods

2.2.1 Study area

Binh Dinh Province is in the South-central coast region of Vietnam and is composed of 11 districts. The province is divided into the highland region along the western border, and lowlands in the center and along the coast (Fig 2.1). Most of the population of the province lives near the coast. The annual average temperature and rainfall are 26 °C and 1935 mm, respectively, with a dry season from January to August, and a rainy season from September to December, with mostly serious flooding in December because of tropical storms. People in the province mainly live on agriculture, including rice cultivation, raising of livestock and poultry, and fish production by sea fishing and aquaculture. Fresh water in the province mainly comes from four big rivers including: the Kon River, Lai Giang River, Ha Thanh River and Latinh River, of which three rivers support fresh water for the lowlands. In addition, 49 artificial freshwater reservoirs support freshwater requirements during the long dry season in the province.

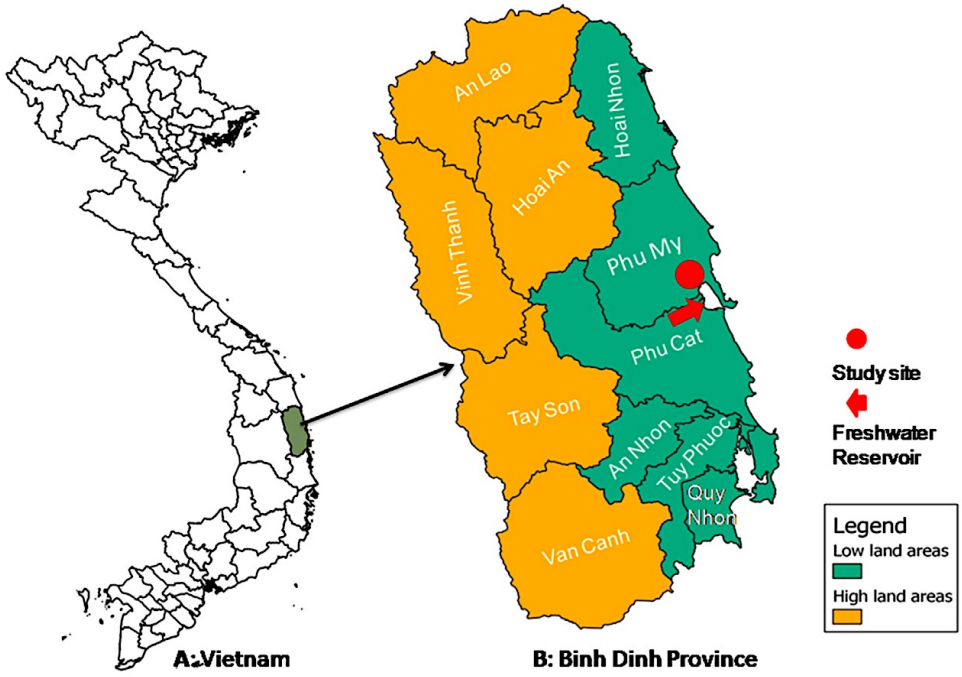

Figure 2.1 Map of Vietnam showing the location of Binh Dinh Province and My Tho commune in Phu My District where the *Opisthorchis viverrini* study took place in June 2015.

Three villages, Chanh Trach 1 (14° 13′ 46″ N, 109° 10′ 25″ E), Chanh Trach 2 (14° 13′ 44″ N, 109° 10′ 39″ E) and Chanh Truc (14° 13′ 06″ N, 109° 10′ 07″ E) of My Tho Commune (14° 13′ 23″ N, 109° 9′ 16″ E), a typical lowland area of Binh Dinh Province, were included in the study. These villages are located northeast of the Freshwater Reservoir in the basins of the Latinh River. These villages were selected based on, (i) previous records on *O. viverrini* infections diagnosed at the IMPE Quy Nhon hospital; (ii) representativeness of typical Central Vietnamese lowland villages characterized by the proximity of irrigation canals from which freshwater fish is caught; (iii) maintenance of the tradition of eating raw fish dishes, which is typical for rural areas in lowland Central Vietnamese provinces (ranging from Quang Tri to Nha Trang and Phan Rang Provinces).

A cross-sectional survey was conducted in June 2015 to investigate the *O. viverrini* infection status in the area and to determine the associated risk factors of the disease.

2.2.2 Sample size and sampling

The sample size was calculated based on an expected prevalence of *O. viverrini* infection of 8% (Nguyen et al., 2009), a confidence level of 95%, a desired absolute precision (d) of 0.05 and using the following formula: $n = p(1 - p) \times 1.96^2 / d^2$ (Thrusfield, 2013). Since individuals have similar raw fish consumption habits within villages, a correction factor of magnitude 2 (Wagner and Salman, 2004) was applied to account for the clustering of individuals within villages. In addition, contingencies were adjusted for by adding another 15% of individuals leading to a total of 254 persons to be fecal sampled. A questionnaire was administered to all individuals to determine the potential risk factors associated with the disease.

The sampling frame was the list of all administrated households in the 3 villages (total: n = 1015): Chanh Trach 1, 165 households, Chanh Trach 2, 239 households, and Chanh Truc, 611 households; from which 254 households were randomly selected by the proportionate stratified sampling of households in each village. One randomly selected member of each household, older than 7 years of age and with the ability to understand and respond to the study requirements was invited to

participate in the study. Each study participant provided one stool sample for coprological examination.

A questionnaire was then administered to the selected members in each household. Researchers in the study read the questions and invited the participants to answer, and to sign the form upon completion of the questionnaire. The questionnaire addressed the following points: name, address, gender (male/ female), age (< 18 years/ 18–60years/ >60 years), occupation (agriculture/ others), knowledge of *O. viverrini* (know *O. viverrini*: yes/ no; know *O. viverrini* transmission: yes/no; know *O. viverrini* harmfulness to human health: yes/ no), eating raw fish (yes/no), how often (usually/ sometimes/ rarely/ never), which type of fish prepared for raw fish dish (small wild caught fresh water fish/ sea fish/ both), where the fish dish was consumed (at home/restaurant/relative & friend home/ both), history of examination and treatment for *O. viverrini* infection (examination or dewormed before: yes/ no). All collected information was used for the evaluation of risk factors associated with *O. viverrini* infection.

2.2.3 Stool examination

Stool samples were examined within 12h after collection by the Kato-Katz technique as described by World Health Organization (1991) (WHO, 1991). In brief, two Kato-Katz smears were prepared from each stool sample. Then, both fecal slides were examined by light microscopy (×100) by the same examiner. *Opisthorchis*-like eggs were identified and enumerated. The average number of counted eggs from two slides was multiplied by 24 to obtain the number of eggs per gram (EPG) of feces. Intensity of *O. viverrini* infection was scaled as follows: light infection: EPG < 1000, moderate infection: EPG from 1000–10,000 and heavy infection: EPG > 10,000 (Maleewong et al., 1992). Prevalence of *O. viverrini* infection is considered low (<10%), moderate (10–15%) or high (>15%) (Sithithaworn et al., 2012).

2.2.4 Adult O. viverrini *expulsion*

Eleven persons who were positive for *Opisthorchis*-like eggs at fecal examination and who were willing to participate in the procedure of worm expulsion were treated under the responsibility of local physicians. The patients were asked to eat a light liquid meal in

the evening before treatment. The following morning, they were given oral praziquantel 400 mg (Distocide®, Shin Poong Phar- maceutical Co. Ltd., Seoul, Korea) at a dosage of 40 mg/ kg; 1h later, they were given a solution of 30 g of $MgSO_4$ dissolved in 100 ml pure water. Subsequently, 3–4 consecutive post-treatment stools were collected. Worms were recovered by a series of washing steps (Chai et al., 2005b; Do et al., 2007). Patients who did not consent to the procedure of adult worm expulsion were given a free praziquantel treatment.

2.2.5 Identification of recovered adult worms

Adult worms were kept in 70% ethanol for morphological and molecular analysis. Twenty recovered worms were randomly selected and stained with Carmine dye (based on Semichon's acetic carmine 1929). Then, morphological identification was made by using published taxonomic references on *O. viverrini* (Bray et al., 2008; Kaewkes, 2003).

The DNA was extracted separately from two fresh worms using the Gentra Puregene Kit (Qiagen, ref: 1042606). Two pairs of primers 3S & BD2 (Bowles et al., 1993) for ITS2 marker and COI- Ov-Hap F & R (Thaenkham et al., 2011b) for COI marker were used to amplify the portions of the ITS2 and the COI gene, respectively. The PCR products were visualized on agarose gel 1.5% and sent to the VIB Genetic Service Facility (University of Antwerp, Belgium) for sequencing. The obtained DNA sequences of the COI and the ITS2 markers after editing in Bioedit (Hall, 1999) were aligned to sequences of *O. viverrini*, which are deposited in Genbank (HQ 328542, the COI DNA sequence & HQ 328548, the ITS2 DNA sequence).

2.2.6 Statistical analyses

Descriptive statistics were used to describe the overall prevalence of *O. viverrini* infection and the prevalence by gender, age groups, occupation, knowledge on *O. viverrini,* habit of eating raw fish dishes, place where the consumption of raw fish took place, type of fish consumed, history of *O. viverrini* infection and treatment, and intensity of EPG from fecal egg count.

Each participant who was positive for *Opisthorchis*-like eggs was assumed to be infected with *O. viverrini*. To determine the potential risk factors associated with *O.*

viverrini infection, a two-stage modeling approach was used: First, a univariate analysis was performed to determine the strength of the association between *O. viverrini* infection status and each risk/ indicator factor using Fisher's exact test (for variables with cell frequencies less than 5 following cross-tabulation with *O. viverrini*-positive/ negative cases) or a Chi square test (for variables with cell frequencies ≥ 5).

Secondly, variables with $p \leq 0.10$ in the univariate analysis were further analyzed using a multivariable Firth's logistic regression analysis to overcome the computational limitations and convergence issues caused by the sparseness (separation) of the data (Heinze and Schemper, 2002). A manual forward stepwise model building approach was employed with the Akaike's Information Criterion (AIC) as the calibrating parameter to select the final model. Age, which was a non-significant variable in the univariate analysis but which has been reported as a strong risk factor for *O. viverrini* infection in previous studies was added to the final model. The models were built using the firthlogit function (Firth, 1993) in STATA, version 13, software (StataCorp LP, College Station, TX). The statistical significance level in the final model was set at $p \leq 0.05$.

2.2.7 Ethical approval

The study was approved by the ethical committee of the Institute of Malariology, Parasitology and Entomology, Quy Nhon, Vietnam (reference number 441/IMPE − IRB) and the Institutional Review Board of ITM Antwerp (reference number 1064/15). Written consent was obtained from each participant during the study. Before sampling in each village, village meetings were organized. An information sheet in local language detailing the aims, procedures, potential risks and benefits of the study was read to the villagers. A member of each household who was willing to participate in the study was asked to sign the consent form. Participants found positive for *O. viverrini*-like eggs by stool examination were invited for a praziquantel and purgation treatment for the recovery of adult worms, as described above. Non-consenting stool- examination positive individuals were given praziquantel tablets for a single dose treatment of 40 mg/kg (WHO, 1991).

2.3 Results

In 29 of the 254 collected stool samples, *O. viverrini*-like eggs were recovered, which is an apparent prevalence of 11.4% (CI: 8–16%) (Table 2.1). All positive cases had light infections as assessed by fecal egg counts, in the range of 96–720 EPG (Mean arithmetic EPG for the entire population = 30.5). Eggs of intestinal nematodes (hookworm and *Ascaris lumbricoides*) were found in only a few samples and at low numbers. From the 29 individuals shedding *O. viverrini*-like eggs, 11 consented to enroll in the treatment and expulsion group. Adult flukes were recovered from the stools of all 11 treated individuals. The number of recovered worms by expulsion ranged from 2 to 44, average of 14.5 worms per expulsion case. The number of recovered worms is positively correlated with the fecal egg output (EPG) (R^2 = 0.806) (Fig 2.3). The adult expelled worms were identified as *O. viverrini* by morphological (Fig 2.2) and by molecular analyses. The ITS2 DNA sequences of the recovered worms (ID numbers: KT 726408 and KT 726409) are 100% identical to the *O. viverrini* ITS2 DNA sequence ID: HQ 328548 after excluding all the gap sites. And the COI sequences of the recovered worms (ID numbers: KT 726410 and KT 726411) are identical to the COI DNA sequence ID: HQ 328548 at 98.8% with 3 different nucleotides from the compared DNA sequence's length of 249 nucleotides.

More than half (53.5%) of the participants were male; the mean age of the participants was 40 (SD = 17.6) years. Most subjects (92.1%) were farmers and most (78.3%, 81.1% and 85.4%) did not have any knowledge of *O. viverrini* infection, *O. viverrini* transmission and *O. viverrini* harmfulness, respectively. Almost half of the participants (45.3%) had the habit of eating raw fish. In contrast, only a small proportion had a history of *O. viverrini* examination (8.7%) and treatment (5.9%).

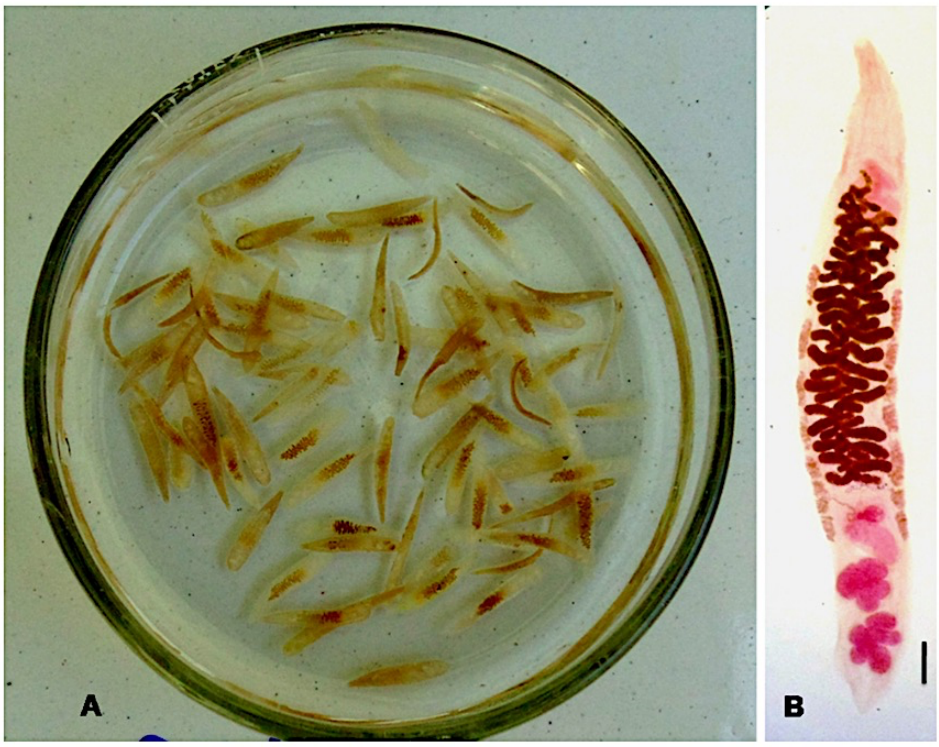

Figure 2.2 Adult *Opisthorchis viverrini* recovered from the patients (ages from 31 to 59) in My Tho Commune of Phu My District, Binh Dinh Province: (A) Fresh worms; (B) Carmine stained worm, with the scale bar = 0.5 mm.

Participants who had eaten raw fish dishes prepared from small wild caught freshwater fish and who had consumed the dish at a friend/relative's home, had the highest percentage positives of *O. viverrini* infection, 35.2% and 37.5%, respectively. People who had eaten raw fish dishes prepared from small wild caught freshwater fish (Fig 2.4) and who had consumed the dish at all places including home, restaurant and friend/relative's home had the highest fecal egg counts (Mean of 89 and 101 EPG, respectively). The highest number of worms recovered fell in the group of participants who ate both types of fish dishes (No. of worms recovered ranged from 36 to 44).

Table 2.1 Prevalence and intensity of *O. viverrini* infections and potential risk factors associated with the infection in My Tho Commune, a lowland area in Binh Dinh Province, Central Vietnam (n = 254)

Category	N (Pos)	N	Prevalence (%)	Intensity of EPG		Univariate analysis	
				Mean ± SD	Max	OR (95% CI)	*p*-value
Gender							
Male	26	136	19.1	52.4 ± 142	720	9.1 (2.7 - 30.6)	< 0.001
Female	3	118	2.5	5.3 ± 37	360	1	
Age (years)							
<18	2	43	4.7	8.4 ± 40	240	1	0.926
18 - 60	22	178	12.4	33.0 ± 115	720	1 (0.2 - 5.2)	
>60	5	33	15.2	46.5 ± 138	624	1.2 (0.6 - 4.0)	
Occupations							
Farmer	27	234	11.5	32.1 ± 113	720	1.2 (0.3 - 5.3)	1
Others	2	20	10.0	12.0 ± 44	192	1	
Knowledge of							
Ov infection	14	55	25.5	65.5 ± 150	624	4.2 (1.9 - 4.4)	< 0.001
None	15	199	7.5	20.9 ± 94	720	1	
Ov transmission	13	48	27.1	67.5 ± 153	624	4.4 (2.0 - 10.0)	< 0.001
None	16	206	7.8	21.9 ± 95	720	1	
Ov harmfulness	12	37	32.4	82.4 ± 169	624	5.6 (2.4 - 13.2)	< 0.001
None	17	217	7.8	21.7 ± 94	720	1	
Eating RFD							
Yes	29	115	25.2	67.4 ± 155	720	Omitted	< 0.001
No	0	139	0	0	0	1	
Which fish							
Sea fish	2	24	8.3	5.8 ± 21	96	Omitted	< 0.001
Small wild fish	25	71	35.2	88.6 ± 162	672	1	
Both	2	20	10.0	66.0 ± 204	720	6 (1.3 - 27.5)	
Where to eat							
Home	10	48	20.9	40.5 ± 112	624	1.2 (0.2 - 9.6)	< 0.001
Restaurant	0	6	0	0	0	1	
Fri/Rel home	6	16	37.5	78.0 ± 160	624	2.3 (0.7 - 7.8)	
All	13	45	28.9	101.3 ± 195	720	1.5 (0.6 – 4.0)	
History of							
Exam with Ov	5	22	22.7	37.1 ± 89	360	2.5 (0.9 - 7.5)	0.081
None	24	232	10.3	30.0 ± 112	720	1	
Ov treatment	3	15	20.0	17.6 ± 43	144	2.0 (0.5 - 7.7)	0.391
None	26	239	10.9	31.3 ± 113	720	1	
Total	**29**	**254**	**11.4**	**30.5 ± 109**	**720**		

Note: (Pos) = positive, Ov =*Opisthorchis viverrini*, EPG = number of eggs per gram in the feces, SD = standard deviation, RFD = raw fish dish, Fri/Rel home = friend or relative's home, OR = odds ratios, 95% CI = 95% confidence intervals.

Results of the univariate logistic regression analysis (Table 2.1) showed that factors including, gender, knowledge about *O. viverrini* and *O. viverrini* infection, habit of eating raw fish dishes, type of fish, place where the fish dishes were consumed, and history of examination for *O. viverrini* infection were significantly associated with *O. viverrini* infection ($p < 0.10$). In contrast, factors such as age, occupations and history of *O. viverrini* deworming were not significantly associated with the infection.

Table 2.2 Associated risk factors for *O. viverrini* infections in a lowland My Tho Commune of Binh Dinh Province, Central Vietnam (n = 254)

Factors		N (Pos)	N	OR	95% CI	*p*-value
Gender						
	Female	3	118	1		
	Male	26	136	5.0	1.48 - 16.96	0.009
Type of fish						
	None	0	139	1		
	Sea fish	2	24	20.1	0.91 - 444.50	0.058
	Small wild fish	25	71	106.8	6.21 - 1801.50	0.001
	Both	2	20	22.2	0.98 - 500.20	0.051
Age						
	< 18	2	43	1		
	18 – 60	22	178	1.1	0.94 - 5.68	0.940
	> 60	5	33	1.1	0.16 - 7.46	0.930

Note: Pos = positive, 95% CI = 95% confidence intervals, OR = odds ratio.

The result of the final model in the multivariate Firth's logistic analysis (Table 2.2) showed that overall, gender and type of fish, which was used for the raw fish dish were significantly associated with *O. viverrini* infections. The odd of being infected by *O. viverrini* for the male gender was 5.0 (95% CI: 1.48–16.96, $p = 0.009$) times higher than for female. People who ate raw fish dishes prepared from small wild caught freshwater fish had odds of being infected with *O. viverrini* that were 106.8 (95% CI: 6.21–1801.5, $p = 0.001$) in comparison to those who had never eaten these dishes. The knowledge on *O. viverrini*, places where the raw fish dishes were

consumed, and a history of *O. viverrini* examination were not significantly associated with *O. viverrini* infection.

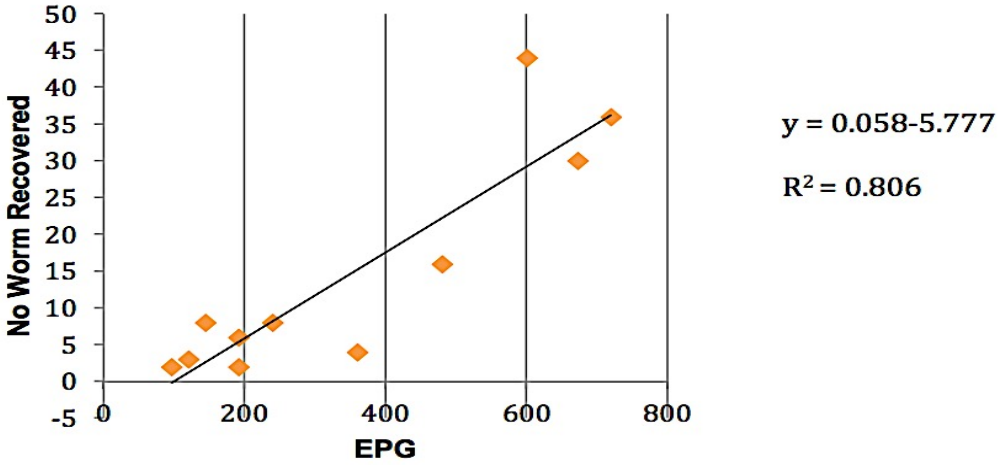

Figure 2.3 Correlation between EPG (eggs per gram of feces) and number of *Opisthorchis viverrini* worms recovered from stools from consenting individuals following praziquantel treatment and purgation, in My Tho Commune, Phu My District, Binh Dinh Province (n = 11 cases).

Figure 2.4 Live small wild caught freshwater fish ('diec' fish-*Carassius auratus*), red arrow, used in a raw fish dish, sold at a local market in Phu My District, Binh Dinh Province, Central Vietnam.

2.4 Discussion

An overall apparent prevalence of 11.4% of *O. viverrini* infections was found in the investigated population in Binh Dinh Province, Central Vietnam, which is considered a moderate prevalence (Sithithaworn et al., 2012). Among the individuals who declared having eaten raw fish dishes, the prevalence was 25.5%. The villages investigated are representative for the lowland areas of the Central Vietnamese coastal provinces in terms of source and preparation of freshwater fish. The prevalence of *O. viverrini* infection in this study is lower than in a rural area in Thailand (21.6%) and lower than the reported very high prevalence of > 50% in Lao PDR and Cambodia (Forrer et al., 2012; Rangsin et al., 2009; Yong et al., 2012). However, those studies only investigated the presence of *O. viverrini* eggs in fecal samples. Eggs of *O. viverrini* can easily be confused with eggs of *C. sinensis* and minute intestinal flukes (Chai et al., 2005b; Do et al., 2007), potentially leading to an overestimation of *O. viverrini* infection.

