Parasitology International 61 (2012) 32–37

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

The systematics and population genetics of *Opisthorchis viverrini sensu lato*: Implications in parasite epidemiology and bile duct cancer

Paiboon Sithithaworn [a,b,*], Ross H. Andrews [a,b], Trevor N. Petney [c], Weerachai Saijuntha [d], Nonglak Laoprom [a,b]

[a] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[c] Institute of Zoology 1: Ecology and Parasitology, Karlsruhe Institute of Technology, Kornblumen Strasse 13, Karlsruhe, Germany
[d] Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand

ARTICLE INFO

Available online 29 July 2011

Keywords:
Opisthorchis viverrini sensu lato
Systematic
Genetic variation
Population genetics
Opisthorchiasis
Cholangiocarcinoma

ABSTRACT

Together with host and environmental factors, the systematics and population genetic variation of *Opisthorchis viverrini* may contribute to recorded local and regional differences in epidemiology and host morbidity in opisthorchiasis and cholangiocarcinoma (CCA). In this review, we address recent findings that *O. viverrini* comprises a species complex with varying degrees of population genetic variation which are associated with specific river wetland systems within Thailand as well as the Lao PDR. Having an accurate understanding of systematics is a prerequisite for a meaningful assessment of the population structure of each species within the *O. viverrini* complex in nature, as well as a better understanding of the magnitude of genetic variation that occurs within different species of hosts in its life cycle. Whether specific genotypes are related to habitat type(s) and/or specific intermediate host species are discussed based on current available data. Most importantly, we focus on whether there is a correlation between incidence of CCA and genotype(s) of *O. viverrini*. This will provide a solid basis for further comprehensive investigations of the role of genetic variation within each species of *O. viverrini sensu lato* in human epidemiology and genotype related morbidity as well as co-evolution of parasites with primary and secondary intermediate species of host.

1. Introduction

Opisthorchiasis is caused by *Opisthorchis viverrini*, one of the most important food-borne liver flukes in Asia. It is endemic in several Southeast Asian countries such as Thailand, the Lao People's Democratic Republic (Lao PDR), Vietnam, and Cambodia. Eight million people in Thailand and 2 million people in Lao PDR are infected with *O. viverrini* [1–3]. In Thailand, the distribution of *O. viverrini* varies throughout the region. High prevalence is found in the northeast followed by the north and central regions, however, there is very low prevalence in the southern region. This geographical distributional pattern is believed to be linked with the distribution of the snails, which act as intermediate hosts.

The life-cycle of *O. viverrini* involves freshwater snails (*Bithynia* spp.) as first intermediate hosts, cyprinid fish as second intermediate hosts, humans as the definitive hosts and cats and dogs as reservoir hosts. Infection in humans is caused by eating raw or undercooked fish containing viable metacercariae of *O. viverrini* [1]. Infection with this liver fluke induces hepatobiliary pathology which can eventually lead to bile duct cancer, cholangiocarcinoma (CCA), the leading cause of death within the region [4]. Interestingly, the prevalence, incidence and symptomatology of the diseases associated with *O. viverrini* infection vary geographically [1,2]. Recently, molecular studies have been employed to examine whether there is a genetic basis for this observed heterogeneity.

In this review, current aspects of the systematics and population genetics of *O. viverrini* as well as potential genetic variation among different hosts which perpetuate the life cycle will be addressed. Gaps of knowledge towards understanding the contribution of parasite genetic variation in pathogenicity and/or virulence in human hosts are explored. We envisage that this may enhance our understanding of the long term dynamics of infection between different host species in the life cycle with respect to the genetic composition of natural species and populations of *O. viverrini sensu lato*.

2. Geographical variation in epidemiology of *O. viverrini* and incidence of CCA

The prevalence, intensity of infection and incidence, and the diseases associated with *O. viverrini* infection vary geographically. Studies on prevalence and intensity of opisthorchiasis in all regions of

* Corresponding author at: Department of Parasitology and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Tel.: +66 43348387; fax: +66 43202475.
E-mail address: paib_sit@hotmail.com (P. Sithithaworn).

1383-5769/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2011.07.020

Thailand have revealed marked regional variation with the greatest prevalence and intensity found in the northeast [5]. Within the northeast, there is variability at the province, district and village level. A recent study in Khon Kaen Province, which examined people from different districts for *O. viverrini* infection, revealed a high incidence of CCA. Surprisingly, however, the prevalence of infection varied within each district. The average crude prevalence of *O. viverrini* infection was 24.5%, ranging from 2.1% to an alarmingly high 70.8% between different districts.