In our study, a confirmatory test was done by morphological and molecular identification of adult worms recovered from stools of patients after treatment. All 11 consenting individuals shed adult *O. viverrini* following praziquantel treatment. Although we were able to confirm *O. viverrini* infection in only 11 of the 29 individuals shedding trematode eggs, our findings strongly suggest *O. viverrini* infections in all those people. We found a strong correlation between the fecal egg output and the number of expelled worms in these consenting individuals. *C. sinensis* has until now only been found in North Vietnam (Do et al., 2007) and *Haplorchis* metacercariae have been found in less than 1% of ‘diec’ fish (*Carassius auratus*) in Binh Dinh Province (unpublished results), suggesting a low prevalence of minute intestinal fluke infections. However, the intensity of *O. viverrini* fecal egg counts was low (EPG < 1000). This may be due to the way the fish is eaten and by infection rates and intensities of metacercariae in fish. The local population believes that eating raw ‘diec’ fish, a small freshwater fish that is caught in irrigation canals or rivers during the first part of the dry season (from January to early March annually) will bring them health and reduce illness. They only eat this fish species during a certain time of the year because of its better taste in that season. This is different from other endemic countries where local people eat raw and undercooked

fish dishes year-round (Grundy-Warr et al., 2012; Phongluxa et al., 2013). In addition, while the infection prevalence of *C. auratus* with *O. viverrini* metacercariae is high, the intensity of infection is low (Dao et al., 2017). Recently, local people have started preparing a new raw fish dish with sea fish and mostly sell it as sashimi in the local restaurants. Until now, no studies have shown the presence of *O. viverrini* metacercariae in sea fish. This may explain why in our study, the participants who ate small freshwater fish ('diec' fish) were more at risk for infection with *O. viverrini* than the people who ate raw fish prepared from sea fish.

In some studies on *O. viverrini* infection and associated risk factors, *O. viverrini* infection was found both in people who either have the habit of eating raw fish and in those who had declared not to eat raw fish (Chaiputcha et al., 2015; Chudthaisong et al., 2015). However, in our study, only participants who ate raw fish dishes were found to have opisthorchiasis. This can be explained by the special way the local people prepare raw fish in Binh Dinh. In North Vietnam (typically for *C. sinensis* endemic areas) and in Lao PDR, Cambodia and Thailand, raw fish is sliced or ground by a knife on a cutting board, giving the chance for *O. viverrini* metacercariae to detach from the fish; people can accidentally be infected through contamination of other foodstuff prepared on the board or by the knives (Grundy-Warr et al., 2012; Songserm et al., 2011). The raw fish dish in Binh Dinh area is prepared from live fish. Live fish bought on the local market (Fig 2.4) is washed several times with fresh water before being transferred into a big bowl or plate; next the consumer picks up a fish into a small bowl adding lemon, chili and some spices; 'cooked' live fish is now ready for consumption. Fish is thus never cut and consequently kitchen materials are not contaminated with metacercariae.

Knowledge on *O. viverrini* and opisthorchiasis had no positive effect on infection rate in the community. Even when people are aware of the risk of infection with liver fluke when eating raw fish, they do not associate it with a serious disease and trust that alcohol and lemon will kill bacteria and parasites. They also rely on anthelmintics, as such they will not change their behavior after having been infected and treated. They are not aware that liver fluke can kill people by causing liver or bile duct cancer. Sriraj et al. (Sriraj et al., 2013a; Sriraj et al., 2013b) demonstrated that alcohol can induce *O. viverrini* metacercariae excystation, leading to early

development of parasites in the hepatobiliary system. The preference of men to eat raw fish and drink alcohol may explain why males are at a significantly higher risk of infection with *O. viverrini*. While age was not a significant risk factor in this study it has been strongly associated with higher infection rates in Thailand (Chaiputcha et al., 2015; Chudthaisong et al., 2015). It would be interesting to investigate other risk factors, such as, latrine availability and use, and assess the relationship between clinical signs and symptoms and opisthorchiasis in endemic areas of Vietnam.

In conclusion, we have demonstrated that opisthorchiasis is currently occurring in a lowland area of Binh Dinh Province, Central Vietnam with a moderate prevalence. Male gender and consumption of raw wild freshwater fish ('diec' fish-*C. auratus*) were found the two main risk factors for *O. viverrini* infection in the communities. Although the investigated villages are representative for lowland areas in central Vietnam, larger scale epidemiological studies in other areas of Central and South Vietnam are needed to estimate the extent of this zoonotic disease in the country. In addition, hospital-based studies should be conducted to assess the public health impact. The role of intermediate hosts (snail and fish) and reservoir hosts (dog, cat and fish eating bird) in the lifecycle should be investigated. This will help establishing a sustainable prevention and control program for this fatal liver fluke. We recently detected an *O. viverrini*-like liver fluke in ducks in Binh Dinh Province with slight morphological and molecular differences compared to the 'human' *O. viverrini* (Dao et al., 2014; Dorny et al., 2015). Although the zoonotic potential of this duck genotype of *O. viverrini* has yet to be demonstrated, the sympatric occurrence of both genotypes in Binh Dinh Province complicates the epidemiological situation and may impact on infections in intermediate and definitive hosts.

CHAPTER 3

DISCOVERY OF A *OPISTHORCHIS VIVERRINI*-LIKE FLUKE IN DOMESTIC DUCK IN AN ENDEMIC AREA OF *OPISTHORCHIS VIVERRINI* IN HUMANS IN CENTRAL VIETNAM

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Opisthorchis viverrini-like liver fluke in birds from Vietnam: morphological variability and rDNA/mtDNA sequence confirmation

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3.1 Introduction

Opisthorchis viverrini, *Opisthorchis felineus* and *Clonorchis sinensis* are liver flukes of man and piscivorous mammals that occur in many parts of East Asia and Eastern Europe. The number of people infected with liver flukes has been estimated at 45 million worldwide and the number of people at risk at over 600 million (Bouvard et al., 2009; Keiser and Utzinger, 2005). Liver flukes are foodborne trematodes that use Bithynia snails and cyprinid fish as first and second intermediate hosts, respectively. The human and animal final hosts become infected by eating raw or undercooked fish infected with metacercariae (Kaewkes, 2003; Nguyen and Le, 2011). In humans, the infection is associated with several hepatobiliary abnormalities. In addition, experimental and epidemiological evidence strongly implicate *O. viverrini* infection in the etiology of cholangiocarcinoma (CCA) or bile duct cancer, which has one of the highest mortality rates of any cancer (Sripa et al., 2012). Dogs and cats are important reservoir hosts of many foodborne trematodes, including *Opisthorchis* spp.; birds are reservoirs of some intestinal fluke species (Aunpromma et al., 2012; Chai et al., 2005a; Nguyen, 2009; Nguyen et al., 2010). In 2003, *Opisthorchis lobatus*, a new species of *Opisthorchis*, was described in the intestines of ducks (*Anas* sp.) in Pakistan, and its metacercariae were found in red-tailed snakehead fish in Laos (Bilqees et al., 2003; Thaenkham et al., 2011b). Up to now, the epidemiology and zoonotic potential of this new species are not known.

In Vietnam, *C. sinensis* is prevalent in the north and 2009, a small *Opisthorchis*-like fluke was found during routine necropsy in the bile ducts of domestic ducks in Binh Dinh province, in the South-central coast region of Vietnam that is endemic for *O. viverrini*. *Opisthorchis* spp. had previously never been recorded in birds in Vietnam. The objective of this study was to establish the taxonomic status of this fluke using morphological and molecular methods.

3.2 Materials and methods

3.2.1 Collection and examination of flukes

Twenty-five adult worms were collected in November 2011 from the bile ducts of two naturally infected domestic ducks (*Anas platyrhynchos)* during routine

necropsy. The ducks originated from Phu My district, Binh Dinh province, Central Vietnam (14.005–14.024' N, 108.057–109.017' E). Eleven worms were flattened between glass slides, fixed in polyvinyl alcohol–fixative–adhesive (AFA) solution and stained with Semichon's acetic carmine solution, before being cleared in xylene and mounted in DPX mountant on a glass slide. Stained worms were examined under a light microscopy: the shape and the sizes of the body, internal organs, suckers and eggs, and the ratios of body length to body width, and egg length to egg width, were considered for morphological examination. The identification key of the Opisthorchiidae family-*Opisthorchis* genus (Bray et al., 2008) and morphological descriptions from several authors (Bilqees et al., 2003; Kaewkes, 2003; Mordvinov et al., 2012; Thaenkham et al., 2011b) were used to compare morphological characteristics of the duck worms with that of liver flukes from humans and other mammals.

3.2.2 Molecular analysis

Genetic markers used in this study include sequences of the second internal ribosomal spacer (ITS2) and the cytochrome c oxidase subunit I (COI) of the mitochondrial DNA, obtained by polymerase chain reaction (PCR) and sequencing. Total genomic DNA was extracted from a single piece of adult worms using the commercial Gentra Puregene Kit, according to the manufacturer's instructions (Qiagen GmbH, Hilden. Germany). Amplification was done from 5 µl of the extracted genomic DNA, using 0.4 µl of each primer: 3S: (5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and BD2 (5' -TATGCTTAAATTCAGCGGGT-3') for the ITS2 marker (Bowles et al., 1993) and COI-OV-Hap-F (5'-GGGTTYGGTATRRTKAGWCAC-3') and COI-OV-Hap-R (5'-AAACCAAGTRTCATGMAACAAAG-3') for the COI marker (Thaenkham et al., 2011b; Thaenkham et al., 2007), and 0.2 µl of GoTaq® DNA polymerase (Promega, Madison, Wisconsin, USA), plus 1.65 µl of $MgCl_2$ 25 mM and 0.2 µl deoxynucleoside triphosphates (dNTP) in a total volume of 25 ml. The PCR amplification was performed as follows: 40 cycles at 94 °C for 15 s for denaturation; annealing 45 s at 50 °C with primers BD2 and 3S and at 52 °C with primers COI-Ov-Hap-F and -R; extension at 72 °C for 45 s, final extension at 72 °C for 10 min. Then, 5 µl of each PCR product was electrophoresed on agarose 2% in 0.5 x TBE buffer at 100 Voltage for 20 min and visualized under ultraviolet light.

Next, PCR amplicons of both markers were purified on a column using QIAquick® PCR Purification Kit (Qiagen), cloned in JM109 Cells of the pGEM® T Vector Systems Kit (Promega) and sent to the VIB Genetic Service Facility (University of Antwerp, Belgium) for sequencing.

The obtained DNA sequences were edited with BioEdit (Hall, 1999), Blasted on NCBI and aligned with sequences from *O. viverrini*, *O. lobatus*, *O. felineus* and *C. sinensis* (Table 3.1). DNA sequences were aligned with the MAFFT L-INS-I algorithm version 7 (Katoh and Standley, 2013). Phylogenetic trees were created with the MAFFT server (http://mafft.cbrc.jp/alignment/server/phylogeny.html) using neighbor joining (NJ; all gap-free sites) and bootstrap resampling set to 1000. The amino acid sequence was derived from the COI DNA sequences using transeq (Rice et al., 2000) to verify whether changes in the DNA also resulted in amino acid changes.

3.3 Results

3.3.1 Morphological analysis

Based on *Opisthorchis*-specific morphological features, such as the presence of two extra-caecal chains of vitelline glands, running between the ventral sucker and the anterior or posterior testes, and the location of the ovary in the posterior third of the body, the duck fluke is to be classified as an *Opisthorchis* species. The duck fluke differs in several morphological characteristics from *O. felineus* and *C. sinensis*, such as the location of the uterus, which overlaps the ventral sucker entirely or partially, and the shape of the testes, which is lobate in *Opisthorchis* spp. and dendritic in *C. sinensis*.

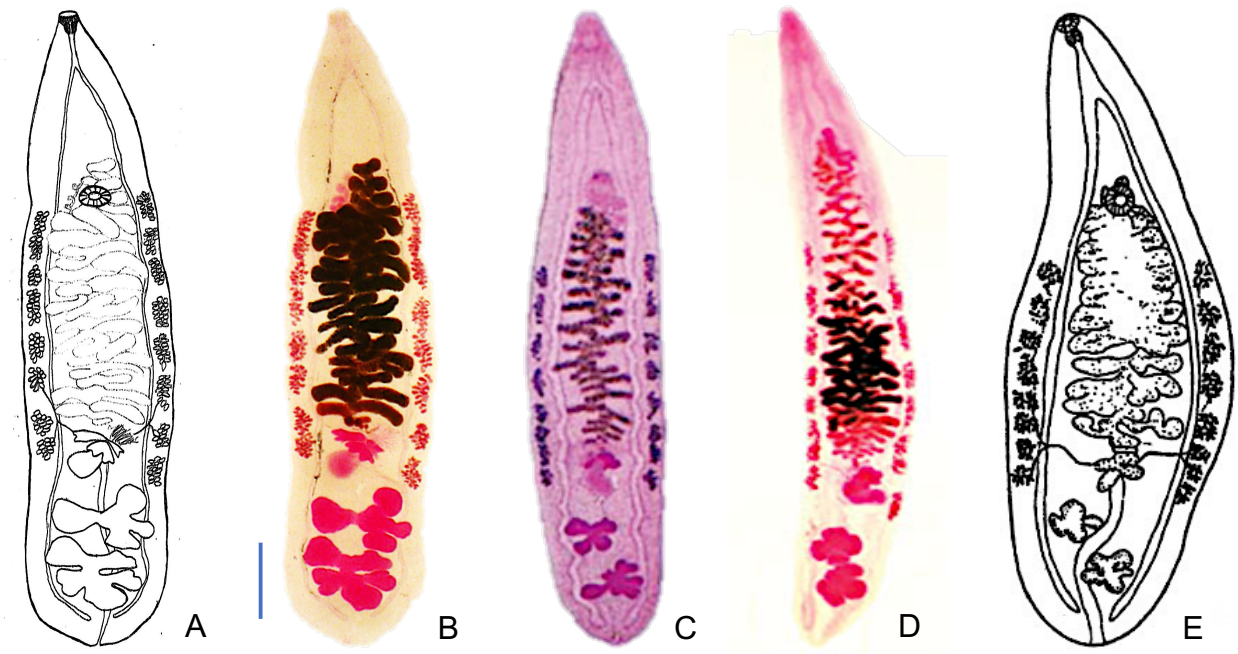

Figure 3.1 Intraspecific morphological variation of *Opisthorchis viverrini* in different hosts.

(A) Schematic drawing of an *Opisthorchis* sp. in birds from Vietnam; (B) *Opisthorchis* sp. in birds from Vietnam(this study); (C) *O. viverrini* in a hamster (Sohn et al., 2011); (D) *O. viverrini* in a man from Cambodia (Sohn et al., 2011); (E) *O. viverrini* in a cat from Vietnam (Le, 2000). Scale bar=1 mm.

The morphological characteristics of the liver flukes of ducks are compatible with those of *O. viverrini* in human hosts, cats and an experimental hamster model (Kaewkes, 2003; Le, 2000; Mas-Coma and Bargues, 1997; Sohn et al., 2011) (Table 3.2 and Fig 3.1). The body of the flukes is transparent and lanceolate; the ventral sucker is larger than the oral sucker, located in the anterior quarter of the body; the uterus, packed with oval-shaped eggs, loops irregularly between the ventral sucker and the ovary; the three-lobed ovary locates slightly sub-median or median in the posterior third of the body; the seminal receptacle is voluminous, sited posteriorly to the ovary. The testes are large and located one after the other (tandem) with their extremities deeply lobed; they occupy almost the entire posterior body's width, overlapping both caeca laterally. The posterior testis is bigger than the anterior one. The excretory bladder lies posteriorly to the testes. The vitelline glands form two chains extra-caecally, and they extend between the ventral sucker and the testis. The caeca end blind in the posterior part of the body.

Table 3.1 DNA sequences of four *Opisthorchis* spp. and *Clonorchis sinensis* used for the alignment and construction of the phylogenetic trees

Species	COI (gi numbers)	ITS2 (gi numbers)
Opisthorchis viverrini	gi|316992170|gb|HQ328542.1| gi|316992172|gb|HQ328543.1| gi|316992174|gb|HQ328544.1|	gi|316992179|gb|HQ328548.1| gi|316992180|gb|HQ328549.1| gi|316992181|gb|HQ328550.1| gi|46411094|gb|AY584735.1|
Opisthorchis lobatus	gi|316992164|gb|HQ328539.1| gi|316992166|gb|HQ328540.1| gi|316992168|gb|HQ328541.1|	gi|316992176|gb|HQ328545.1| gi|316992177|gb|HQ328546.1| gi|316992178|gb|HQ328547.1|
Opisthorchis felineus	gi|151175844|gb|EF688123.1| gi|151175852|gb|EF688127.1| gi|151175854|gb|EF688128.1|	gi|151175869|gb|EF688140.1| gi|151175871|gb|EF688142.1| gi|151175865|gb|EF688136.1|
Clonorchis sinensis	gi|238625314|gb|FJ965384.1| gi|238625328|gb|FJ965391.1|	gi|151175872|gb|EF688143.1| gi|151175873|gb|EF688144.1|
Bird *Opisthorchis* sp.	VN11 (this study) VN12 (this study)	VN1 (this study) VN2 (this study)

However, morphometric data of the liver fluke of ducks are larger than those of *O. viverrini* in mammal hosts, except for the body length (Table 3.2). The testes of the bird fluke are two to four times larger. Although the size of the oral and ventral suckers of the bird fluke is considerably larger, ratios of ventral to oral sucker are similar, with values of 1.36, 1.30 and 1.39 for bird flukes and *O. viverrini* in cat and hamster, respectively. The ratio of uterus egg length/width of the bird fluke is similar to that of *O. viverrini* in humans (ratio=1.85 and 1.86, respectively). This ratio is higher in cat (ratio=2.5) and lower in hamster (ratio=1.27) flukes. These morphological variations may fall into the intraspecific variability of *O. viverrini* in different hosts.

Table 3.2 Morphological and morphometric comparison (in µm unless stated) of *Opisthorchis* sp. in ducks and *O. viverrini* in a man (Sohn et al., 2011), cat (Le, 2000) and hamster (Thaenkham et al., 2011b); all flukes are lanceolate in shape

	Opisthorchis sp. in ducks	*O. viverrini* in man	*O. viverrini* in cat	*O. viverrini* in hamster
Body length × width (mm)	6.0–9.2 × 1.4–2.2	6.5–12.0 × 1.5–1.7	4.9–6.3 × 1.4–1.7	4.2–7.0 × 1.2–1.8
Length/width ratio	4.1–4.3	4.4–7.1	3.6–3.7	3.5–3.9
Oral sucker (OS)	187–284 × 243–306	No data	150 × 180–200	160–240 × 100–220
Ventral sucker (VS)	214–349 × 274–349	No data	180–200	160–260
VS/OS ratio	1.36	No data	1.30	1.39
Testes	4–5 deep lobes	4–5 deep lobes	4–5 deep lobes	4–5 deep lobes
Anterior testis	622–1060 × 1078–1277	No data	300–360 × 390–450	300–520 × 260–420
Posterior testis	690–1195 × 989–1538	No data	350–400 × 420	320–480 × 260–400
Ovary	3 deep lobes	3 deep lobes	3 deep lobes	3 deep lobes
	291–677 × 459–848	No data	400–500 × 340–450	240–500 × 240–360
Egg (*in utero*)	23–27 × 12–15	25–29 × 13–16	25 × 10	12.5–22.5 × 10.0–17.5
Egg length/width ratio	1.85	1.86	2.50	1.27

3.3.2 *Molecular analysis*

BLASTN analysis and MAFFT alignments of VN1 and VN2 (ITS2) showed a high degree of identity to the other *O. viverrini* sequences. A total of two mismatches (positions 145 and 329) and two gaps (six nucleotides) were observed. BLASTN reports 99.4% identity (no gaps, 355 aligned bases) and 97.8% identity (2 gaps, 361 aligned bases).

BLASTN analysis of VN11 and VN22 (COI) revealed a lower degree of identity to the other *O. viverrini* sequences, ranging from 89.3 to 90.6%. No gaps were observed, but BLASTN reported mutations all over the DNA sequence as well as 2 – 3 unaligned bases. The many mutations that were observed in the DNA sequences resulted in 3 – 5 amino acid changes.

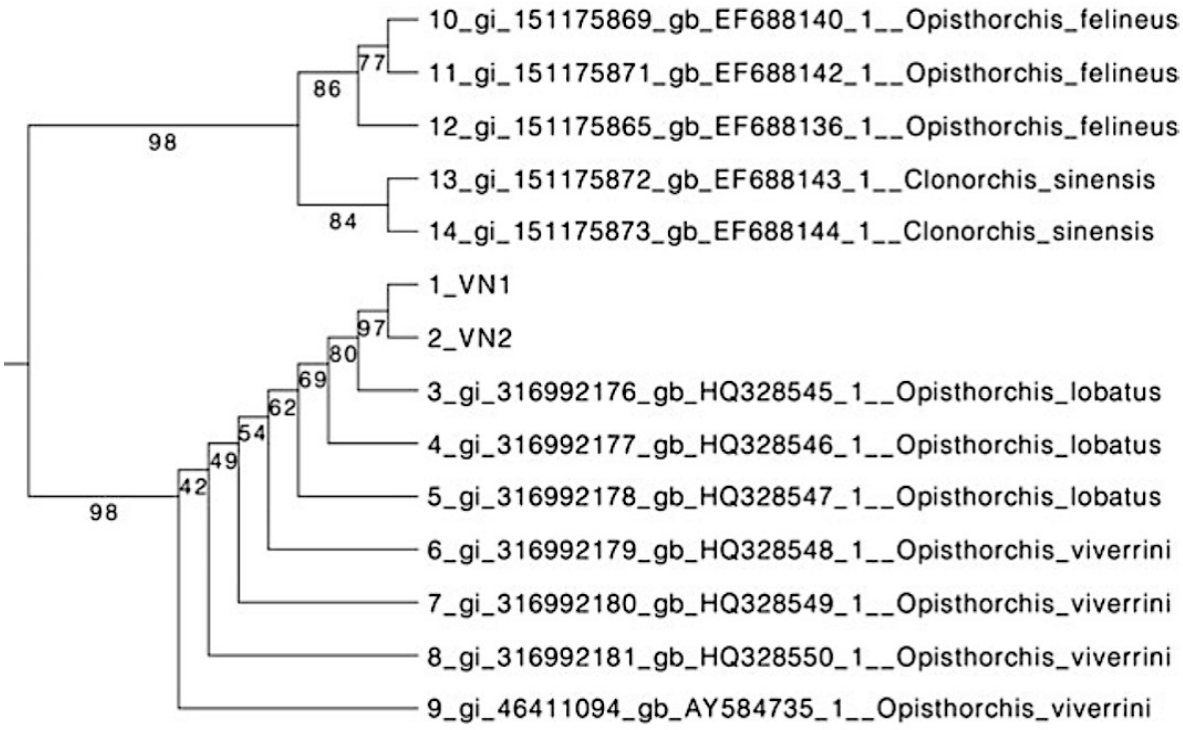

Figure 3.2 Phylogenetic tree of the partial DNA sequences of ITS2 from the *Opisthorchis* sp. in ducks and *O. viverrini*, *O. lobatus*, *O. felineus* and *Clonorchis sinensis* sequences submitted to Genbank (Table 3.1).

Analysis of the ITS2 and COI phylogenetic trees reveals close resemblance between *O. viverrini, O. lobatus* and the unknown species; however, in both cases, the unknown species is always in a separate clade (Figs 3.2 and 3.3).

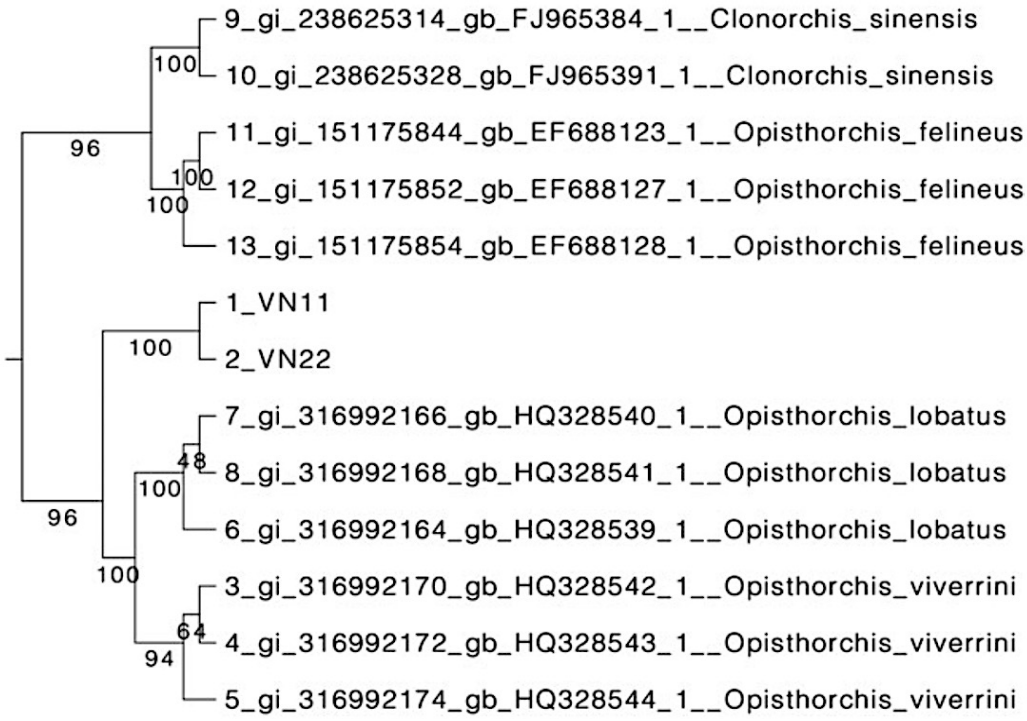

Figure 3.3 Phylogenetic tree of the partial DNA sequences of COI from the *Opisthorchis* sp. in ducks and *O. viverrini, O. lobatus, O. felineus* and *Clonorchis sinensis* sequences submitted to Genbank (Table 3.1).

3.4 Discussion

Morphological examination and gene analysis of the liver flukes found in ducks in Central Vietnam show close similarity with *O. viverrini* and *O. lobatus*. Based on genetic analysis of isolates from different geographical areas, *O. viverrini* may be divided into at least two or six cryptic species; however, morphological variations within the species were not described until now (Laoprom et al., 2009; Sithithaworn et al., 2007). In this study, we found some morphometric differences between the flukes of ducks and *O. viverrini* isolated from cat, human and hamster (Fig 3.1, Table 3.2). In other trematode species, such as *Fasciola hepatica*, the final host species may influence both the morphology and the morphometric characteristics of adult worms and eggs, and the genetic characteristics (Valero et al., 2001). In our

study, analysis of the partial sequence of ITS2 of the rDNA, shows very close resemblance (97.4–99.4%) between the duck fluke and *O. viverrini*. On the mtDNA COI marker, a lower degree of identity between the duck fluke and published sequences of *O. viverrini* was found (~90%).

Although the observed differences in morphology and COI and ITS2 DNA sequences may suggest that the liver flukes are a separate species in the *Opisthorchis* genus, they may also be due to adaptation of *O. viverrini* to a new/different host and the differences are then to be considered intra-specific variations. Further studies on pooled genes of this *Opisthorchis viverrini*-like parasite in ducks should be done to clarify its position the phylogenetic tree of the Opisthorchiidae.

CHAPTER 4

CLARIFICATION OF THE POSITION OF AN *OPISTHORCHIS VIVERRINI*-LIKE FLUKE IN THE PHYLOGENETIC TREE OF THE OPISTHORCHIIDAE FAMILY

Based on the accepted paper in Parasites and Vectors:

Updated molecular phylogenetic data for the *Opisthorchis* species (Trematoda: Opisthorchioidea) from ducks in Vietnam

Dao Thi Ha Thanh, Thi Giang Thanh Nguyen, Sarah Gabriël, Khanh Linh Bui, Pierre Dorny, Thanh Hoa Le

4.1 Introduction

The family Opisthorchiidae (Digenea: Opisthorchioidea) consists of 33 genera considered valid including the genera *Opisthorchis* and *Clonorchis*, in which *Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis* are species known to infect humans (Bray et al., 2008). Humans become infected by eating uncooked cyprinoid fish containing metacercariae. *Opisthorchis viverrini* has been reported in Central Vietnam, where Binh Dinh and Phu Yen Provinces are highly endemic for human opisthorchiasis (Dao et al., 2016b; Petney et al., 2013; Pham and Nawa, 2016).

In 2013, Dao et al. found adults of an opisthorchiid species in ducks (*Anas platyrhynchos*) in areas of Binh Dinh Province where there are many human opisthorchiasis cases. This parasite was then given the working name '*Opisthorchis viverrini*-like', because of its close similarity to *O. viverrini* (Dao et al., 2014; Dao et al., 2016a). Subsequently, there has been a debate about the identity of this worm. Nawa et al. (Nawa et al., 2015) argued that the duck liver fluke not be *O. viverrini*, but is most likely *O. parageminus* that was previously reported from ducks in Vietnam (Le, 2000; Nguyen et al., 2017; Oshmarin, 1970). However, Dorny et al. (Dorny et al., 2015) considered that their "*Opisthorchis viverrini*-like" species exhibited some morphological differences from *O. parageminus*. We now propose to use the working name "*Opisthorchis* sp. BD2013" instead of the earlier "*Opisthorchis viverrini*-like".

Molecular phylogenetic/systematic studies are excellent aids for taxonomy (Kostadinova and Pérez-del-Olmo, 2014; Le et al., 2017; Olson et al., 2003; Tkach et al., 2016). Such studies require homologous sequences from as many taxa as possible within the group of interest. In the genus *Opisthorchis*, a number of genetic markers from complete mitochondrial sequences and the nuclear ribosomal transcription units including, ITS1, ITS2, 18S rDNA and partial 28S rDNA have been generated for *O. viverrini, O. felineus* and *C. sinensis*. These genetic markers have greatly contributed to molecular diagnostic, epidemiological, phylogenetic and evolutionary studies of the species in Opisthorchiidae and trematodes (Cai et al., 2012a; Olson et al., 2003; Pham and Nawa, 2016; Shekhovtsov et al., 2009; Thaenkham et al., 2012; Thaenkham et al., 2011a). However, *Opisthorchis* is a very large genus (Nawa et al., 2015) and molecular data are available for only a few

species. Moreover, given difficulties with the morphological taxonomy within the genus, it is not always certain that names assigned to samples are accurate. The only molecular data claimed to be from *O. parageminus* consist of two sequences recently deposited in Genbank (accession numbers: KX258656, KX258657) by Nguyen and Nguyen (otherwise unpublished data). Although their worms came from ducks in Vietnam, no information is available on the morphological basis for the identification. Both of these sequences (mitochondrial partial mt *co*1 and nuclear ribosomal ITS2) are very similar to earlier sequences available for *Opisthorchis* sp. BD2013 published by (Dao et al., 2014). Here, we provide additional mitochondrial sequences, i.e. complete cytochrome b (*cob*), nicotinamide dehydrogenase subunit 1 (*nad*1) and cytochrome oxidase subunit 1 (*co*1) genes, and near-complete 18S rDNA and partial 28S rDNA sequences in an effort to better resolve the affinities of *Opisthorchis* sp. BD2013 within the family Opisthorchiidae and superfamily Opisthorchioidea.

4.2 Materials and methods

4.2.1 Opisthorchis sp. BD2013 samples collected from the field

Adult specimens and eggs of *Opisthorchis* sp. BD2013 were collected from naturally infected domestic ducks (*Anas platyrhynchos*) originating from 4 localities (Phu Cat, Phu My, An Nhon and Tuy Phuoc Districts) in Binh Dinh Province of Central Vietnam (Dao et al., 2017; Dao et al., 2016a) (Table 4.1). Each adult worm, unstained or stained with acetic carmine, was morphologically identified by light microscopy (Dao et al., 2014). Up to three adult worms from each locality were individually fixed in 70% ethanol and one or two worms from each locality were separately subjected to genomic DNA extraction and molecular analysis.

Fishes (harboring metacercariae) and snails (shedding cercariae) were collected from My Tho Lake in the lowlands of Binh Dinh Province (Dao et al., 2017). Infected fishes were identified as *Puntius brevis, Esomus metallicus, Rasbora aurotaenia*, and the snail as *Bithynia funiculata* (Dao et al., 2017) (Table 4.1). For molecular analysis, metacercariae and cercariae were individually fixed in RNAlater^{TM} buffer (Qiagen, Texas USA, cat no./ID: 76104) at 4 °C. Individual

parasites from each intermediate host and each locality were used for extraction of DNA and molecular study.

Eggs were individually collected from the gall bladder of naturally infected ducks by washing and centrifuging the bile 10 times in normal saline (0.9% NaCl), then three times in phosphate buffered saline (PBS) before storage at -20 °C until use (Table 4.1).

4.2.2 Genomic DNA extraction and primers

Total genomic DNA was extracted from individual adults, metacercariae, cercariae or pooled eggs (approximately 2000 - 3000 eggs) using the GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., MA, USA), according to the manufacturer's instructions. A slight modification applied for eggs was to increase the incubation period by 3 - 4 hours after enzymatic lysis. Genomic DNA was eluted in 50 μl of the elution buffer provided in the kit and stored at -20 °C. The DNA concentration was estimated using a GBC UV/visible 911A spectrophotometer (GBC Scientific Equipment Pty. Ltd., Australia) and diluted to a working concentration of 50 ng/μl (about 10 ng/μl for DNA from eggs). From this genomic DNA, 2 - 3 μl was used as template in a PCR of 50 μl volume.

Primers used both for amplification and sequencing of the mitochondrial and nuclear ribosomal genes are listed in Table 4.2. The primer pair OACOBF/OACO1R amplified approximately 7.8 kb of mtDNA. Based on the sequence obtained from this amplicon, three primer pairs specific for the individual target protein-coding genes were designed. Primer pairs OACOBF/OACOBR, OAND1F/OAND1R, OACO1F/OACO1R amplified complete *cob*, *nad*1 and COI genes, respectively. The primer pairs U18SF/U18SR were used for obtaining major fragments of ribosomal 18S and U28SF/U28SR for 28S, respectively (Le et al., 2017). Additional internal primers were designed and used as needed (Table 4.2).

4.2.3 Amplification of mitochondrial and ribosomal genes

The 7.8 kb mt genomic region: long PCR reactions of 50 μl were prepared using 25 μl of Fusion High-Fidelity PCR Master Mix (2x) (Thermo Fisher Scientific Inc., MA, USA) and 2 μl of each primer (10 pmol/μl), 2 μl DNA template of the adult

sample (50 ng/µl), 2 µl DMSO (dimethyl sulfoxide) and 17 µl H_2O. All PCRs of 50 µl were performed in a MJ PTC-100 thermal cycler with initiation at 98 °C for 30 seconds, followed by 35 cycles consisting of denaturation for 10 s at 98 °C, annealing at 56 °C for 30 s, extension at 72 °C for 6 min.

Individual mt and ribosomal DNA genes: PCR reactions of 50 µl were prepared using 25 µl of DreamTaq PCR Master Mix (2x) (Thermo Fisher Scientific Inc., MA, USA) 2 µl of each primer (10 pmol/µl), 2 µl DNA template (50 ng/µl for adults; metacercariae 10-20 ng/µl for cercariae and eggs), 2 µl DMSO (dimethyl sulfoxide) and 17 µl H_2O. All PCRs of 50 µl were performed in a MJ PTC-100 thermal cycler with initiation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94 °C, annealing at 56 °C for 30 s, extension at 72 °C for 3 min.

4.2.4 Sequencing and sequence analyses

PCR products were obtained from at least two individual samples for each template (i.e. adults, metacercariae, cercariae and eggs) originating from different geographical localities. The PCR products (10 µl of each) were examined on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec, USA).

All the purified or gel-extracted amplicons were subjected to direct sequencing by automated sequencers using amplifying/flanking and internal primers (Table 4.2) by primer-walking in both directions (Macrogen Inc., South Korea). Sequences (two from each sample) were aligned to obtain the final sequence for characterization. All sequences of *Opisthorchis* sp. BD2013 were identical, regardless of the life-cycle stage or locality.

The concatenated nucleotide and amino acid sequences of three protein-coding genes, i.e., *cob*+*nad*1+*co*1, were used to infer the pairwise genetic distances between 10 opisthorchiids within Opisthorchiidae (Table 4.3). These isolates included *Opisthorchis* sp. BD2013 and the reference sequences of *O. viverrini* from Laos (JF739555); Vietnam (MF287777; MF287778; MF287779) and Thailand (MF287780; MF287781; MF287782). The genetic distances were inferred by pairwise analysis using the MEGA6.0 software and the number of base

substitutions per site was calculated by the most simplified method (uncorrected *p*-distance) (Tamura et al., 2013).

4.2.5 Phylogenetic analysis

4.2.5.1 Preparation of DNA sequences

A phylogenetic analysis using three mitochondrial protein-coding (*cob*, *nad*1, *co*1) and two nuclear ribosomal (18S and 28S rDNA) genes was conducted to examine the taxonomic placement of *Opisthorchis* sp. BD2013 from ducks within the superfamily Opisthorchioidea. Sequences of trematode species/isolates of Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (as the outgroup) were used. Summary data of species/isolates, mainly from the available complete mitochondrial genomes are presented in Table 4.3. Accession numbers for the target and reference 18S and 28S rDNA sequences are listed in Table 4.4. For *Opisthorchis* sp. BD2013, we decided to use only 2 sequences of adults, and one each from metacercariae, cercariae and eggs for phylogenetic analyses.

Concatenated nucleotide sequences of mt protein-coding genes (*cob*, *nad*1, *co*1) from adults, metacercariae, cercariae, and eggs of *Opisthorchis* sp. BD2013, and from additional taxa (available in Genbank; see Table 4.3) were imported into GENEDOC 2.7 (available at: http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html) and aligned for phylogenetic analysis. Additionally, the sequences of opisthorchiids were translated (using the echinoderm/flatworm mitochondrial genetic code: translation Table 9 in Genbank), and the deduced amino acid sequences were aligned for pairwise genetic distance analysis.

Table 4.1 List of field samples used in this study, their geographic collection site in Binh Dinh province and their hosts

Life-cycle stage	Site collected (district)	Host	Scientific name	Sample abbreviation for use in this study
Adult worm	Phu Cat	Duck	*Anas platyrhynchos*	*Opisthorchis* sp. BD2013-PC6aduBD
Adult worm	Phu My	Duck	*Anas platyrhynchos*	*Opisthorchis* sp. BD2013-PM10aduBD
Adult worm	An Nhon	Duck	*Anas platyrhynchos*	
Adult worm	Tuy Phuoc	Duck	*Anas platyrhynchos*	
Metacercariae	Phu My	Fish	*Puntius brevis*	*Opisthorchis* sp. BD2013-PCmetaBD
Metacercariae	Phu My	Fish	*Rasbora aurotaenia*	
Metacercariae	Phu My	Fish	*Esomus metallicus*	
Cercariae	Phu My	Snail	*Bithynia funiculata*	*Opisthorchis* sp. BD2013-PCcercaBD
Eggs	Phu My	Duck	*Anas platyrhynchos*	*Opisthorchis* sp. BD2013-PCeggBD

Table 4.2 Primers for amplification and sequencing of the mitochondrial protein-coding and nuclear ribosomal genes used in this study

Primer name	Sequence (5'–3')	Target gene	Amplicon by PCR	Length of sequence (bp)	Reference
OACOBF	AGCCGGAGAGTCATTGTGTG	*cob*	1.4 kb	1,110 bp	This study
OACOBR	TGAATCCCACAACCGCGTTA				
OACOBR2*	TACGTTGAAGGACGGGTTGG				
OAND1F	CGTGTGGTGGGGCAAGATAG	*nad*1	1.2 kb	903 bp	This study
OAND1R	CCACACAGCCTTCTCAAGGT				
OACO1F	GAGGGTTACGTGGGTTGGAG	*co*1	1.8 kb	1,551 bp	This study
OACO1R	CAACCCTACTAAGCACCACAGC				
OACO1R2*	GGATCCCAAAAACGCTCACG				
U18SF	GCGAATGGCTCATTAAATCAGC	18S	1.8 kb	~1,790 bp	[1]
U18SR	GGAACCAATCCGAGGACCTTGC				
NS2F*	GCAAGTCTGGTGCCAGCAGCC				
U28SF	CTAACAAGGATTCCCTTAGTAAC	28S	1.3 kb	~1,100 bp	[1]
U28SR	GTCTTTCGCCCCTATACTCAC				

Abbreviations F, forward; R, reverse; *Internal primer used for sequencing, [1] (Le et al., 2017).

Table 4.3 Summary data for complete mitochondrial genomes of species providing cytochrome b (*cob*), nicotinamide dehydrogenase subunit 1 (*nad*1) and cytochrome oxidase subunit 1 (*co*1) used in the phylogenetic analysis including *Opisthorchis* sp. BD2013 in ducks in Vietnam

Family/Species	Isolates/Strains	Country	Genbank nos.	Reference
Opisthorchiidae				
Opisthorchis sp. BD2013	PC6aduBD	Vietnam	MF287762 - MF287764	This study
Opisthorchis sp. BD2013	PM10aduBD	Vietnam[b]	MF287765 - MF287767	This study
Opisthorchis sp. BD2013	PCmetaBD	Vietnam	MF287768 - MF287770	This study
Opisthorchis sp. BD2013	PCcercaBD	Vietnam	MF287771 - MF287773	This study
Opisthorchis sp. BD2013	PCeggBD	Vietnam	MF287774 - MF287776	This study
Opisthorchis viverrini	n/a	Laos[b]	JF739555	[2]
	Binh Dinh 1	Vietnam[b]	MF287777 - MF287779	This study
	Khon Kaen	Thailand[b]	MF287780 - MF287782	This study
Opisthorchis felineus	Ust-Tula (Novosibirsk)	Russia[b]	EU921260	[3]
Clonorchis sinensis	Nam Dinh	Vietnam[c]	MF287783 - MF287785	This study
	Guangdong	China[b]	JF729303	[2]
	n/a	South Korea[b]	JF729304	[2]
	Amur -Khabarovsk	Russia[b]	FJ381664	[3]
Metorchis orientalis	Heilongjiang	China[b]	KT239342	[4]
Heterophyidae				
Haplorchis taichui	n/a	Laos	KF214770	[5]
	Quang Tri 3	Vietnam	MF287786 - MF287788	This study
Metagonimus yokogawai	n/a	South Korea	KC330755	
Fasciolidae				
Fasciola hepatica	Geelong	Australia	AF216697	[6]
Fasciola gigantica	Guangxi	China	KF543342	[7]
	Thua Thien-Hue	Vietnam	MF287789 - MF287791	This study
Fasciola sp. (intermediate form)	GHL-Heilongjiang	China	KF543343	[7]
Fasciolopsis buski	Jiangxi	China	KX169163	[8]
	Ha Tay	Vietnam	MF287792 - MF287794	This study
Fascioloides magna	Kokořínsko	Czek	KU060148	[9]
Schistosomatidae				
Schistosoma haematobiumi[a]	N10 Village	Mali	DQ157222	[10]

Note: [(a)]Sequence used as the outgroup.

[(b)]Sequences of the opisthorchiids used for pairwise genetic distance calculation (Table 4.5 and 4.6).

[2] (Cai et al., 2012a); [3] (Shekhovtsov et al., 2010); [4] (Na et al., 2016); [5] (Lee et al., 2013); [6] (Le et al., 2001); [7] (Liu et al., 2014); [8] (Ma et al., 2017); [9] (Ma et al., 2016); [10] (Littlewood et al., 2006);

DNA sequences of 18S rRNA and 28S rRNA genes (listed in Table 4.4) were aligned separately using GENEDOC 2.7. The sequences were trimmed at both ends to the shortest length for the representative sequences. For 18S rDNA, in this study, the final alignment was 2005 nucleotides (nt) long of which 87 nt positions were trimmed at 5' end and 114 nt at 3' end, leaving 1804 characters for analyses. For 28S rDNA, the final alignment was 1449 nt long of which 122 nt at 5' end and 123 nt at 3' end, leaving 1202 characters for analyses. The two sequences were then concatenated as indicated in Table 4.4, preferably from the same strains/isolates. The concatenated 18S + 28S rDNA sequences representing species/isolates were imported in GENEDOC 2.7 and phylogenetic analysis and tree construction were done by MEGA6.0 (Tamura et al., 2013).

4.2.5.2 Phylogenetic construction

The alignments of the concatenated nucleotide sequences of (*cob*, *co*1, *nad*1) and 18S rDNA+28S rDNA, respectively, were trimmed to the length of the shortest sequence and imported into the MEGA 6.06 software (Tamura et al., 2013). Maximum likelihood (ML) analyses were performed in each case. For DNA sequences, we used the general time-reversible model of evolution with gamma distributed rate heterogeneity and a proportion of invariant sites (GTR) + Γ + I). This model was given the best Bayesian information criterion score by MEGA. For amino acid sequences, the Jones-Taylor-Thornton (JTT) model with uniform rates and Nearest-Neighbor-Interchange (NNI) method was used. The confidence in each node was assessed using 1000 bootstrap resamplings (Tamura et al., 2013).

Table 4.4 Accession numbers of the reference 18S and 28S rDNA sequences and their species information used for phylogenetic analysis with those derived from *Opisthorchis* sp. 2013 in ducks in the present study

Family/Species	18S rDNA Genbank ID (isolate)[b]	28S rDNA Genbank ID (isolate)[b]	Origin of sequences	References
Opisthorchiidae				
Opisthorchis sp.	MF077358 (PC6aduBD)[b]	MF110001 (PC6aduBD)	Vietnam	This study
	MF077359 (PCcercaBD)	MF110002 (PCcercaBD)	Vietnam	This study
	MF077360 (PCeggBD)	MF110003 (PCeggBD)	Vietnam	This study
	MF077361 (PCmetaBD)	MF110004 (PCmetaBD)	Vietnam	This study
	MF077362 (PM10aduBD)	MF110005 (PM10aduBD)	Vietnam	This study
Opisthorchis viverrini	HM004211 (SK)	HM004188 (SK);	Thailand	[11]
	JF823987 (THASK)	JF823990 (THASK)	Thailand	[12]
	MF077364 (PY2)	MF099792 (PY2)	Vietnam	Genbank
	MF077363 (BD1)	KY369165 (BD1)	Vietnam	Genbank
Opisthorchis felineus	MF077357 (Ust-Tula)	MF099790(Ust-Tula)	Russia	Genbank
Clonorchis sinensis	JF823988 (VNM)	JF823989 (VNM)	Vietnam	[11]
	JF314770 (GD)	JF823989 (VNM)	China; Vietnam	Genbank [11]
	MF077353 (NH)	MF099784 (NH)	Vietnam	Genbank
Heterophyidae				
Haplorchis pumilio	HM004194 (HpNP1)	HM004186 (HpNP1)	Thailand	[13]
	KX815125 (HPU8)	KX815125 (HPU8)	Vietnam	[14]
Haplorchis taichui	KX815126 (QT3)	KX815126 (QT3)	Vietnam	[14]
	HM004201 (NA3)	HM004187 (NA3)	Thailand	[11]
Haplorchis yokogawai	HM004207 (CP1)	HM004178 (CP1)	Thailand	[13]
	HM004208 (CP2)	KY369160 (An394)	Thailand; Vietnam	[13] [14]
Procerovum varium	HM004199 (PvNP1)	HM004182 (PvNP1)	Thailand	[11]
	MF077365 (HspND)	KY369161 (HspND)	Vietnam	Genbank [14]
Stellantchasmus falcatus	HM004202 (VN1)	HM004174 (VN1)	Vietnam	[12]
	MF077366 (QN2)	KY369164 (QN2)	Vietnam	[14]
Metagonimus takahashii	HQ832629 (Mt3)	HQ832638 (Mt3)	Japan	[15]
Metagonimus yokogawai	HQ832630 (My1)	HQ832639 (My1)	Japan	[15]
Metagonimus miyatai	HQ832626 (Mm3)	HQ832635 (Mm3)	Japan	[15]
Fasciolidae				
Fasciolopsis buski	AY311386 (Vinh)	EU025870 (NA)	Vietnam	[16]
Fasciola gigantica	MF077354 (NB)	MF099787 (NB)	Vietnam	Genbank
Fasciola hepatica	MF077355 (Geelong)	MF099788 (Geelong)	Australia	Genbank
Fascioloides magna	EF051080	EU025872	United States	Genbank [17]
Schistosomatidae				
Schistosoma haematobium[a]	Z11976	AY157263	Mali	[18] [19]

Note: ([a])Sequence used as the outgroup. [(b)]Abbreviation for isolares are given in parenthese

[11] (Thaenkham et al., 2010); [12] (Thaenkham et al., 2011a); [13] (Thaenkham et al., 2012); [14] (Le et al., 2017); [15] (Pornruseetairatn et al., 2016); [16] (Le et al., 2004); [17] (Lotfy et al., 2008); [18] (Johnston et al., 1993); [19] (Lockyer et al., 2003)

4.3 Results

4.3.1 Mitochondrial cob, nad1, co1 and genetic distances among opisthorchiid species/sequences

For *Opisthorchis* sp. BD2013, lengths of the complete *cob*, *nad*1 and *co*1 genes were 1110, 903 and 1551 nucleotides, respectively. Among opisthorchiid species, *cob* genes ranged in length from 1110 to 1116 nt and *co*1 genes were 1551 to 1563 nt in length. The primer pairs U18SF/U18SR were used for obtaining major fragments of ribosomal 18S and U28SF/U28SR for 28S rDNA.

Nucleotide and amino acid pairwise comparisons of the concatenated mt genes among ten opisthorchiid isolates/species are presented in Table 4.5 and 4.6. The concatenated *cob*+*nad*1+*co*1 nucleotide sequences of *Opisthorchis* sp. BD2013 differed at 14.4% - 14.5% of nucleotide sites and 10.3% – 10.6% of amino acids positions from the reference sequences of *O. viverrini* (Vietnam, Thailand and Laos isolates) (Cai et al., 2012a); 17.9% - 18.2% for nucleotides and 13.3% – 13.7% for amino acids from *C. sinensis* (Russia, China, South Korea and Vietnam isolates); 18.1% (nucleotides) and 13.7% (amino acids) from *O. felineus* (a Russian isolate) (Shekhovtsov et al., 2010) and 15.4% (nucleotides) and 11.6% (amino acids) from *Metorchis orientalis* (China isolate) (Na et al., 2016).

Within each opisthorchiid taxon, pairwise genetic distances are small, only 0.4 – 0.7% for nucleotides and 0.5 to 0.6% for amino acids within *O. viverrini*; 0.3 – 0.6% (nucleotides) and 0.2 – 0.8% (amino acids) within *C. sinensis*. This means that *Opisthorchis* sp. BD2013 in ducks differs from *O. viverrini* by an inter-species distance of more than 10%, a figure comparable to those separating species within the genus *Opisthorchis* and family Opisthorchiidae (Tables 4.5 and 4.6).

Table 4.5 Pairwise genetic distance (%) between *Opisthorchis* sp. BD2013 sample from ducks in Vietnam and sequences for *O. viverrini*, *C. sinensis*, *O. felineus* and *M. orientalis* of the concatenated mitochondrial genes *cob*, *nad*1 and *co*1

	Nucleotide sequences	Accession No	1	2	3	4	5	6	7	8	9	10
1	*Opisthorchis* sp. BD2013 (PM10aduBD/Vietnam)	MF287767	-									
2	*O. viverrini* (Binh Dinh1/Vietnam)	MF287779	14.4	-								
3	*O. viverrini* (Khon Kaen/Thailand)	MF287782	14.5	0.4	-							
4	*O. viverrini* (Laos)	JF739555	14.4	0.5	0.7	-						
5	*Clonorchis sinensis* (Amur Khabarovsk/Russia)	FJ381664	17.9	18.1	18.1	17.9	-					
6	*C. sinensis* (Guangdong/China)	JF729303	18.0	18.1	18.1	17.9	0.4	-				
7	*C. sinensis* (South Korea)	JF729304	18.2	18.2	18.3	18.0	0.5	0.3	-			
8	*C. sinensis* (Nam Dinh/Vietnam)	MF287784	18.0	18.1	18.2	18.0	0.5	0.5	0.6	-		
9	*O. felineus* (Ust-Tula/Russia)	EU921260	18.1	18.8	18.9	18.7	15.4	15.6	15.8	15.5	-	
10	*Metorchis orientalis* (Heilongjiang/China)	KT239342	15.5	13.7	13.7	13.5	17.0	17.2	17.2	17.0	16.8	-

Table 4.6 Pairwise genetic distances (%) between *Opisthorchis* sp. BD2013 sample from ducks in Vietnam and sequences for *O. viverrini*, *C. sinensis*, *O. felineus* and *M. orientalis* of the concatenated mitochondrial amino acid sequence of cob, nad1 and *co*1

	Nucleotide sequences	Accession No	1	2	3	4	5	6	7	8	9	10
1	*Opisthorchis* sp. BD2013 (PM10aduBD/Vietnam)	MF287767	-									
2	*O. viverrini* (Binh Dinh 1/Vietnam)	MF287779	10.6	-								
3	*O. viverrini* (Khon Kaen/Thailand)	MF287782	10.6	0.5	-							
4	*O. viverrini* (Laos)	JF739555	10.3	0.6	0.6	-						
5	*Clonorchis sinensis* (Amur-Khabarovsk/Russia)	FJ381664	13.3	12.4	12.4	12.4	-					
6	*C. sinensis* (Guangdong/China)	JF729303	13.5	12.8	12.8	12.8	0.3	-				
7	*C. sinensis* (South Korea)	JF729304	13.7	12.7	12.7	12.7	0.3	0.2	-			
8	*C. sinensis* (Nam Dinh/Vietnam)	MF287784	13.6	12.6	12.6	12.6	0.4	0.8	0.8	-		
9	*O. felineus* (Ust-Tula/Russia)	EU921260	13.7	13.8	13.9	13.9	9.3	9.7	9.7	9.5	-	
10	*Metorchis orientalis* (Heilongjiang/China)	KT239342	11.6	8.8	8.8	8.7	9.8	10.2	10.2	10.1	11.0	-

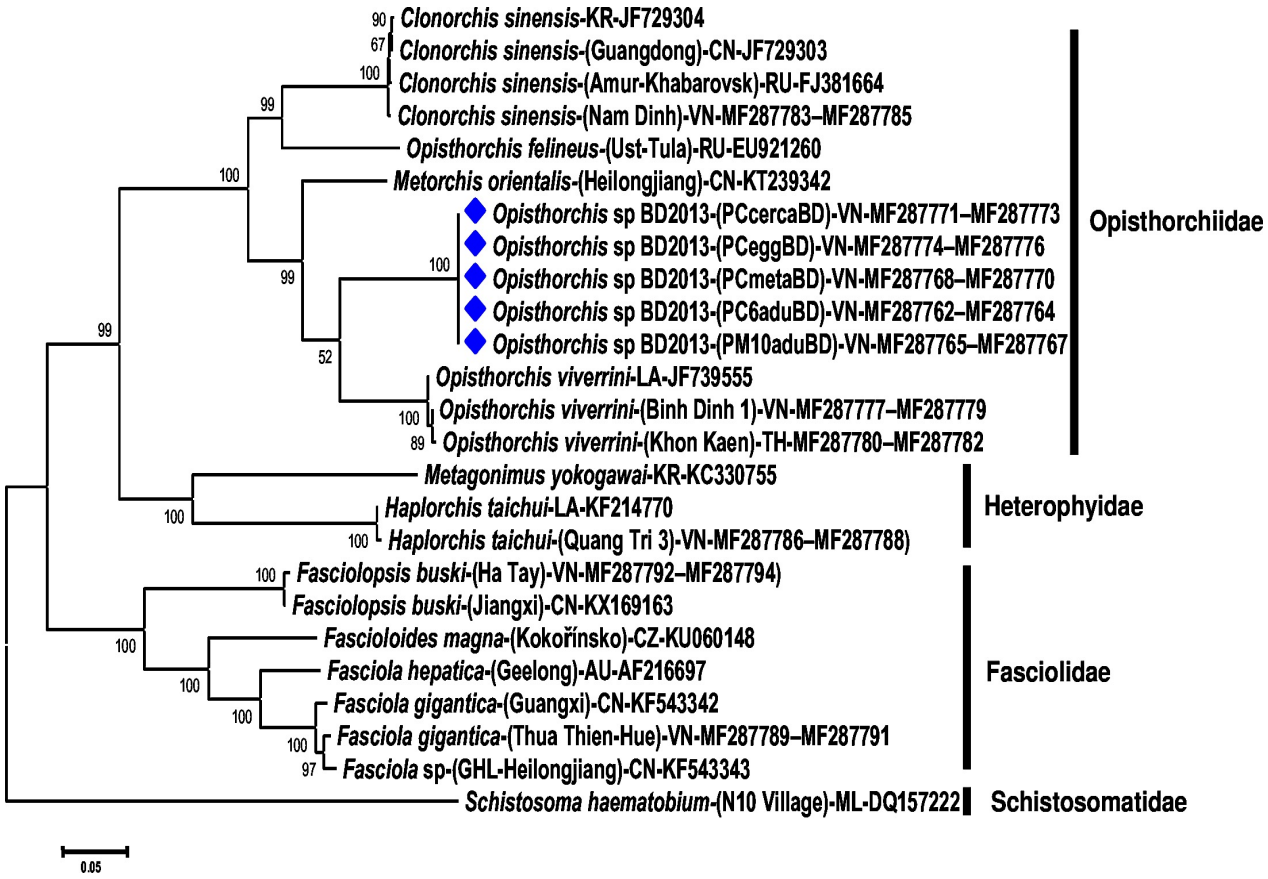

Figure 4.1 Phylogenetic tree of *Opisthorchis* sp. BD2013 (indicated by diamond symbol) and other opisthorchiids and representive trematodes from 4 families (Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (the later used as an outgroup), based on cancatenated nucleotide sequences of complete cytochrom b (*cob*), nicotinamide dehydrogenase subinit 1 (*nad*1) and cytochrome c oxidase subinit 1 (*co*1) genes. Phylogenetic reconstruction was performed using maximum likelihood analysis with the general time - reversible model with a gamma distributed rate heterogeneity and a proportion of invariant sites (GTR) +Γ+ I) in the MEGA6.06 software package. Support for each node was evaluated using 1000 bootstrap resamplings (Tamura et al., 2013). The scale-bar indicates the number of substitutions per site. Accession numbers (where available) are given at the end of each sequence name. Isolates/geographical localities are given in parentheses (if available). Country abbreviation codes (2-letter) given prior to the accession numbers: AU, Australia; CN, China; CZ, Czech Republic; KR, Korea; LA, Lao PDR; RU, Russia; TH, Thailand; VN, Vietnam.

4.3.2 Phylogenetic analysis

4.3.2.1 Phylogenetic construction based on the complete cob+nad1+co1 amino acid sequences

A phylogenetic tree was constructed from 25 nucleotide sequences inferred from complete *cob*+*nad*1+*co*1 of 13 trematode species belonging to 4 families with *Schistosoma haematobium* of Schistosomatidae as the outgroup (Table 4.3; Fig 4.1). The superfamily Opisthorchioidea in this study comprises Heterophyidae and Opisthorchiidae (no appropriate sequences from the third family, Cryptogonimidae were available), with strong nodal support of 99%, clearly separate from the family Fasciolidae. The *Opisthorchis* sp. BD2013 clade was placed as a sister[2] of *O. viverrini* from Thailand, Vietnam and Laos. The genus *Opisthorchis* appears as paraphyletic with respect to *C. sinensis*, *O. felineus* and *M. orientalis* (Fig 4.1).

4.3.2.2 Phylogenetic construction based on partial 18S rDNA+28S rDNA sequences

Five concatenated 18S+28S rDNA sequences of Vietnamese *Opisthorchis* sp. BD2013 (from eggs, cercariae, metacercariae and adults) were aligned with 26 available sequences representing 17 trematode species of Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (outgroup) (Table 4.4). The nuclear ribosomal dataset from the Opisthorchioidea included available sequences of the 18S+28S rDNA of 12 taxa only from the Opisthorchiidae and Heterophyidae (data from the Cryptogonimidae were not available). The combined length of alignment in use was between 2940 and 2960 nt. The inferred phylogenetic tree (Fig 4.2) again placed *Opisthorchis* sp. BD2013 in a sister position with *O. viverrini* from Thailand and Vietnam. Again, the genus *Opisthorchis* appeared as paraphyletic. Monophyly of the superfamily Opisthorchioidea was strongly supported (Fig 4.2).

[2] closest relative among the species that are included in the phylogenetic analysis

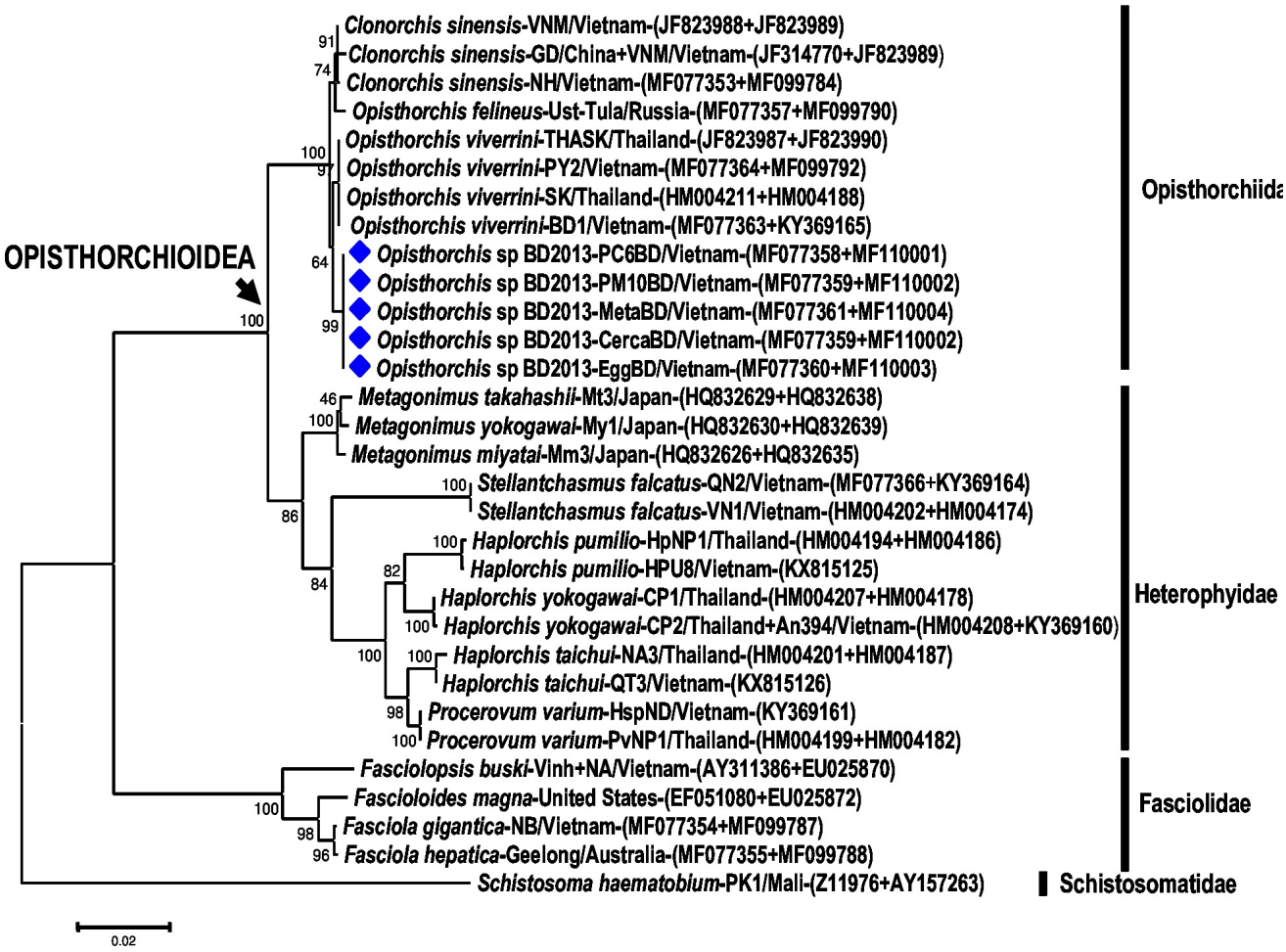

Figure 4.2 Phylogenetic tree of Opisthorchis sp. BD2013 (indicated by diamond symbol) and other opisthorchiids and representative trematodes from 4 families (Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (the latter used as the outgroup)), based on combined nucleotide sequences of nuclear small ribosomal subunit (18S rDNA) and large ribosomal subunit (28S rDNA). Phylogenetic reconstruction was performed using maximum likelihood analysis with the general time-reversible model and a gamma distributed rate heterogeneity and proportion of invariant sites (GTR + Γ+ I) in the MEGA6.06 software package. Support for each node was evaluated using 1000 bootstrap resamplings (Tamura et al., 2013). The node for the superfamily (infraorder) Opisthorchioidea is indicated by an arrow. The scale-bar indicates the number of substitutions per site. Accession numbers are given at the end of each sequence name. Isolates or geographical localities and country isolated are given in the between (if available).

4.4 Discussion

In this study, we used two concatenated datasets to infer the molecular phylogenetic position of *Opisthorchis* sp. BD2013 (formerly named '*Opisthorchis viverrini*-like' or as *O. parageminus* by several authors). We did not have samples of *O. lobatus* (Thaenkham et al., 2011b) and the so-called *O. parageminus* (Le, 2000; Oshmarin, 1970) for analysis in the present study, therefore, we were not able to establish the relationship between *Opisthorchis* sp. BD2013 and these species.

The genus *Opisthorchis* is very large (Nawa et al., 2015), but relevant sequence data are limited to only a few species. It was necessary to determine whether *Opisthorchis* sp. BD2013 from ducks is distinct from *O. viverrini*, a zoonotic liver fluke known to infect and to cause cholangiocarcinoma in humans (Sripa et al., 2012). The data presented in this study strongly imply that the two are distinct species. The sister-species relationship demonstrated between *Opisthorchis* sp. BD2013 and *O. viverrini* might simply be because *O. felineus* is the only other member of the genus for which data are available. *Opisthorchis felineus* renders *Opisthorchis* paraphyletic in our trees, indicating that much systematic work remains to be done in the Opisthorchiidae. A further unresolved question is the relationship between *Opisthorchis* sp. BD2013 and *O. parageminus*. Both were found in ducks in Vietnam, but some morphological differences seem to exist (Dorny et al., 2015). At this stage, we prefer to leave the question open, pending future morphological and molecular work.

Our previous phylogenetic analysis using short sequences of ITS2 and *co*1, revealed close affinities between *O. viverrini*, *O. lobatus* and *Opisthorchis* sp. BD2013 (Dao et al., 2014). In the current study, we are unable to resolve the status of *O. lobatus* compared to *Opisthorchis* sp. BD2013 and other opisthorchiids.

Conclusions

Based on mitochondrial *co*b + *nad*1 + *co*1 and ribosomal 18S + 28S rDNA sequence analyses, *Opisthorchis* sp. BD2013 was distinct from *O. viverrini*, although the two species are closely related. The genus *Opisthorchis* itself appears as paraphyletic. Data from additional *Opisthorchis* species are vital to create a phylogeny with higher resolution within *Opisthorchis* and the Opisthorchiidae.

CHAPTER 5

PREVALENCE AND INTENSITY OF INFECTION OF *OPISTHORCHIS VIVERRINI*-LIKE FLUKES IN DOMESTIC DUCKS IN BINH DINH PROVINCE, CENTRAL VIETNAM

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BRIEF COMMUNICATION

Prevalence of *Opisthorchis viverrini*-Like Fluke Infection in Ducks in Binh Dinh Province, Central Vietnam

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5.1 Introduction

Opisthorchiasis is a neglected tropical disease caused by the Asian liver fluke, *Opisthorchis viverrini*. The life cycle of *O. viverrini* involves freshwater snails and fish as intermediate hosts. Humans acquire the infection by consumption of raw or undercooked fish (Kaewkes, 2003). This hepatobiliary parasitic disease affects millions of people in the greater Mekong delta region and is linked to the development of malignant liver cancer (cholangiocarcinoma). In Thailand, where the disease has been studied extensively, the economic loss caused by opisthorchiasis is estimated at 120 million USD annually (Andrews et al., 2008; Sithithaworn et al., 2012; Sripa et al., 2007). While dogs and cats have been identified as *O. viverrini* reservoir hosts (Aunpromma et al., 2012), the potential role of fish-eating birds as reservoir hosts has received less attention (Kaewkes, 2003). In Vietnam, Binh Dinh Province (Fig 5.1) is considered an endemic area for opisthorchiasis with an infection rate of 11.4% in humans in lowland Phu My district (Dao et al., 2016b). Interestingly, *O. viverrini* infection was reported in domestic ducks (*Anas platyrhynchos*) in this area (Dao et al., 2014). Although morphological and genetic characteristics of the duck isolate are slightly different from those of human isolates from this area and from other endemic countries, a possible role of aquatic birds as reservoir hosts for this zoonotic fluke was suggested (Dao et al., 2014; Dorny et al., 2015). The validity of a *O. viverrini* duck genotype has been contested (Nawa et al., 2015; Pham and Nawa, 2016), but Dorny et al. (2015) argued that the high similarly of the ITS-2 partial sequence of the duck isolates to human isolates of *O. viverrini* justifies the classification of the duck flukes as *O. viverrini*-like. Two genotypes of a species occurring in the same endemic area and sharing the same intermediate hosts may lead to hybridization/introgression (Le et al., 2008) and make the epidemiology of the disease more complex.

Following the first record of *O. viverrini* infection in ducks in 2009 (Dao et al., 2014), many cases have been reported in other areas of the province, including 4 lowland districts, namely, Phu My, Phu Cat, An Nhon, and Tuy Phuoc. In these districts, ducks are reared for dual purposes; meat and egg production. During the day ducks scavenge on rice-fields, irrigation canals, streams or rivers, and may feed on small freshwater fish. Infected ducks contaminate the water bodies by passing

parasite eggs with their feces. In the water, prevailing *Bithynia* spp. snails and Cyprinid fish, 2 common intermediate hosts of *O. viverrini*, can complete the life cycle of this parasite. The aim of this study was to clarify the role of domestic ducks as the final hosts of *O. viverrini* by determining the prevalence and intensity of infection of *O. viverrini* in ducks in Binh Dinh Province. Information obtained in this study will contribute to a better understanding of the epidemiological situation of small liver fluke infections in Central Vietnam.

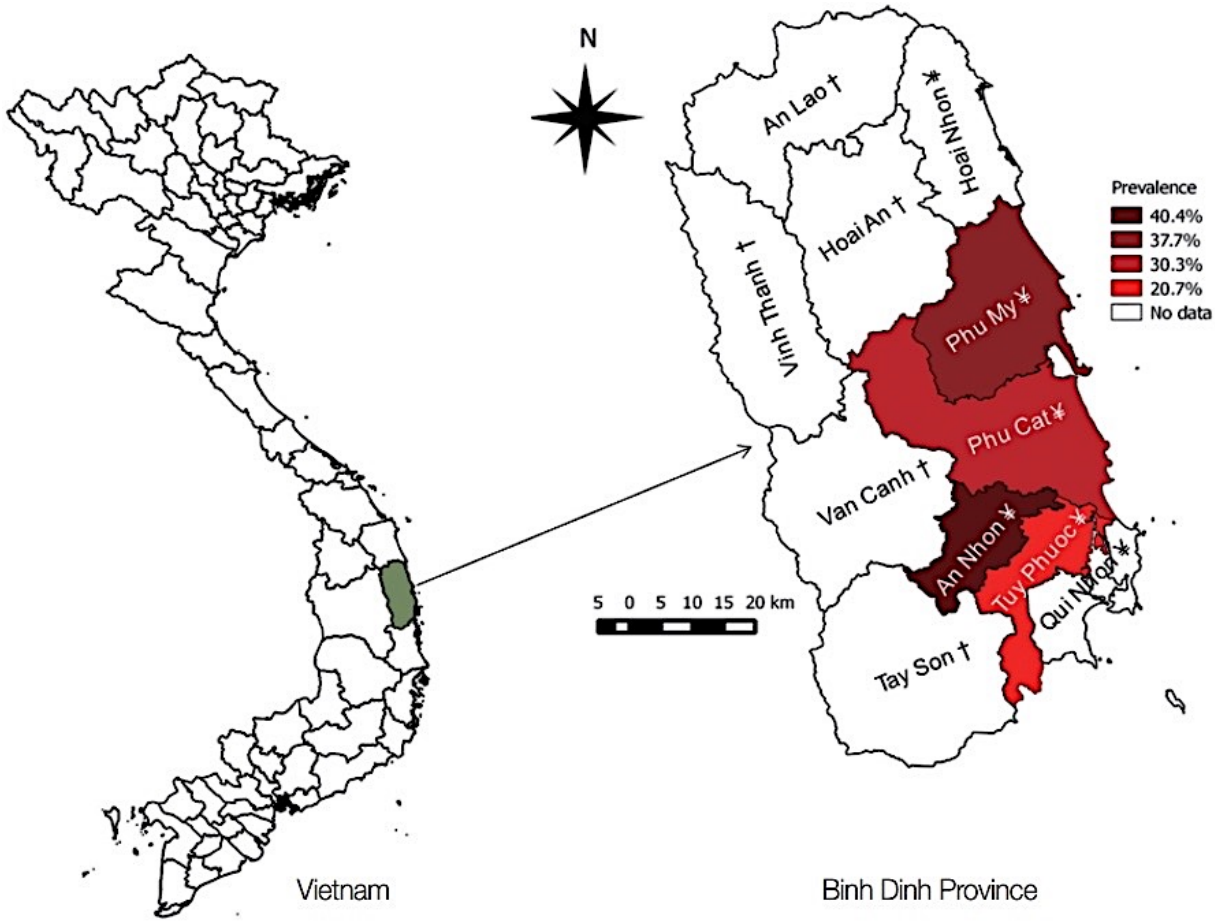

Figure 5.1 Study areas and distribution of the prevalence of *Opisthorchis viverrini* infections in ducks (*Anas platyrhynchos*) in 4 lowland districts of Binh Dinh Province, Central Vietnam. † highland areas and ¥ lowland areas of the province.

5.2 Materials and methods

5.2.1 Study area

Binh Dinh Province is in the South-central coast region of Vietnam and is composed of 11 districts, 5 of which are in the highland and 6 in the lowland along the coast (Fig 5.1). Most of the population of the province lives near the coast. The annual average temperature and rainfall are 26 ˚C and 1,935 mm, respectively, with a dry season from January to August, and a rainy season from September to December. Serious flooding may occur, mostly in December as a result of tropical storms. Fresh water in the province mainly comes from 4 big rivers of which 3 rivers support fresh water for the lowlands (Latinh River in Phu My and Phu Cat districts, Kon River in An Nhon district, and Ha Thanh River in Tuy Phuoc district and Quy Nhon city). In addition, 49 artificial freshwater reservoirs support freshwater requirements during the long dry season in the province. People in the province mainly live on agriculture, including rice cultivation, raising livestock and poultry, and fish production by sea fishing and aquaculture. Ducks are mostly raised in the lowland districts, which have been reported as the endemic area of opisthorchiasis in humans and ducks. Ducks are kept on water bodies during the day and in duck houses at night where they also lay their eggs. Guided by the farmers, duck flocks are taken to water bodies in the morning; they consist of streams or rivers on which ducks scavenge in a fenced area. Only after harvest, ducks are also taken to rice fields where they can feed on seed leftovers. Ducks are fed 3-4 times/day with commercial feed. Duck production contributes to more than 45 million US dollars per year of the provincial income (Binh Dinh Statistical Office, 2015).

5.2.2 Duck sampling

From December 2013 to March 2015, 34 duck-farms in districts of Phu My (14.250˚N-109.083˚E), Phu Cat (14.000˚N-109.000˚E), An Nhon (13.917˚N-109.083˚E), and Tuy Phuoc (13.833˚N-109.167˚E) were randomly selected from the registries supported by the Sub-Department of Animal Health of Binh Dinh Province. From each farm, 2-8 ducks were randomly purchased depending on the size of the flock. A total of 178 ducks from 20 communes (a lower geographical unit of district) were examined for adult worms in the liver and the gall bladder.

5.2.3 Recovery of worms from duck livers

The ducks were killed by exsanguination from the neck vein and their livers and gall bladders were removed in separate dishes containing buffered saline. The liver was opened by following the main tributaries of the biliary duct. The gall bladder was opened and turned inside out to examine for any visible worms. Next, the liver was cut into small and thin pieces, and placed in buffered saline for 5 min, then crushed and filtered through a tea strainer. All visible flukes were collected in separate Petri dishes and washed several times with buffered saline before being counted and fixed in 70% ethanol for morphological and molecular identification.

5.2.4 Morphological and molecular examinations of recovered worms

Forty recovered worms were randomly selected and stained with carmine dye (based on Semichon's acetic carmine 1929). Morphological identification was done using published taxonomic references on *O. viverrini* (Dao et al., 2014; Kaewkes, 2003). DNA was separately extracted from 4 fresh randomly selected worms using the Gentra Puregene Kit (Qiagen, Germantown, Maryland, USA). Two pairs of primers 3S & BD2 (Bowles et al., 1993) for ITS2 marker and COI-Ov-Hap F & R (Thaenkham et al., 2011b) for COI marker were used to amplify portions of the ITS2 and the COI genes, respectively. The PCR products were visualized on agarose gel 1.5% with the Mupid® One electrophoresis system and sequenced (University of Antwerp, Antwerp, Belgium). The obtained DNA sequences of the COI and ITS2 markers, after editing (Hall, 1999) were aligned to sequences of *O. viverrini*, which are deposited in Genbank (No. KF 557572 & KF 557573 for the COI DNA sequence and KF 577570 & KF 577571 for the ITS2 DNA sequence). All collected data were recorded in Excel and transferred to STATA, version 11.2, software (StataCorp LP, College Station, Texas, USA) for statistical analysis. Descriptive statistics were used to summarize the overall prevalence and the intensity of *O. viverrini* infection by district, age, production type (egg or meat production), places where ducks were scavenging, and knowledge of the duck owners on the small liver fluke infection of their ducks.

5.3 Results

Small liver fluke infection was found in the liver and gall bladder of 61 ducks from 178 ducks examined, with the number of worms recovered per duck ranging from 1 to 100. The overall infection rate of *O. viverrini* infection was 34.3% (Table 5.1) and the mean intensity of worms recovered was 13.8 worms/ positive duck. The worms were morphologically identified as *O. viverrini* duck type (Fig 5.3), with larger testes in comparison with those of the *O. viverrini* human type (Dao et al., 2014). The ITS2 DNA sequences of the worms (No. KT 894940, KT 894941, KT 894942, and KT 894943) were 100% identical to those of the *O. viverrini* duck genotype (No. KF 577570, KF 577571). Furthermore, the COI sequences of the recovered worms (No. KT 894944, KT 894945, KT 894946, and KT 894947) were identical to the COI DNA sequences (No. KF 577572, KF 577573) of the *O. viverrini* duck genotype at 99.7% (1 nucleotide differences) and 98.8% (4 nucleotides difference), respectively from 322 compared nucleotides. Ducks in An Nhon district were the most infected (40.4%) with the highest intensity (mean no. of worms=18.9/ positive duck). The prevalence and intensity of *O. viverrini* infection in ducks reared for egg production were much higher (46.2%; 16.4 worms/ positive duck) than those of ducks for meat production (16.7%; 3.4 worms/ positive duck). It was also observed that the prevalence and intensity of infection was significantly higher in older ducks (Fig 5.2). Ducks scavenging on streams were the most infected (44%) whereas ducks scavenging on rivers presented the highest mean intensity of 17.3 worms/ positive duck.

Table 5.1 Opisthorchiasis in ducks and related information in Binh Dinh Province

Factor	Categories	No. Positive	No. Examined	Apparent Prevalence (%)	95%CI (%)
Total		**61**	**178**	**34.3**	**27 – 42**
District	Phu My	26	69	37.7	26 – 50
	Phu Cat	10	33	30.3	16 – 49
	An Nhon	19	47	40.4	26 – 56
	Tuy Phuoc	6	29	20.7	8 – 40
Age	< 3 months	11	76	14.5	7 – 24
	3 - 12 months	20	47	42.6	28 – 58
	> 12 months	30	55	54.5	41 – 68
Type of production	Meat	12	72	16.7	9 – 27
	Eggs	49	106	46.2	36 – 56
Scavenging on	Rice field	57	166	34.3	27 – 42
	River	25	73	34.2	24 – 46
	Stream	33	75	44.0	33 – 56
	Irrigation canal	60	159	37.7	30 – 46
Knowledge*	Yes	31	61	50.8	38 – 64
	No	30	117	25.6	18 – 35

Note: AP = apparent prevalence, (*) = Knowledge of duck owner on opisthorchiasis in their duck; CI = Confidence Intervals.

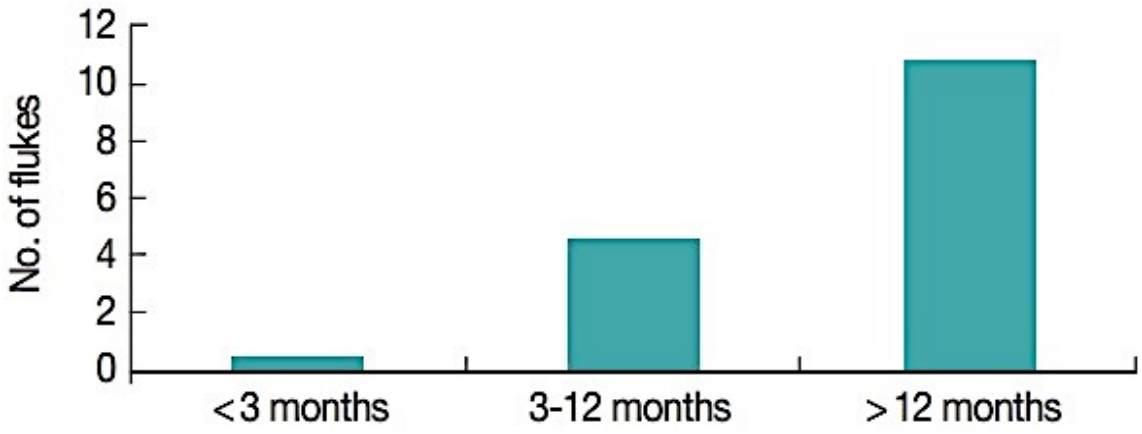

Figure 5.2 Average intensity of *Opisthorchis viverrini* infection in ducks in function of age.

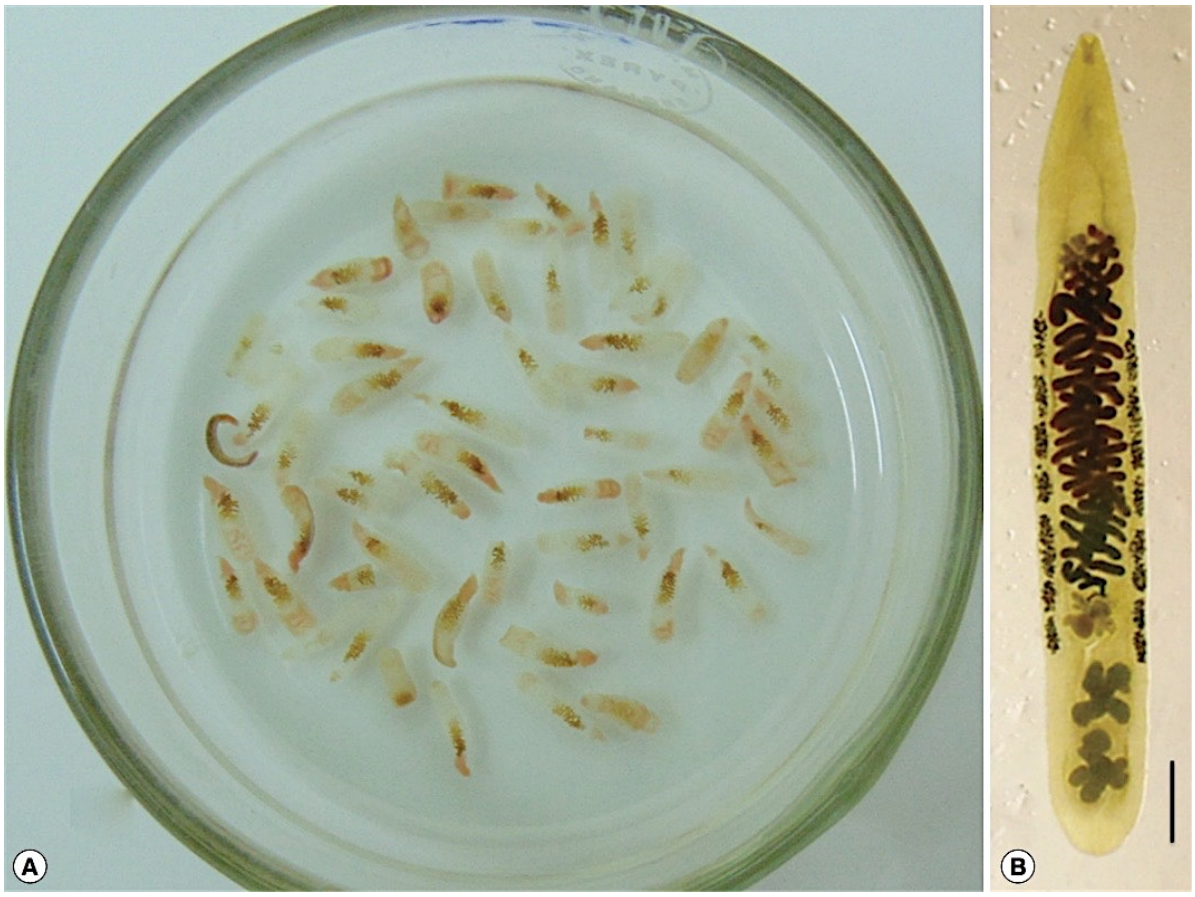

Figure 5.3 Worms recovered from domestic ducks (*Anas platyrhynchos*) in Binh Dinh Province, Central Vietnam. (A) Worms recovered from the liver. (B) Worm recovered from the gall bladder, unstained. Scale bar=1 mm.

5.4 Discussion

The prevalence of *O. viverrini* of 34.3% was high when compared to the infection rate of humans with *O. viverrini* (Sithithaworn et al., 2012), which is considered low if <10%, moderate if = 10-15%, or high if >15%. Because of its dependence on freshwater bodies, the domestic duck can feed on small freshwater fish, which might be highly infected with fishborne zoonotic trematodes (Nguyen et al., 2007; Nguyen et al., 2015). The role of ducks as reservoir hosts of some zoonotic small intestinal flukes was confirmed (Nguyen et al., 2010), and its infection with *O. viverrini* (small liver fluke) was reported (Dao et al., 2014). Although the zoonotic potential of the *O. viverrini* duck genotype has yet to be confirmed (Dorny et al., 2015), the high prevalence and intensity of *O. viverrini* in ducks indicate that large numbers of parasite's eggs are spread into water bodies on which ducks scavenge

every day. These eggs can be dispersed with water to wide geographical areas by the strong flow of streams, irrigation canals, and by annual flooding of rice fields or rivers. The two kinds of *O. viverrini* intermediate hosts, *Bithynia* snails and cyprinid fish are highly abundant in all freshwater bodies in lowland districts of the province (unpublished results). Infection of a *Bithynia* snail with a single *O. viverrini* egg can result in shedding of up to 27,692 *O. viverrini* cercariae during two months (Phongsasakulchoti et al., 2005). This presents the parasite with favorable conditions for completing its life cycle and explains why both prevalence and intensity of infection were high in the four investigated districts. Keeping ducks on rivers was associated with a significantly higher intensity of *O. viverrini* infection in ducks compared to other places ($p < 0.001$). During the dry season, streams, rice fields, and irrigation canals dry out while there is still water in rivers. Ducks therefore tend to scavenge more on rivers during this period in which small freshwater fish are present with the highest intensity of *O. viverrini* infection (Dung et al., 2014). In addition, infection of ducks with the liver fluke is an accumulative infection. Both the prevalence and intensity of *O. viverrini* infection in ducks were increased as the age of ducks increased (Fig 5.2). The accumulative infection can also explain the higher prevalence and intensity of infection of ducks for egg production in comparison with those of ducks for meat production. Ducks for meat purpose are sold on the local markets at the age of three months and as a result are only briefly exposed to infection. In contrast, ducks for egg production are culled at the age of two years, allowing them a much longer exposure. The significant positive relationship between knowledge of *O. viverrini* infection in ducks and prevalence (Table 5.1) can be explained by the fact that more farmers who raise ducks for eggs know about the infection than duck meat farmers. Some duck egg farmers treat their ducks with praziquantel, which may explain the lower intensity of infection in this group.

In conclusion, while Binh Dinh Province in Central Vietnam was confirmed as an endemic area of *O. viverrini* human genotype (Dao et al., 2016b), the findings of the present study confirmed this area to be hyper-endemic of the *O. viverrini* duck genotype. The co-existence of two *O. viverrini* genotypes in an endemic area, probably sharing the same intermediate hosts may render the epidemiological status of opisthorchiasis more complex. The sympatric occurrence of *O. viverrini*

human and duck genotypes in Binh Dinh Province may result in hybridization (interbreeding) or introgression (gene flow) between these two genotypes. Evidence of hybridization/introgression among species in the same genus was found in zoonotic *Fasciola gigantica* in Vietnam (Le et al., 2008). More studies on the genome and epidemiology of these two *O. viverrini* genotypes and on the intermediate hosts in Binh Dinh Province should be performed for a better understanding of this cryptic parasite (species complex).

CHAPTER 6

OPISTHORCHIS VIVERRINI INFECTION IN SNAILS AND FISH INTERMEDIATE HOSTS IN CENTRAL VIETNAM

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Opisthorchis viverrini infection in the snail and fish intermediate hosts in Central Vietnam

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6.1 Introduction

The Southeast Asian liver fluke *Opisthorchis viverrini*, a fish-borne trematode, is the causative agent of opisthorchiasis. This condition is a major public health problem in the Greater Mekong Region, including northeast Thailand, and parts of Cambodia, Lao PDR and Vietnam (Bouvard et al., 2009; WHO, 2008). Although most cases of opisthorchiasis are asymptomatic, the condition can lead to cholangitis, cholecystitis and even cholangiocarcinoma, a malignant tumor of the bile duct (Sripa, 2003; Sripa et al., 2012). It is estimated that more than 10 million people are infected with *O. viverrini*. In Thailand, where the disease has been studied extensively, the economic loss caused by opisthorchiasis is estimated at 120 million USD annually (Andrews et al., 2008; Sithithaworn et al., 2012; Sripa et al., 2007).

The life cycle of *O. viverrini* involves bithyniid freshwater snails (Ditrich et al., 1990) as the first intermediate hosts and more than 80 species of Cyprinid fish and at least 13 fish species belonging to other families of freshwater fish as the second intermediate hosts (Sithithaworn and Haswell-Elkins, 2003). Humans acquire the infection by consumption of raw or undercooked fish that contains metacercariae (Kaewkes, 2003). Dogs and cats and other fish-eating mammals can act as reservoir hosts (Aunpromma et al., 2012; Kaewkes, 2003). The disease is strongly related to the culinary tradition of raw fish consumption. In Vietnam, *O. viverrini* is present in the central and southern parts of the country, while the related *Clonorchis sinensis* occurs in the North (Nguyen and Le, 2011). The first cases of opisthorchiasis were identified in Central Vietnam in the 90 s (Nguyen, 2005). However, the epidemiology of this parasitic disease has received little attention in this region. In a recent study, we estimated the prevalence of opisthorchiasis at 11.4% in a commune of Binh Dinh Province; almost half of the local population, reporting eating raw fish dishes was at risk for the infection (Dao et al., 2016b). Few data are available on the intermediate host species responsible for the transmission of *O. viverrini* in Central Vietnam. The epidemiological situation in Central Vietnam is further complicated by the existence of a *O. viverrini*-like species that was found in the liver of ducks in this region (Dao et al., 2014; Dorny et al., 2015). Given the close genetic similarity of this parasite to human isolates of *O. viverrini*, sharing of the same intermediate host species is plausible. A better knowledge of the

epidemiology, including transmission by intermediate hosts is needed for identifying risk factors and developing control measures for opisthorchiasis.

The aim of the current study was to identify the snail and fish intermediate host species and investigate the prevalence and intensity of *O. viverrini* infection in these intermediate hosts in an opisthorchiasis endemic area in Central Vietnam. In addition, the association between prevalence and intensity of *O. viverrini* infection, and host size as well as snail habitat was determined.

6.2 Materials and methods

6.2.1 Study area

Bau My Tho (My Tho Lake) (14°13′23″N 109°9′16″E), located in Binh Dinh Province (Fig 6.1), an endemic area of opisthorchiasis in Central Vietnam (Dao et al., 2016b), was selected for investigating *O. viverrini* infection in snails and fish.

'Bau' or lake is a small freshwater reservoir commonly present in lowland communes in the central coastal area of Vietnam and consists of a complex of irrigation canals, rice fields and small ponds. Flooding occurs all over the 'bau' in the rainy season that spans from September to December, turning the area into a big lake during this period. Freshwater activities of the commune, such as rice and vegetable production, and duck farming, typically occur in the 'bau'. A wide diversity of freshwater snails and wild fish species inhabit the 'bau'. One of these fish species, the 'diec' (*Carassius auratus*) is preferred for preparation of the traditional raw fish dish in Binh Dinh province (Dao et al., 2016b).

In 2014 (March to May) and 2015 (January to May), snails and wild fish were collected twice a month in Bau My Tho for identification and examination of *O. viverrini* (-like) cercariae and metacercariae.

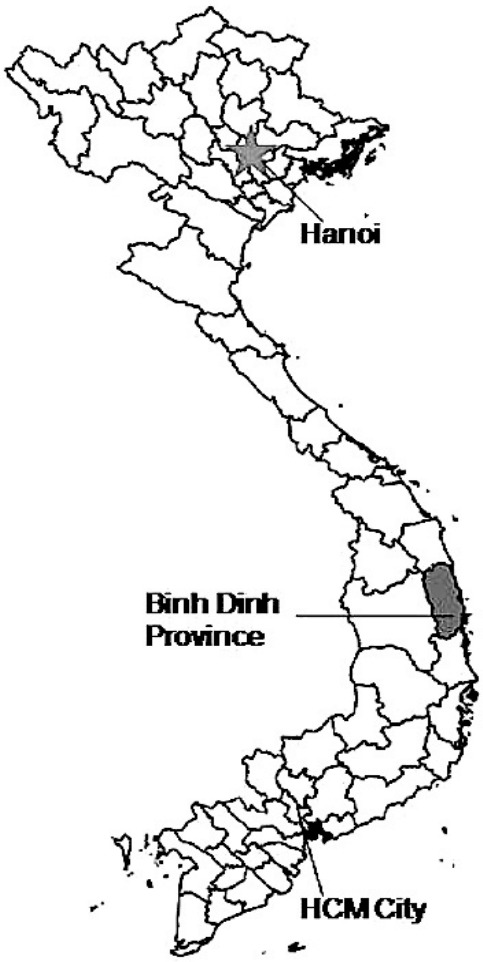

Figure 6.1 Map of Vietnam showing Binh Dinh Province where Bau My Tho (My Tho Lake) is located.

6.2.2 Collection and examination of snails

Snails were collected by hand picking on the banks of irrigation canals, rice fields and ponds in the Bau My Tho following the snail sampling method of Bui et al. (2010). A distance of approximately 3 km around the lake was covered for snail sampling; every 10 m, an area of about one square meter was inspected and all visible snails were collected. Snails from each collection point were kept separately for examination. Snails were transported in a cool box to the laboratory of the Sub-Animal Health Department of Binh Dinh province where they were identified using keys for freshwater snails (Chichamvong, 1992). Bithynia snails were identified at sub-species level. *Bithynia siamensis goniomphalos* and *Bithynia funiculata* have a sub-oval, conic shell. The umbilicus of *B. s. goniomphalos* is relatively wide and

deep with a weak carina, whereas the umbilicus of *B. funiculata* is funnel-shaped. Then, the shell sizes (length and width) of snails were measured. All collected snails, both Bithynia and other species were transferred to transparent plastic cups containing tap water. First, 10 snails of the same species were pooled for observing cercarial shedding. If pleurolophocercous cercariae were seen, individual snails were transferred to cups for shedding. A snail was considered infected with *O. viverrini* (-like) when pleurolophocercous cercariae (Adam et al., 1995; Kiatsopit et al., 2016) were observed. Pleurolophocercous cercariae were differentiated from parapleurolophocercous cercariae from *Haplorchis* spp. and other cercariae based on morphological differences (Nissen, 2012; Schell, 1985). For molecular confirmation, pleurolophocercous cercariae from each infected snail were randomly selected and preserved in RNAlater™ buffer (Qiagen, Texas USA, cat No./ID: 76104) at 4 °C in pools of one, five, or 100 (Webster, 2009). The total cercarial shedding over 24 h from each positive snail was collected and counted.

6.2.3 Collection and examination of fish

Five fishermen were active at Bau My Tho at the times of the fish collections. Fishermen use small boats from which they catch fish with nets. During the sampling days, all caught fish were gathered and approximately 50 fish were randomly picked for examination. Fish were transported in a cool box to the laboratory of the Sub-Animal Health Department of Binh Dinh Province, where they were identified (FIMSEA, accessed on 23/06/2016-http://ffish.asia/?p=h; (Rainboth, 1996; Tran et al., 2013), measured and weighed. Next, each individual fish was cut, grinded and digested in pepsin 1% for release of metacercariae (WHO, 1995). Metacercariae recovered from each fish were washed several times with normal saline before being examined under a stereomicroscope at a magnification of 80x, and with a light microscope at 100x and 400x. All *O. viverrini* metacercariae present in each fish were identified based on morphological characteristics (Sithithaworn and Haswell-Elkins, 2003), collected and counted. The presence of other types of metacercariae was recorded. *O. viverrini* and *O. viverrini*-like metacercariae from each infected fish species were randomly selected and individually preserved in RNAlater™ buffer (Qiagen, Texas USA, cat No./ID: 76104) at 4 °C for PCR confirmatory testing (Webster, 2009).

6.2.4 Molecular analysis

DNA was extracted from individual and pools of pleurolophocercous cercariae, and from individual *O. viverrini* metacercariae and *O. viverrini*-like metacercariae (See result section) using the Gentra Puregene Kit (Qiagen, Maryland, USA) following the manufacturer's instructions. The COI marker was used to amplify a portion of the COI gene. Two pairs of primers were used to amplify the genomic DNA extracted from pleurolophocercous cercariae in each infected snail: the COI-Ov-Hap F & R primers for *O. viverrini* (Thaenkham et al., 2011b) and the DOV-COI F (5′ CTTTATTCGGTTATGGCGGG 3′) and DOV-COI R (5′ CGAAGCAGAAAGCACTATACC 3′) primers, designed from the DNA sequence of adult *O. viverrini*-like adult worms, collected from ducks (Genbank number KF 577572, later referred to as *O. viverrini* − duck genotype). On the other hand, the primers of COI-Ov-Hap F & R were used to amplify the COI portion of *O. viverrini* metacercariae and DOV-COI F & R were used for *O. viverrini*-like metacercariae. The PCR products were visualized on agarose gel (Eurogentec, Liège, Belgium) 1.5% in TAE buffer 1x, using the Mupid®-One electrophoresis system and sent to the University of Antwerp, Belgium for purifying and sequencing. The obtained DNA sequences of the COI markers, after editing in Bioedit (Hall, 1999) were aligned to the DNA sequences of adult *O. viverrini*, deposited in Genbank (KF 557572 and KF 557573), and adult *O. viverrini*-like (KF 577572 and KT 894946) to evaluate identity among the COI DNA sequences of each developmental stage in the life cycle of each *O. viverrini* genotype.

6.2.5 Statistical analysis

All collected data were entered in Excel (Microsoft Office version 2007) and transferred to STATA, version 11.2, software (StataCorp LP, College Station, TX) for statistical analysis. Descriptive statistics were used to summarize the overall prevalence and the mean intensity of *O. viverrini* infection (for snail: number of cercariae/day/snail, for fish: number of metacercariae/fish) by snail and fish species and snail size. Shell lengths of Bithynia snails were divided into three groups: < 8.0 mm, 8.0 −< 10.0 mm and ≥ 10.0 mm.

An exact logistic regression was chosen to analyze the association of *O. viverrini* infection in snails with species, shell size and habitat, motivated by the low number

of infected snails per class (less than five infected cases). Logistic regression was applied to study the association of *O. viverrini* infection in fish with species. A negative binomial regression was used to analyze the association of the *O. viverrini* infection intensity in fish with species, and was preferred over Poisson regression based on the likelihood ratio test. For modelling purposes, only fish species having more than thirty fish examined were included in the analyzes. Univariate analyses were run first and subsequently multivariate analyzes. Models of the latter were evaluated based on the likelihood ratio test. The statistical significance level was set at $p \leq 0.05$.

6.3 Results

6.3.1 O. viverrini infection in Bithynia snails

A total of 12,000 snails were collected from Bau My Tho of Binh Dinh Province. These snails belonged to six families: Bithyniidae (*B. s. goniomphalos* and *B. funiculata*), Planorbidae (*Indoplanorbis* sp.), Lymnaeidae (*Lymnaea* spp.), Thiaridae (*Melanoides tuberculata*), Viviparidae (*Angulyagra* spp., *Cipangopaludina* spp. and *Sinotoia* sp.) and Ampullariidae (*Pila* sp.). *M. tuberculata* was the most abundant snail species (data not shown); 923 and 693 *B. s. goniomphalos* and *B. funiculata* were collected, respectively.

Pleurolophocercous cercariae (body size = 110 × 50 μm, tail size = 400 × 25 μm) were only found in Bithynia snails. The prevalence of *O. viverrini* infection in *B. s. goniomphalos* and *B. funiculata* was 0.87% (8/923) and 0.14% (1/693), respectively. The obtained COI DNA sequences from pleurolophocercous cercariae isolated from *B. s. goniomphalos* were all from *O. viverrini*: the sequences showed 98.86% identity on 260 nucleotides compared to those of the adult stage. No *O. viverrini* duck-genotype was found in *B. s. goniomphalos*. However, in *B. funiculata* both *O. viverrini* and *O. viverrini* duck-genotype were found. The COI DNA sequences of *O. viverrini* showed 98.86% identity, and those of *O. viverrini*-duck genotype revealed 100% identity with those of its adult stages. The DNA sequences from this study were deposited in Genbank with the ID numbers: KX 394630 and KX 394631 for *O. viverrini*, and KY 009926 and KY 009927 for *O. viverrini*-duck genotype.

Table 6.1 Prevalence and intensity of *O. viverrini* infection in *Bithynia* snails collected from My Tho Lake, Binh Dinh Province, Central Vietnam

Factor		Prevalence (%)				Intensity (±SD)
		(N pos/ N)	OR	*p*-value	95% CI	No.cer/24h/snail
Species	*B. funiculata*	0.14 (1/693)	1			638.0 (NA)
	B.s. goniomphalos	0.87 (8/923)	3.02	0.449	0.17 - 53.00	472.5 (±262.72)
Shell size	< 8 mm	0.65 (2/308)	1			140.0 (±56.57)
	8 -< 10 mm	0.90 (5/553)	0.31	0.212	0.05 - 1.93	544.0 (±186.23)
	≥ 10 mm	0.27 (2/746	0.60	0.639	0.47 - 7.62	709.0 (NA)
Collection site	Rice field	0.11 (1/934)	1			180.0 (NA)
	Irrigation canal	1.17 (8/673)	13.60	0.029	1.31 - 141.25	529.8 (±238.68)

Note: OR = odds ratios, CI = confident interval, SD = standard deviation, NA = not available

The mean number of cercariae shed per *B. s. goniomphalos* in 24h was 472.5 and the number of cercariae shed in 24h was 638 in the one *B. funiculata* infected snail (Table 6.1). In the multivariate analysis, neither species nor shell length were significantly associated with *O. viverrini* infection. In contrast, the habitat was associated with infection (p = 0.029), with *Bithynia* spp. collected in irrigation canals having a 13.6 higher odds (95% confidence interval (CI): 1.31- 141.25) of being infected compared to those collected in rice fields (Table 6.1).

6.3.2 O. viverrini infection in freshwater fish

A total of 754 fish belonging to 12 species from six families were identified from Bau My Tho. In Table 6.2, fish families and species, as well as number of fish examined and number of fish in which *O. viverrini* metacercariae were found are presented. *O. viverrini* metacercariae were recovered from 10 fish species belonging to five families. Mixed infections of *O. viverrini*, *Haplorchis* spp., *Centrocestus* spp. and *Echinostoma* spp. were observed in most infected fish species except for *C. auratus* (data not shown). Infections with *Haplorchis* spp., *Centrocestus* spp. and *Echinostoma* spp. were most frequently observed, at rates of 80–90%. Cercariae of *Haplorchis* spp. were found in 2.5% of *Melanoides tuberculata* (data not shown). Metacercariae of both *O. viverrini* and *O. viverrini*-duck genotype were found in three fish species, *P. brevis*, *R. aurotaenia* and *Esomus metallicus*. Metacercariae of *O. viverrini*-duck genotype are smaller (168 ± 4.79 × 82 ± 4.85 μm) and more ellipsoidal compared to the larger and oval-shaped *O. viverrini* metacercariae (225 ± 59.16 × 208 ± 83.17 μm). The obtained COI DNA sequences of *O. viverrini* and *O. viverrini*-duck-genotype metacercariae showed 100% identity with those of their respective adult stages, confirming the morphological identification of *O. viverrini* and *O. viverrini*-duck genotype metacercariae. The COI DNA sequences of the metacercariae in this study were deposited in Genbank with the ID numbers: KX 394632 and KX 394633 for *O. viverrini* and KY 009923 and KY 009924 for *O. viverrini*-duck genotype.

Table 6.2 Fish species collected from Bau My Tho

Fish species	Numbers of fish		Mean of body weight (g) (±SD)
	Examined	Positive	
Family Cyprinidae	626	311	
Carassius auratus	254	188	10.8 (±3.46)
Rasbora aurotaenia	104	58	4.7 (±0.88)
Puntius brevis	158	50	3.5 (±1.44)
Esomus metallicus	81	11	1.0 (±0.55)
Hampala dispar	14	4	9.0 (±0.48)
Cyprnius carpio	15	0	4.8 (±5.81)
Family Cichlidae			
Oreochromis niloticus	48	12	9.9 (±11.12)
Family Anabantidae			
Anabas testudineus	22	7	5.7 (±1.90)
Family Bagridae			
Mystus carasius	30	8	4.6 (±0.95)
Family Osphronemidae			
Trichopsis vittatus	9	2	1.0 (±0.23)
Trichogaster trichoptenis	7	2	1.9 (±0.90)
Family Channidae			
Channa orientalis	12	0	38.0 (±2.98)
Total	**754**	**339**	

Note: [a] = positive for *O. viverrini* metacercariae, SD = standard deviation

The prevalence of *O. viverrini* metacercariae in the six most abundant fish species is shown in Table 6.3. *C. auratus*, *Rasbora aurotaenia*, and *Puntius brevis* had the highest prevalence of infection with *O. viverrini* metacercariae, of 74.0%, 55.8% and 31.6%, respectively. The presence of *O. viverrini* was significantly associated with fish species ($p \leq 0.005$), except for *Mystus carasius* and *Oreochromis niloticus*. The most abundant fish species, *C. auratus* had an 18.1 higher odds (95% CI: 9.39–38.1) of being infected with *O. viverrini* than *E. metallicus*. Fish species was not significantly associated with the intensity of infection with *O. viverrini* metacercariae (Table 6.4).

Table 6.3 Prevalence of *O. viverrini* infection in fish species collected from My Tho Lake, Binh Dinh Province, Central Vietnam

Factors	Prevalence (95% CI)	OR (95% CI)	*p*- value
Species			
Esomus metallicus	13.6 (6.1 - 21.1)	1	
Oreochromis niloticus	18.8 (7.6 - 29.9)	1.47 (0.55 – 3.86)	0.434
Mystus carasius	26.7 (10.5 - 42.8)	2.31 (0.81 – 6.74)	0.110
Puntius brevis	31.6 (24.4 - 38.9)	2.95 (1.48 – 6.31)	0.003
Rasbora aurotaenia	55.8 (46.2 - 65.4)	8.02 (3.94 – 17.60)	< 0.001
Carassius auratus	74.0 (68.6 - 79.4)	18.10 (9.39 – 38.10)	< 0.001

Note: OR = odds ratios obtained from univariate logistic regression, CI = confidence interval

Table 6.4 Intensity of *O. viverrini* metacercariae infection in fish species collected from My Tho Lake, Binh Dinh Province, Central Vietnam

Factors	No. metacercariae/fish (±SD)	IRR (95% CI)	*p*-value
Species			
Carassius auratus	6.4 (±5.75)	1.2 (0.95 – 1.50)	0.133
Rasbora aurotaenia	5.7 (±3.39)	1.1 (0.80 – 1.40)	0.694
Puntius brevis	5.4 (±3.36)	1	

Note: IRR = incidence rate ratios obtained from univariate negative binominal regression, CI = confidence interval, SD = standard deviation

6.4 Discussion

O. viverrini cercariae were found in two Bithynia species, *B. s. goniomphalos* and *B. funiculata* collected from Bau My Tho in Binh Dinh Province. This finding confirms the role of these species as the first intermediate hosts of *O. viverrini*; yet, it is the first identification of *O. viverrini* in Bithynia snails in Vietnam. Three Bithynia species and sub-species are known to be susceptible for *O. viverrini*: *B. s. goniomphalos*, *B. s. siamensis* and *B. funiculata* (Andrews et al., 2008). The prevalence of *O. viverrini*

in those (sub)species is typically low, ranging from 0.03–1.3% in Thailand, comparable to the 0.87% prevalence in *B. s. goniomphalos* and 0.14% in *B. funiculata* in our study, to 2.01–3.04% in a hyper-endemic wetland area at the border between Thailand and Lao PDR (Kiatsopit et al., 2012).

While the number of infected snails in an endemic area is low, infected snails can shed several ten thousand of cercariae over a period of several months (Phongsasakulchoti et al., 2005). In this study, *B. s. goniomphalos* and *B. funiculata* shed an average of 472 cercariae and 638 cercariae per day, respectively. Transmission from the first to the second intermediate host of *O. viverrini* is very effective. In an experimental study, 95% and 100% *Barbonymus gonionotus* (Java barb) fingerlings challenged with *O. viverrini* cercariae became infected after exposure with low (25 cercariae) and high (200 cercariae) doses, respectively (Donthaisong et al., 2014). Those figures explain why the prevalence of *O. viverrini* cercariae in the first intermediate host is low, while that in the second intermediate fish host is usually high, up to 90–95% in endemic areas (Sithithaworn and Haswell-Elkins, 2003). Also in this study occurrence of *O. viverrini* metacercariae in wild caught fish was high. Ten of twelve wild caught fish species, belonging to five families, were infected. The Cyprinidae family was the most frequently caught (626/754 fish examined) and infected. The finding of *O. viverrini* metacercariae in both cyprinid and non-cyprinid fish in our study is similar to findings from other opisthorchiasis endemic areas in Thailand, Cambodia and Lao PDR. Indeed, over 80 species of the Cyprinidae family and at least 13 other families have been shown to serve as the second intermediate host of *O. viverrini* (Eom et al., 2015; Sithithaworn and Haswell-Elkins, 2003; Touch et al., 2009).

Bau My Tho in Binh Dinh province supports an abundant population and a wide variety of wild fish species. Fishing is the main activity of several families living around the lake. Wild caught fish sold at the local market is a rich and cheap source of proteins for the local communities. In Central Vietnam, *C. auratus* (‘diec’ fish) is the favorite fish used to prepare the traditional live fish dish (Dao et al., 2016b). This fish species was found to have the highest prevalence (74%) of infection with *O. viverrini* metacercariae in Bau My Tho. Similar high prevalence and intensity of infection with *O. viverrini* metacercariae in fish were found in other wetland

opisthorchiasis endemic areas of Southeast Asia (Eom et al., 2015; Sithithaworn and Haswell-Elkins, 2003).

Other species of cyprinid and non-cyprinid infected fish in the lake are not used to prepare the traditional raw fish dish in this area. Nevertheless, they are abundant in freshwater bodies, and because of their small size (average weight of fish examined = 7.92 ± 2.56 g), they can maintain the life cycle through infection of reservoir final hosts, such as dogs, cats and possibly also ducks (Dao et al., 2014; Dorny et al., 2015).

Examination of snails and fish in My Tho Lake showed that not only (meta)cercariae of *O. viverrini* were found but also (meta)cercariae of the *O. viverrini*-duck genotype (Dao et al., 2014; Dorny et al., 2015). This finding reveals the potential of hybridization or introgression of these two *O. viverrini* genotypes by its sharing of the same intermediate hosts, similar as to what was shown for other trematodes and cestodes (Henrich et al., 2013; Le et al., 2008). In addition, cercariae and metacercariae of other zoonotic intestinal fishborne trematode species were also observed in snails and fish.

In conclusion, our study is the first to show the role of *B. s. goniomphalos* and *B. funiculata* as the first intermediate snail hosts of *O. viverrini* in Vietnam. It also demonstrated the role of the second intermediate fish hosts for *O. viverrini* of a wide range of wild fish species, including five species of cyprinid fish and five species belonging to four non-cyprinid families. *B. s. goniomphalos* and *C. auratus* seem to play the most important role as the first and second intermediate hosts of *O. viverrini* in Central Vietnam, respectively. Our findings also showed the complex epidemiology of opisthorchiasis in this region by demonstrating the presence of (meta)cercariae of *O. viverrini* and the *O. viverrini*-duck genotype in the same snail and fish intermediate host species. Further studies are needed in the region on the possibility of hybridization/introgression between these genotypes and on the role of potential reservoir final hosts, such as dogs, cats, and fish-eating birds for a better understanding of the epidemiology and for developing an effective control programme.

CHAPTER 7

SUMMARY OF THE LIFE CYCLE OF THE *OPISTHORCHIS* SP. BD2013

Opisthorchis sp. BD2013 isolated from the domestic duck (*Anas platyrhynchos*) requires Bithynia snails and freshwater fish as the first and second intermediate hosts, respectively, to complete its life cycle:

7.1 Domestic duck final hosts of *Opisthorchis* sp. BD2013

Domestic ducks are freely looking for food on freshwater reservoirs. They defecate in the water and may contaminate freshwater reservoir with fluke eggs.

Figure 7.1 Domestic ducks are freely looking for food on freshwater reservoirs.

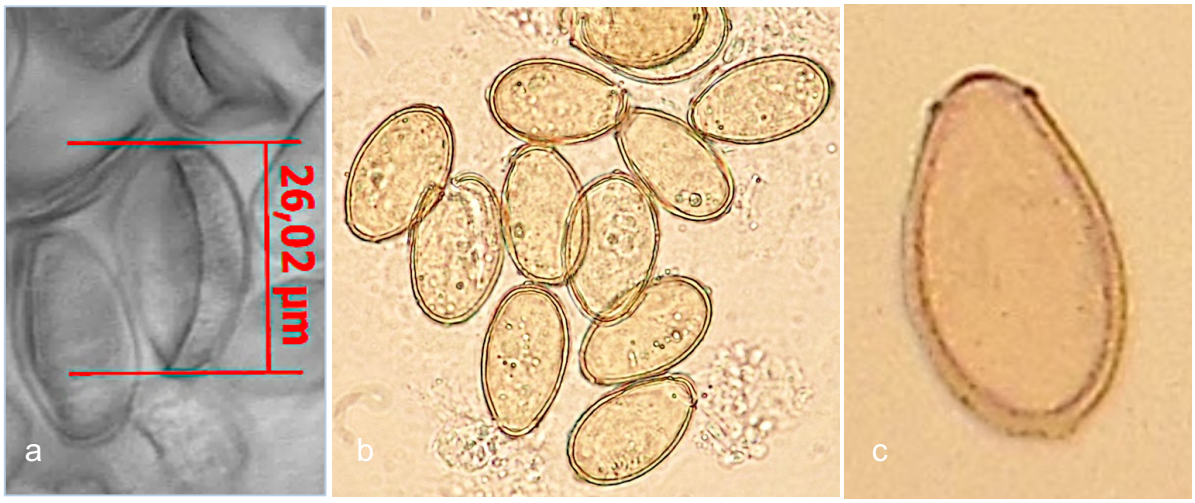

Figure 7.2 The eggs from *Opisthorchis* sp. BD2013. (a) eggs in the uterus, (b) eggs in the gall bladder, (c) egg in feces.

7.2 The Bithynia first intermediate snail hosts

The Bithynia first intermediate host ingest *Opisthorchis* sp. BD2013 eggs; in the snail host the parasite eggs develop and multiply in sporocysts and rediae resulting in the development of pleurolophocercous cercariae that are shed into the water.

Figure 7.3 *Bithynia funiculata* snails, the first intermediate host of *Opisthorchis* sp. BD2013.

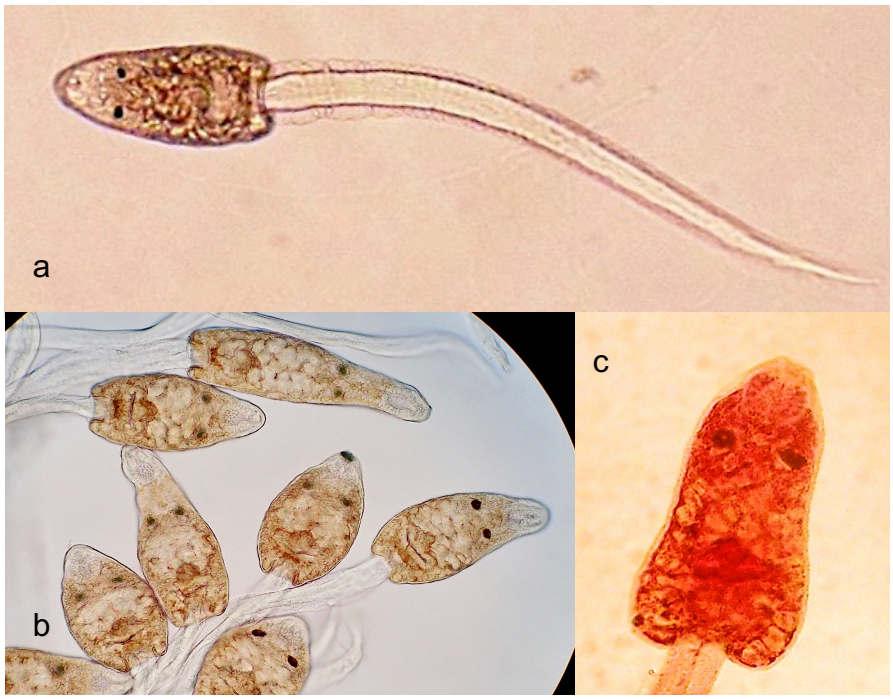

Figure 7.4 Pleurolophocercous cercariae. (a) Fresh cercaria: body size = 110 x 50 µm, tail size = 400 x 25 µm; (b) cercariae in formaldehyde 10%; (c) cercaria stained with Carmine dye.

7.3 The second intermediate hosts of *Opisthorchis* sp. BD2013

The shed cercariae attach to the freshwater fish second intermediate host and develop into metacercariae in muscle and skin of freshwater fish.

Figure 7.5 The second intermediate hosts of *Opisthorchis* sp. BD2013.

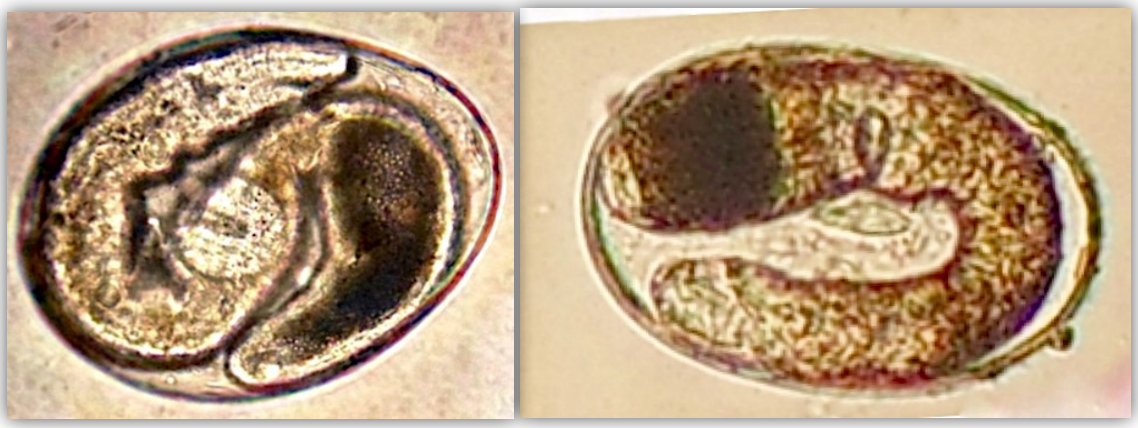

Figure 7.6 Metacercariae of *Opisthorchis* sp. BD2013, average size: 168.5 x 82.4 µm.

7.4 The adult *Opisthorchis* sp. BD2013

By consuming the raw fish containing metacercariae of *Opisthorchis* sp. BD2013, a range of final hosts including ducks become infected with the fluke. The metacercariae excyst in the duodenum and migrate to the bile ducts in the liver to mature into adult hermaphrodite worms and start laying eggs.

Figure 7.7 The adult *Opisthorchis* sp. BD2013. (a) fresh worm in gall bladder, (b) fresh worm in the bile duct - scale bar = 1mm, (c) Carmine dye-stained worm.

CHAPTER 8

GENERAL DISCUSSION: THE COMPLEXITY OF THE EPIDEMIOLOGY OF OPISTHORCHIASIS IN CENTRAL VIETNAM AND OPTIONS FOR A CONTROL PROGRAM

8.1 Introduction

The recent findings of new species within the genus *Opisthorchis* may complicate the epidemiology of opisthorchiasis in Southeast Asia. Indeed, besides the description of *Opisthorchis lobatus* in Lao PDR in 2011 we have described in this thesis the finding and characterization of a new species, *Opisthorchis* sp. BD2013 in Central Vietnam (Dao, 2012; Laoprom et al., 2009; Thaenkham et al., 2011b). These new *Opisthorchis* species are sympatric with *O. viverrini,* a potentially fatal liver fluke in the Greater Mekong region and share first and second intermediate hosts. In this final chapter, we discuss the potential consequences of the co-existence of these sister species on the epidemiology of opisthorchiasis in the endemic area of Binh Dinh Province in Central Vietnam. In addition, we propose options for a One Health control program for opisthorchiasis in Vietnam and we give recommendations for future research.

8.2 Opisthorchiasis in Central Vietnam: complex epidemiology

Opisthorchiasis caused by the potentially fatal *O. viverrini* has been reported in central Vietnam for the last decades (Nguyen, 2005). Results presented in chapter 2 of this thesis showed that the current prevalence of *O. viverrini* in the investigated human population in Central Vietnam was 11.4%. Almost half of the participants (45.3%) in our study population declared maintaining the habit of eating raw fish namely, 'goi ca' prepared from a freshwater crucian carp ('diec' fish – *Carasius auratus*), a key risk factors of the infection. Humans and cats were confirmed as the final/reservoir hosts of *O. viverrini* in Vietnam. Infection rates of *O. viverrini* in cats in southern provinces were 2.5% in Dong Thap and 21.43% in Tay Ninh. The role of dogs in the life cycle has not been confirmed yet (Le, 2012; Nguyen, 2005). While many studies from Thailand report on the severe consequences of chronic opisthorchiasis, namely the development of bile duct cancer, until now, no studies on the clinical importance of this parasite were conducted in Vietnam (Bouvard et al., 2009; Sripa et al., 2012) where more attention has gone to *Clonorchis sinensis*, which is endemic in the northern part of the country (Nguyen and Le, 2011).

In 2012, we reported on the finding of an *Opisthorchis* sp. (*Opisthorchis* sp. BD2013) in the bile ducts of the domestic duck (*Anas platyrhynchos*) in an *O. viverrini* endemic area in Central Vietnam; subsequently, we found a high prevalence of 34.3% in the local duck population (Chapters 3 and 5). While some controversy remains on the scientific name of the duck *O. viverrini* genotype (Dorny et al., 2015; Nawa et al., 2015; Pham and Nawa, 2016), the duck *Opisthorchis* isolate was confirmed as a sister species of *O. viverrini* (Chapter 4). These two *Opisthorchis* species were found in the same *B. funiculata* snail first intermediate host and in three fish second intermediate host species, namely *Puntius brevis*, *Rasbora aurotaenia* and *Esomus metallicus* (Chapter 6, Fig. 7.5).

Interaction among different parasite species within both intermediate and final hosts can be important factors shaping the evolution of parasite and host populations. Within snail intermediate hosts, antagonistic interaction among trematode species, such as competition and predation, can influence the parasite abundance and diversity (Keeney et al., 2008). An example in trematodes is that in co-infection of *Biomphalaria glabrata* with *Schistosoma mansoni* and *Echinostoma liei*, *S. mansoni* sporocysts show a very strong decrease of their infectivity in mice (Jourdane et al., 1990). The co-infection of two *Opisthorchis* spp. in fish hosts may increase the chance of co-infection with these two species in the final/reservoir hosts such as, humans, dogs, cats, aquatic poultry and fish-eating birds. The interactions between parasite and host and also between different closely related parasite species infecting the same host can result in hybridization/introgression (Leger and Webster, 2017; Webster et al., 2013). Experimental studies revealed that hybridization of trematodes not only increases fitness by extending the range of suitable (intermediate) host species but that hybrids also have a higher fecundity, a faster maturation time and a higher infectivity (Henrich et al., 2013; Huyse et al., 2009; Leger and Webster, 2017; Webster et al., 2013). For instance, natural introgression/hybridization of *Fasciola hepatica* and *F. gigantica* was demonstrated in goats in Central Vietnam (Le et al., 2008). A well studied example from West Africa is the hybridization of the human parasite *Schistosoma haematobium,* the cause of urinary bilharziosis with the cattle parasite *Schistosoma bovis.* The finding of this hybrid may have serious consequences on the epidemiology and burden of schistosomiasis in the region. It was suggested that the acquisition of new genes

may generate new phenotypes that might differ in virulence, resistance, pathology, and host use, ultimately leading to the emergence of new diseases (Huyse et al., 2009; Leger and Webster, 2017; Webster et al., 2013), new reservoir hosts potentially impacting on the effectiveness of current control programs.

To date, the zoonotic status of *Opisthorchis* sp. BD2013 has not been shown yet, although there exists a potential of co-infection of these two *Opisthorchis* spp. by its sharing of the same fish intermediate hosts. Indeed, three of the most abundant fish species in My Tho Lake were found infected with metacercariae of both *O. viverrini* and *Opisthorchis* sp. BD2013 at a high prevalence: *R. aurotaenia* 55.8%, *P. brevis* 31.6%, and *E. metallicus* 13.6%. These fish species have a small size and weigh less than five grams. They are abundant in shallow freshwater reservoirs in Central Vietnam (Chapter 6). Dogs, cats, ducks and other wild fish-eating birds (storks, egrets) were observed on the banks or in the water of freshwater reservoirs and rice-fields looking for food and they may catch these fishes (See Fig 1.9, Chapter 1). Interestingly, we only found metacercariae of *O. viverrini* and not of *Opisthorchis* sp. BD2013 in 'diec' fish (*Carasius auratus*), the wild freshwater fish used for the traditional raw fish dish consumed in the region. This finding, together with the unknown final host range of *Opisthorchis* sp. BD2013 may be one of the reasons for not finding *Opisthorchis* sp. BD2013 in humans. In Central Vietnam, other fish species than 'diec' fish are generally well cooked before consumption.

In this research, we attempted to sample potential reservoir final hosts of *Opisthorchis* spp. in our study region. Unfortunately, this turned out to be a difficult enterprise. During our visits, we could sample around 100 fecal samples from dogs for microscopic examination of helminth eggs. Some samples were positive for *Opisthorchis*-like eggs. Since eggs of *Opisthorchis* spp. and minute intestinal flukes that are very prevalent in the region (Do et al., 2007) cannot be differentiated by morphology we intended to perform additional studies but unfortunately the dogs were lost to follow up (stolen). Cats are free roaming and absent from home all day long; even cat owners could not help catching their cats for fecal sampling. We tried to catch wild fish-eating birds but could trap only three storks and five egrets that were euthanized and necropsied. In these wild birds, we did not find *Opisthorchis* spp. but could recover an *Echinostoma* sp. from the intestines of one stork. It is

clear that more work should be done in the region to explore the host range of the two sister *Opisthorchis* spp.

We started the study of the life cycle of *Opisthorchis* sp. BD2013 on a duck farm from which the first case of infection with this new species originated. On this farm snail and fish species were collected from the freshwater reservoir on which ducks were roaming, for examination of cercariae and metacercariae; however, none were found. Parapleurolophocercous cercariae were found in *Melanoides* sp. sampled from a neighboring rice field. These were confirmed as *Haplorchis* spp. by sequencing (unpublished data). Consequently, we expanded the sampling of snails and fish to adjacent communes and districts but could still not identify cercariae or metacercariae of *Opisthorchis* sp. BD2013, while adult worms were isolated from almost every examined duck population in those communities in those districts of Binh Dinh Province, Central Vietnam (Chapter 5). *Opisthorchis* sp. BD2013 cercariae and metacercariae were found only in My Tho Lake of My Tho Commune, an endemic commune of opisthorchiasis caused by *O. viverrini*. These findings raise many questions on the epidemiology of *Opisthorchis* sp. BD2013 in the region.

8.3 Towards an integrated control programme for opisthorchiasis in Central Vietnam

Opisthorchis spp. require a complex life cycle involving two intermediate hosts, freshwater snails and fish, and a range of final/ reservoir hosts including, humans and wild and domestic fish-eating birds and animals. The culinary habit of eating raw fish dishes is defined as the key risk factor for infection with *Opisthorchis* spp. (Kaewkes, 2003); Chapter 2 & 6 & 7). To prevent and control this parasite, an integrated control programme using an eco-health/ one-health approach involving different parties including, public health, animal health and environmental sectors must be built (Sripa et al., 2015; Sripa et al., 2017). In an attempt to control small liver flukes in Vietnam, the Vietnamese Government issued decision No. 1931/QD_BYT (date: 19/05/2016) (https://thuvienphapluat.vn/van-ban/The-thao-Y-te/Quyet-dinh-1931-QD-BYT-huong-dan-tay-san-la-gan-nho-tai-cong-dong-2016-311913.aspx, in Vietnamese). However, the control highlights of this directive are mostly based on the administration of anthelmintics to infected cases and on convincing the local people to give up their habit of consuming raw fish. The conditions of opisthorchiasis in Central Vietnam are characterized by the

typical way of eating live fish, the lack of clinical and epidemiological data on the parasite and the infection it causes in man; and the fact that in this region many men are frequently absent from home because of their fishing activities on the sea. This important population group is difficult to access for public health services or education. Therefore, we propose a control programme adapted to Central Vietnamese conditions:

8.3.1 Epidemiological and clinical studies

The results from our studies have revealed the evidence of occurrence of opisthorchiasis in the investigated population in Central Vietnam, as well as in snail and fish hosts (Chapters 2 & 6). However, the study area in our research was limited. More data on the prevalence of opisthorchiasis in humans, domestic animals and birds, and in snail and fish intermediate host species from a broader geographical area in the central part of the country are needed. This will require collaborations among the departments of public health, animal health and environment and are essential for the delineation of a control strategy and evaluation.

Our studies have shown no difference in the risk of acquiring infection between participants with or without knowledge on opisthorchiasis, its transmission pathway and its harmfulness (Chapter 2). This could in part be due to the perception of the local communities that the infection can easily be cured using praziquantel and a limited believe that the parasite is a cause of the much more serious liver of bile duct cancer. Results from clinical studies conducted in the area, clearly identifying the potential clinical impact of this infection, will help convincing the local communities about the seriousness and economic impact of the disease. This will increase their perception of being at risk due to their behavior/practices i.e., raw fish consumption, allowing dogs and cats to roam freely, etc. Increased perception of risk is one of the key factors leading to behavior change.

8.3.2 Anthelmintics treatment for both humans and domestic animals

In our study, while infection with *O. viverrini* was found in all age classes, children in the age class less than 18 were less infected, as well as females compared to males. No infected cases were found in the ages less than 12 years old (Chapter 2). Based on these epidemiological findings, the WHO (WHO, 1995) and Vietnamese MOH guidelines (https://thuvienphapluat.vn/van-ban/The-thao-Y-te/Quyet-dinh-1931-QD-

BYT-huong-dan-tay-san-la-gan-nho-tai-cong-dong-2016-311913.aspx), and taking into account the potential adverse reactions, we suggest that mass administration of praziquantel should be applied only on individuals older than 12 years and not on pregnant women, diseased or old people. The climate in Central Vietnam comprises a dry and wet season; during the end of the dry season, from end of May to July, no rain and strong sunshine are recorded. Freshwater reservoirs in the region tend to dry up in this season, dramatically reducing the snail and fish populations. This period would be the best time for praziquantel administration in both humans and reservoir hosts, i.e., dogs and cats in order to minimize contamination with fecally excreted parasite eggs in the environment and consequently minimalize transmission. Also, by drying under strong sunlight the parasite eggs show an increased mortality resulting in decreased infectivity in snails (Echaubard et al., 2017). The outcome of this strategic intervention on transmission should be verified.

8.3.3 Sanitation and environmental strategies

Our results and observations in the study area in Central Vietnam showed that freshwater reservoirs are mostly located around villages and contain abundant populations of freshwater snail and fish species. Most available wild fish species were infected with *O. viverrini* and *Opisthorchis* sp. BD2013 metacercariae (Chapter 6). In this region, local people have the tradition of using fresh stools to fertilize rice fields, creating opportunities for closing the parasite's life cycle. In recent years, so-called sanitary toilets have replaced the traditional latrines, which allowed collection of fresh feces for agricultural use. Unfortunately, those local sanitary toilets do not provide conditions to deactive parasite eggs, because they consist of simple septic tanks and not of the required multi-layered sediment tanks. The content of these septic tanks, which may contain viable parasite eggs is poured directly into the lakes and freshwater reservoirs around the villages. Because of the high price for building a good sanitary toilet, the Vietnamese Government should financially support a "Parasite eggs free toilets" campaign as part of an opisthorchiasis control programme.

Another action that they may reduce the parasite's transmission is the control of floating grass and aquatic plants such as, water hyacinth in freshwater reservoirs

and on lakes in the endemic areas during and after the rainy season aiming at reducing the habitat of *Bithynia* snails. In Central Vietnam this is typically done by manual removal of aquatic plants by the community. The impact of this intervention or of other environmental or chemical methods aiming at reducing the snail population on the prevalence of *Opisthorchis* needs to be investigated.

8.3.4 Health education

Health education aimed at giving the local population a solid background knowledge on the parasite, the way it is being acquired and its consequences for health is a key factor in any control programme in the endemic area. All parties of the public health, animal health and environmental sectors need to be involved in campaigns, of which the public health department of the MOH should play a core role.

From our study results, it appeared that half of opisthorchiasis patients had knowledge on *O. viverrini* and opisthorchiasis. However, these patients did not change their behavior of eating raw fish after anthelmintic administration, because they were not aware that liver fluke infection can actually kill people by causing liver or bile duct cancer (Chapter 2). Therefore, more information on the consequences of repeated exposure and chronic infection with *O. viverrini* must be provided as part of a health education campaign. An example of a successful control programme is the “Lawa model” in Thailand (Sripa et al., 2017) that provided color cartoon books, leaflets, posters, etc., including easy understandable but strong information on the life cycle and the disease caused by the liver fluke to the population. Similar educational materials adapted to the local conditions should be designed and distributed for free to the local population in endemic areas. In addition, health messages should be spread in schools and by doctors and nurses. A special notice for the south-central coastal population needs to be addressed to fishermen on the sea. These men, who are the most vulnerable with the parasite from our researched result, are frequently absent from home for periods of up to one month. On the sea, they can only access the FM radio. So, a special radio programme addressing health messages on opisthorchiasis needs to be built for this group.

8.4 Recommendations for future works

One of the main gaps identified in our study is data on the host range of *Opisthorchis* sp. BD2013. A large sample from a greater diversity of wild and domestic aquatic fish-eating bird species, and from dogs and cats should be collected for morphological and molecular analyses of liver flukes. This should also add data on the reservoir host status for *O. viverrini* of several fish-eating mammals and birds. More data on the prevalence of opisthorchiasis in humans should be collected to define its overall prevalence. Particularly, the clinical importance of opisthorchiasis in Central Vietnam should be identified, and the burden of disease and the economic consequences calculated. It is recommended to also molecularly characterize *Opisthorchis* spp. isolated from humans in order to study the possibility of infection with *Opisthorchis* sp. BD2013 or hybrids. These studies should not only be concentrated in Binh Dinh province but be expanded to other Central Vietnamese provinces. These suggested epidemiological and clinical studies should be complemented by experimental studies, such as establishing the full lifecycle of *Opisthorchis* sp. BD2013 in the laboratory, including *Bithynia* and fish colonies and an animal model, e.g. the hamster that has shown to be a suitable laboratory host for several *Opisthorchis* spp. (Bouvard et al., 2009; Dung et al., 2014). An animal model would offer the possibility to perform co-infections of the two *Opisthorchis* species and study the possibility of hybridization and introgression and their consequences.

Other studies include answering the questions on the taxonomic position of *Opisthorchis* sp. BD2013 versus *O. parageminus*, and research on the pathological and economic consequences of infection of ducks with *Opisthorchis* sp. BD2013. Until now it is unclear whether infection of ducks are the cause of disease and losses in meat and egg production.

8.5 General conclusions

In conclusion, we found a *O. viverrini* sister species in domestic ducks in an *O. viverrini* endemic area in Central Vietnam. This finding confirmed the definite host role of fish-eating birds (domestic ducks) in the lifecycle of an *Opisthorchis* sp. Two *Bithynia* species, *B. s. goniomphalos* and *B. funiculata* were for the first time confirmed as the

first intermediate hosts of *O. viverrini* in Vietnam. Co-infection of two *Opisthorchis* spp. was for the first time found in both first and second snail and fish intermediate hosts. This finding reveals the possibility of exposure to both *O. viverrini* and the duck *O. viverrini*-like species in a range of reservoir/final hosts, including humans. Our findings demonstrate the complexity of the epidemiology of opisthorchiasis in Vietnam and suggest the potential of hybridization/ introgression of these two species. While many research questions remain to be answered we recommend the implementation of an integrated control program for opisthorchiasis using a One health/Eco-health strategy adapted to the local epidemiology of the parasite and its hosts.

SUMMARY

The South East Asian liver fluke *Opisthorchis viverrini* causes serious morbidity and mortality in the greater Mekong region. People infected with *O. viverrini* are at risk of developing cholangiocarcinoma (CCA) an aggressive cancer that is fatal in advanced stages. While this fluke has been known to occur in Central and South Vietnam for many decades, little attention has been paid on the epidemiology and on the burden of disease that it causes. Recently, our research group discovered a *O. viverrini* sister species in domestic ducks in a *O. viverrini* endemic area in Binh Dinh Province, in Central Vietnam. This thesis aimed to study the occurrence and the life cycle of both *O. viverrini* and the *O. viverrini-like* fluke, thereby assessing the epidemiological complexity of the co-existence of the human and duck *O. viverrini* genotypes in Binh Dinh Province Central Vietnam. First, we estimated the prevalence and associated risk factors of opisthorchiasis in humans in Central Vietnam. We then characterized the new isolate of *Opisthorchis* sp. in ducks by morphological and molecular characteristics. We also clarified the position of the duck *O. viverrini*-like genotype in the phylogenetic tree of the Opisthorchiidae family. In this summary we further refer to *Opisthorchis* sp. BD2013 when describing the isolate from ducks. Next we estimated the prevalence and intensity of infection of *Opisthorchis* sp. BD2013 in different duck populations in the region. Finally, we identified the first and second intermediate hosts of the duck genotype and summarized the life cycle of the *O. viverrini*-like fluke.

In the **first chapter** a general review of the literature of the *Opisthorchis* genus and opisthorchiasis is given, focusing on the morphology and the molecular biology of the genus, and on the epidemiology, diagnosis and the situation of opisthorchiasis in the Greater Mekong Basin, with a special focus on Vietnam. The chapter also includes a brief description of the history of the finding of an *Opisthorchis* sp. in domestic ducks in Central Vietnam.

Following this chapter, the **rationale** and **objectives** of the thesis are given.

The **second chapter** describes the current status of opisthorchiasis in a Central Vietnamese community aiming at better understanding the opisthorchiasis epidemiology in the region. A cross-sectional survey was conducted in June 2015 in a lowland rural area of Binh Dinh Province in Central Vietnam to investigate the

apparent prevalence of *O. viverrini* infection in the population and the associated risk factors. A total of 254 stool samples were collected and examined by the Kato Katz method. Consenting people shedding *Opisthorchis*-like eggs with their stools were treated with praziquantel and adult worms were collected from stools for morphological and molecular identifications. Risk factors were studied with a structured questionnaire and the association with infection was evaluated by univariate and multivariate Firth's logistic regression analysis. The apparent prevalence in the investigated population determined by stool examination was 11.4% (CI: 8–16%). Infection with *O. viverrini* was confirmed in all 11 individuals consenting to receive treatment and subsequent worm recovery from stools. The mean number of worms recovered after treatment was 14.5 (range 2–44). Male gender and the consumption of dishes prepared from raw small wild-caught freshwater fish (*Carassius auratus*) were found to be significant risk factors associated with opisthorchiasis in the area.

In the **third chapter,** morphological and molecular identification was performed of *Opisthorchis* sp. found in the bile ducts of domestic ducks (*Anas platyrhynchos*), necropsied in the Binh Dinh province of Central Vietnam. Morphological characteristics of the bird flukes were compatible with *O. viverrini*, although some characteristics differed from those described in specimens collected from mammal hosts. Computation of the phylogenetic trees on the partial sequences of the second internal ribosomal spacer (ITS2) of the ribosomal DNA and cytochrome c oxidase subunit I (COI) markers of the mitochondrial DNA showed close similarity of the 'bird' *Opisthorchis* sp. with *O. viverrini*. We speculated that these bird flukes were *O. viverrini* that showed intra-species morphological and molecular variability compared to isolates from mammals. These findings demonstrated the complex epidemiological situation of opisthorchiasis in Vietnam and urged to investigate the potential of birds as a reservoir host of this zoonotic fluke.

During our study on this *Opisthorchis* sp. in duck, there was some controversy on the scientific name of the fluke. In the **fourth chapter** we provide new sequence data from the mitochondrial (mt) genome and the nuclear ribosomal transcription unit of the *Opisthorchis* sp. BD2013. A phylogenetic analysis was conducted to clarify the basal taxonomic position of this species from ducks within the genus *Opisthorchis* of

Opisthorchiidae, superfamily Opisthorchioidea (Platyhelminthes: Trematoda: Digenea). Adults and eggs of liver flukes were collected from ducks, metacercariae from fishes (*Puntius brevis, Rasbora aurotaenia, Esomus metallicus)* and cercariae from snails (*Bithynia funiculata)* in different localities in Binh Dinh Province. From four developmental life stage samples (adults, eggs, metacercariae, cercariae), the complete cytochrome b (*cob*), nicotinamide dehydrogenase subunit 1 (*nad*1) and cytochrome oxidase subunit 1 (*co*1) genes; and near-complete 18S and partial 28S ribosomal DNA (rDNA) sequences were obtained by PCR-coupled sequencing. The alignments of nucleotide sequences of concatenated *cob*+*nad*1+*co*1, and of concatenated 18S rDNA+28S rDNA were separately subjected to phylogenetic and other analyses. Homologous sequences from other trematode species were included in each alignment. Phylogenetic trees were inferred from concatenated (*cob*+*nad*1+*co*1) nucleotide sequences and from combined 18S+28SrDNA nucleotide sequences of five *Opisthorchis* sp. BD2013 samples and additional reference taxa. Both trees demonstrated the anticipated clustering of taxa within the Opisthorchioidea, the paraphyly of the genus *Opisthorchis* and the sister-species relationship of *Opisthorchis* sp. BD2013 with *O. viverrini*. These results demonstrate the co-existence of two *Opisthorchis* spp. in Central Vietnam.

Following the first report of infection with *Opisthorchis* sp. BD2013 in domestic ducks in Phu My District of Binh Dinh Province, Central Vietnam, many other cases were observed in the province. In the **fifth chapter,** we determined the infection rate and intensity of infection with *Opisthorchis* sp. BD2013 in ducks in 4 districts of the province. A total of 178 ducks were randomly selected from 34 farms for examination of flukes in the liver and gall bladder. An infection rate of 34.3% (range 20.7 - 40.4% among districts) was found; the intensity of infection was 13.8 worms per infected duck (range 1-100). These findings showed the role of ducks as a host for *Opisthorchis* sp. BD2013, which is sympatric with *O. viverrini* in this province. It also stresses the need to investigate the zoonotic potential and the life cycle of this parasite; and also the need to clarify the taxonomic position of this *O. viverrini*–duck genotype in the Opisthorchiidae family for more understanding on its evolution in the genus and family.

The **sixth chapter** describes a study on the occurrence of *O. viverrini* and *Opisthorchis* sp. BD2013 in the snails and fish intermediate hosts in the endemic area. A total of 12,000 snails belonging to six families, of which 1616 Bithynia snails representing *Bithynia siamensis goniomphalos* and *Bithynia funiculata*; as well as 754 fish representing 12 species were examined. Shedding of *O. viverrini* cercariae was observed only in *B. s. goniomphalos* and *B. funiculata*, at infection rates of 0.86% and 0.14%, respectively. *O. viverrini* infection in *Bithynia* spp. was significantly associated with the habitat but not with the species and the shell size of *Bithynia* spp. *O. viverrini* metacercariae were found in 10 fish species representing both Cyprinidae and non-Cyprinidae families. The prevalence of *O. viverrini* infection in fish was significantly associated with species. *Carassius auratus*, a fish species commonly eaten raw, *Rasbora aurotaenia* and *Puntius brevis* had the highest prevalence of 74.0%, 55.8% and 31.6%, respectively. Sharing of the same snail and fish intermediate host species was found for *O. viverrini* and *Opisthorchis* sp. BD2013. This study is the first to report on the intermediate host species of *O. viverrini* in Central Vietnam and indicates a high risk of acquiring opisthorchiasis when eating raw fish dishes.

In the **seventh chapter**, the life cycle of new finding of *Opisthorchis* sp. BD2013 was developed. *Bithynia funiculata*, an abundant freshwater snail in the region acts as the first intermediate host of the fluke. This snail species sheds pleurolophocercous cercariae, which are characteristic for the *Opisthorchis* genus. Three freshwater fish species (*Rasbora aurotaenia*, *Puntius brevis*, and *Esomus metallicus*) were found to host the metacercariae of the fluke. Adult worms were isolated from every examined duck population all over the province at a high prevalence (34.3% on average). Ducks and other domestic animals and wild fish-eating birds were observed roaming around and on the freshwater reservoirs and rice fields in the region looking for food. These conditions may create a chance for the parasite to accomplish its life cycle.

In the **eighth chapter,** we discuss the findings of our research on *Opisthorchis* sp. BD2013 in the context of its co-existence with *O. viverrini* in in Central Vietnam and of the shared snail and fish intermediate hosts of these two *Opisthorchis* spp. Depending of the host specificity of the two *Opisthorchis* spp. these conditions may

result in co-infection in final hosts and potentially lead to introgression/ hybridization of these species, increasing the epidemiological complexity of opisthorchiasis in Vietnam. There is a need to perform studies to define the host range of *Opisthorchis* sp. BD2013: by experimental infections of various birds and mammalian hosts and by surveys including molecular characterization of *Opisthorchis* spp. in both humans and domestic and wild animal species. Meanwhile, a One Health/ Eco-health control program involving animal health, public health and environment control sectors should be built for prevention and control of the neglected *O. viverrini* in the region.

SAMENVATTING

De Zuidoost Aziatische leverbot *Opisthorchis viverrini* is de oorzaak van ernstige morbiditeit en mortaliteit in de grotere Mekong regio. Mensen geïnfecteerd met *O. viverrini* lopen het risico om een cholangiocarcinoom (CCA) te ontwikkelen, een agressieve kanker die fataal is in het geavanceerde stadium. Terwijl het bestaan van *O. viverrini* in Centraal en Zuid Vietnam reeds tientallen jaren is gekend werd er tot nu toe weinig aandacht besteed aan de epidemiologie en de ziektelast die deze parasiet veroorzaakt. Enkele jaren geleden ontdekte onze onderzoeksgroep het bestaan van een *O. viverrini* zuster species bij tamme eenden in een *O. viverrini* endemisch gebied in de Binh Dinh provincie in Centraal Vietnam. Het onderwerp van dit doctoraal proefschrift is de studie van het voorkomen en de levenscyclus van zowel *O. viverrini* en het *O. viverrini* zuster species in de Binh Dinh provincie in Centraal Vietnam, met speciale aandacht voor de epidemiologische complexiteit die het samen voorkomen van een humaan en een eend *O. viverrini* genotype met zich meebrengt. We startten met een studie naar de prevalentie van opisthorchiasis bij de mens in Centraal Vietnam en de risicofactoren voor het verwerven van een infectie in deze regio met als doel de epidemiologische status van deze ziekte in het gebied te bepalen. Vervolgens karakteriseerden we het nieuwe *Opisthorchis* eend genotype aan de hand van morfologische en moleculaire parameters. We bepaalden eveneens de positie van het *Opisthorchis* sp. eend genotype in de fylogenetische boom van de Opisthorchiidae familie. In deze samenvatting refereren we naar *Opisthorchis* sp. BD2013 wanneer het om het eenden isolaat gaat. Vervolgens schatten we de prevalentie en de intensiteit van infectie van *Opisthorchis* sp. BD2013 bij verschillende eenden populaties in de regio. Tenslotte identificeerden we de eerste en tweede tussengastheren van *Opisthorchis* sp. BD2013 en vatten de levenscyclus van deze trematode samen.

In het **eerste hoofdstuk** wordt een algemeen overzicht gegeven van de literatuur over het *Opisthorchis* genus en over opisthorchiasis, waarbij dieper wordt ingegaan op de morfologie en de moleculaire biologie van het genus, en op de epidemiologie, diagnose en de situatie van opisthorchiasis in het groter Mekong gebied, met een speciale focus op Vietnam. In dit hoofdstuk wordt eveneens een korte beschrijving gegeven van de geschiedenis van de ontdekking van een nieuw *Opisthorchis* sp. bij tamme eenden in Centraal Vietnam.

Dit hoofdstuk wordt gevolgd door de **rationale** en de **objectieven** van deze thesis.

Het **tweede hoofdstuk** beschrijft de huidige status van opisthorchiasis in een Centraal Vietnamese gemeenschap met als objectief de epidemiologische situatie van opisthorchiasis in de regio beter te begrijpen. Een transversale studie werd uitgevoerd in juni 2015 in een landelijk gebied in het laagland van de Binh Dinh provincie in Centraal Vietnam om de apparente prevalentie van infectie met *O. viverrini* in de populatie te onderzoeken en de risicofactoren voor infectie te identificeren. Er werden 254 stoelgangstalen verzameld en onderzocht met de Kato-Katz methode. Aan personen in wiens stoelgang wormeieren werden teruggevonden die op deze van *Opisthorchis* leken werd gevraagd om deel te nemen aan verder onderzoek. Personen die dit goedkeurden kregen een behandeling met praziquantel toegediend waarna volwassen wormen uit de stoelgang konden verzameld worden voor morfologisch en moleculair onderzoek. Risicofactoren werden onderzocht aan de hand van een gestructureerde vragenlijst en associatie tussen factoren en infectie werd nagegaan aan de hand van univariate en multivariate Firth's logistische regressie analyse. De apparente prevalentie van opisthorchiasis in de onderzochte populatie aan de hand van het stoelgangonderzoek was 11.4% (CI: 8–16%). Infectie met *O. viverrini* werd bevestigd bij alle 11 personen die toestemming gaven voor praziquantel behandeling gevolgd door het verzamelen van adulte wormen in de stoelgang. Het gemiddeld aantal wormen dat werd teruggevonden na behandeling bedroeg 14,5 (van 2 tot 44). Mannelijke geslacht en de consumptie van gerechten bereid met rauwe vis afkomstig van wild gevangen kleine zoetwatervissen (*Carassius auratus*) werden als de belangrijke risicofactoren voor opisthorchiasis in deze regio geïdentificeerd.

In het **derde hoofdstuk** werden morfologische en moleculaire identificaties gedaan van een *Opisthorchis* sp. dat gevonden werd bij autopsie in de levergangen van tamme eenden (*Anas platyrhynchos*) in de Binh Dinh provincie in Centraal Vietnam. Morfologische karakteristieken van de leverbotten van de vogels waren compatibel met deze van *O. viverrini*, alhoewel er sommige karakteristieken enigszins verschillend waren van wormen die bij zoogdieren werden verzameld. De berekening van de fylogenetische bomen van de partiële sequenties van de

tweede interne ribosomale spacer (ITS2) van het ribosomaal DNA en de cytochroom c oxidase subunit I (COI) markers van het mitochondriaal DNA toonde sterke verwantschap aan van het eend *Opisthorchis* sp. en *O. viverrini*. We speculeerden dat deze leverbotten van vogels tot het species *O. viverrini* behoren die morfologische en moleculaire variabiliteit vertonen met botten van dit species geïsoleerd bij zoogdieren. Deze bevindingen tonen de ingewikkelde epidemiologische situatie van opisthorchiasis aan in Vietnam en sporen onderzoek aan naar het potentieel van vogels als mogelijk reservoir van deze zoönotische leverbot.

Tijdens onze studie werd in de wetenschappelijke wereld een debat gevoerd over de identiteit en de wetenschappelijke naam van het *O. viverrini* eend-genotype. In het **vierde hoofdstuk** refereren we naar **'***Opisthorchis* sp. BD2013' voor de leverbot bij de eend en verstrekken we nieuwe data van sequenties van het mitochondriaal (mt) genoom en van de nucleaire ribosomale transcriptie unit. Een fylogenetische analyse werd uitgevoerd met als doel het ophelderen van de taxonomische positie van het species van eenden binnen het genus *Opisthorchis* van de Opisthorchiidae, superfamily Opisthorchioidea (Platyhelminthes: Trematoda: Digenea). Volwassen wormen en eieren van leverbotten werden verzameld bij eenden, metacercariae bij zoetwatervissen (*Puntius brevis, Rasbora aurotaenia, Esomus metallicus)* en cercariae bij zoetwaterslakken (*Bithynia funiculata)* op verschillende plaatsen in de Binh Dinh provincie. De complete sequenties van de cytochroom b (*cob*), nicotinamide dehydrogenase subunit 1 (*nad*1) en cytochroom oxidase subunit 1 (*co*1) genen; en bijna-complete sequenties van 18S en partiële sequenties van het 28S ribosomaal DNA (rDNA) werden bekomen door PCR-gekoppeld sequeneren van 4 ontwikkelingsstadia van de parasiet, namelijk de adulten, eieren, metacercariae en cercariae. De opstelling van de sequenties van nucleotiden van aaneengeschakelde *cob*+*nad*1+*co*1 en aaneengeschakelde 18S rDNA+28S rDNA werden afzonderlijk onderworpen aan fylogenetische en andere analyses. Homologe sequenties van andere trematode species werden opgenomen in elke opstelling. Fylogenetische bomen werden afgeleid van aaneengeschakelde (*cob*+*nad*1+*co*1) nucleotidesequenties en van gecombineerde 18S+28SrDNA nucleotidesequenties van 5 *Opisthorchis* sp. BD2013 monsters en van bijkomende referentie taxa. Beide bomen toonden de geanticipeerde groepering aan van de taxa binnen de

Opisthorchioidea, de paraphyla van het genus *Opisthorchis* en de zuster-species relatie van *Opisthorchis* sp. BD2013 met *O. viverrini.* Deze resultaten tonen de co-existentie aan van twee *Opisthorchis* spp. in Centraal Vietnam.

Volgende op deze eerste waarneming van een infectie met *Opisthorchis* sp. BD2013 bij tamme eenden in het Phu My district in de Binh Dinh provincie werden er vele andere gevallen geobserveerd in deze provincie. In het **vijfde hoofdstuk** van dit proefschrift bepaalden we de prevalentie en de intensiteit van infectie met *Opisthorchis* sp. BD2013 bij eenden in 4 districten van deze provincie. We verzamelden willekeurig 178 eenden in 34 bedrijven die we onderwierpen aan een onderzoek van de lever en de galblaas. We vonden een prevalentie van 34,3%, variërend van 20,7% tot 40,4% naargelang het district; het gemiddeld aantal wormen per geïnfecteerde eend bedroeg 13,8 (variërend tussen 1 en 100 wormen). Deze studie toonde de rol van eenden aan als de gastheer van *Opisthorchis* sp. BD2013, dat samen voorkomt in deze provincie met *O. viverrini*. De bevindingen sporen verder onderzoek aan naar het zoönotisch potentieel en naar de levenscyclus van *Opisthorchis* sp. BD2013; en naar het ophelderen van de taxonomische positie van deze worm voor een beter begrip van de evolutie van het *Opisthorchis* genus en de familie Opisthorchiidae.

Het **zesde hoofdstuk** beschrijft een studie naar het voorkomen van *O. viverrini* en *Opisthorchis* sp. BD2013 in de zoetwaterslak en-vis tussengastheren in het endemisch gebied. In totaal werden 12000 zoetwaterslakken behorende tot 6 families onderzocht, waaronder 1616 *Bithynia* slakken geïdentificeerd als *Bithynia siamensis goniomphalos* en *Bithynia funiculata*; en 754 zoetwatervissen behorende tot 12 species. Het uitscheiden van *O. viverrini* cercariae werd enkel bij *B. s. goniomphalos* en *B. funiculata* geobserveerd, aan infectiegraden van respectievelijk, 0,86% en 0,14%. Er werd een significante associatie gevonden tussen *O. viverrini* infectie in *Bithynia* spp. en de habitat maar niet met het species en de grootte van de schelpen. *O. viverrini* metacercariae werden bij 10 vissen species gevonden behorende tot zowel Cyprinidae en niet-Cyprinidae families. Er werd een significante associatie gevonden van de prevalentie van *O. viverrini* infectie in de vissen en het visspecies. De hoogste prevalenties werden gevonden bij *Carassius auratus*, een species dat rauw wordt gegeten in de regio, *Rasbora aurotaenia* en *Puntius brevis*, respectievelijk 74,0%,

55,8% en 31,6%. Een opmerkelijke bevinding was dat *O. viverrini* en *Opisthorchis* sp. BD2013 zowel slak als vis species delen als tussengastheren. Deze studie is de eerste die de tussengastheren van *O. viverrini* beschrijft in Centraal Vietnam en toont aan dat er een groot risico bestaat voor het verwerven van opisthorchiasis bij het eten van gerechten die rauwe vis bevatten.

In het **zevende hoofdstuk** wordt de levenscyclus van het nieuwe *Opisthorchis* sp. BD2013 ontwikkeld. *Bithynia funiculata*, een veelvoorkomende zoetwaterslak in de regio doet dienst als eerste tussengastheer van deze leverbot. Deze slak scheidt pleurolophocercus type cercariae uit die karakteristiek zijn voor het *Opisthorchis* genus. In 3 species zoetwatervissen (*Rasbora aurotaenia*, *Puntius brevis*, and *Esomus metallicus*) werden metacercariae gevonden van *Opisthorchis* sp. BD2013. Adulte wormen werden in elke eenden farm die werd onderzocht gevonden aan een hoge prevalentie (34,3% gemiddeld). In de regio werden eenden, andere gedomesticeerde dieren en wilde visetende vogels rond en op de zoetwaterreservoirs en rijstvelden gezien op zoek naar voedsel. Deze condities maken het voor de parasiet mogelijk om zijn levenscyclus te voltooien.

In het **achtste hoofdstuk** worden de bevindingen van ons onderzoek van het nieuwe *Opisthorchis* sp. BD2013 besproken in de context van de co-existentie met *O. viverrini* in Centraal Vietnam en van het delen door deze twee *Opisthorchis* spp. van dezelfde eerste en tweede tussengastheren. Afhankelijk van de gastheerspecificiteit van deze twee *Opisthorchis* spp. kunnen zich omstandigheden voordoen die leiden tot co-infectie in de eindgastheer met mogelijke hybridisatie/ introgressie van deze species, met potentiële toename van de epidemiologische complexiteit van opisthorchiasis in Vietnam. Er is behoefte aan het uitvoeren van studies die de gastheerspecificiteit van *Opisthorchis* sp. BD2013 bepalen: door experimentele infectie van verschillende vogel en zoogdieren species en door veldstudies waarbij wormen verzameld bij mensen en van gedomesticeerde en wilde zoogdieren en vogels worden onderworpen aan morfologische en moleculaire onderzoeken voor het vaststellen van het species. Er dient in de regio eveneens een One Health/ Eco-health controleprogramma worden ontwikkeld en uitgerold waarbij de sectoren actief in de volksgezondheid, diergezondheid en milieu moeten samenwerken voor de bestrijding van de verwaarloosde *O. viverrini*.

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