As well as the incidence of CCA, truncated age-standardized incidence of CCA at ages >35 years varied threefold between districts, from 93.8 to 317.6 per 100,000 person-years [6]. Interestingly, this report also showed that there was no correlation between prevalence of *O. viverrini* and incidence of CCA within various districts in Khon Kaen Province. A lack of such a correlation is not unexpected since different species of hosts, the environment as well as parasite factors may contribute to this finding. The extent to which genetic variation of *O. viverrini* within the Chi River wetland in Khon Kaen Province and their correlations with disease epidemiology and CCA remains to be elucidated. Further examination is required on the incidence of CCA at sub-district and village levels within Khon Kaen Province to determine whether a similar pattern exists. For example, the crude incidence rate (per 100,000 person-years) of CCA in different sub-districts within Khon Kaen District, Khon Kaen Province during 1985–2009 showed large variation ranging from 8.0 to as high as 38.1 [7].

3. Systematics and genetic variation of *O. viverrini*

The first report of genetic variation was on *O. viverrini* collected from an autopsy subject with CCA, and was based on 3 enzyme markers by the technique of enzyme electrophoresis [8]. It was not until 2001, that genetic variation of *O. viverrini* was examined again but this time it was based on DNA markers [9]. Partial mitochondrial DNA cytochrome c oxidase subunit 1 (*cox*1) gene and the full length of internal transcribed spacer 2 (ITS2) gene of adult *O. viverrini* from different geographical areas, including from an autopsy in northeast Thailand were analyzed. Intra- and inter-specific variations of *cox*1 sequence were found yielding 5 haplotypes based on the variation of 4 nucleotide positions. The ITS2 sequences, however, were identical among *O. viverrini* from different geographical localities [9]. Application of random amplified polymorphic DNA (RAPD) in a pilot study, suggested that genetic variation of *O. viverrini* may be associated with different species of fish intermediate hosts leading to the hypothesis that genetic variability may be related to these hosts [10]. Further analysis by the RAPD technique revealed different banding patterns between the isolates of *O. viverrini* from Thailand and those from Lao PDR [11]. Subsequently, a comprehensive analysis by multilocus enzyme electrophoresis (MEE) was undertaken for the first time to examine the systematics of *O. viverrini* [12]. We defined and established 32 enzyme loci as genetic markers to examine genetic variation of natural populations of *O. viverrini* isolates from Thailand and Lao PDR [12–14].

The MEE data showed that 15 *O. viverrini* isolates which were from different geographical areas were delineated into 2 major lineages (>30% fixed genetic differences). These lineages could be further subdivided into 6 distinct genetic groups (≥15%) which correlated with 5 different wetland systems, i.e. the Chi, Mun, Songkram and Wang River wetlands in Thailand, and the Nam Ngum River wetland in Lao PDR. Within the Chi River wetland 2 distinct clusters of *O. viverini* were detected one that occurred upstream and the other downstream of the River. In addition, data suggested the possibility of co-evolution between *O. viverrini* and its snail host (*Bithynia siamensis goniomphalos*) as there was a high concordance of lineages and specific genetic groups of *O. viverrini* and *Bithynia* snails [14]. A more recent study by Kiatsopit et al., examined the genetic relationships of isolates of *O. viverrini* from Savannakhet Province, Lao PDR and Thailand by MEE at 20 enzyme markers. The isolate from Savannakhet Province closely aligned to the isolates from the Songkram River wetland in Thailand and the Nam Ngum River wetland in Lao PDR. These results suggested that genetic differentiation and/or gene flow of *O. viverrini* populations may be affected by biotic and/or abiotic factors within each different wetland system [15].

O. viverrini is a sexually reproducing diploid parasite. It is important to note that allozyme analyses of sexually reproducing diploid vertebrate and invertebrate organisms, including parasites, have shown that greater than 15% fixed genetic differences typically reflect the presence of morphologically similar, yet genetically distinct hence "cryptic" species. On the other hand, at a single enzyme locus fixed genetic differences among two sympatric populations indicate lack of gene flow providing evidence for the existence of two species. Two or more fixed genetic differences at more than one enzyme locus are unequivocal evidence of the existence of two sympatric cryptic species (for detailed review see [16]). When samples from allopatric populations are being examined, however, it is wise to obtain data from independent methods (e.g. biological, epidemiological, ecological, molecular etc.), to confirm the existence of cryptic species [16].

In our subsequent study, 2 mitochondrial genes, *cox*1 and NADH dehydrogenase subunit 1 (*nad*1) were used to analyze 14 isolates of *O. viverrini* collected from natural sources from Thailand and Lao PDR. Another 5 sequences retrieved from GenBank were included for comparison [17]. Two and 8 haplotypes were inferred from 2 (0.51%) and 9 (1.35%) nucleotide positions of the *cox*1 and *nad*1 sequences, respectively. A recent study by Thaenkham et al. (2010) detected 19 haplotypes from the variation of 20 (3.10%) nucleotide positions of the *nad*1 sequence of 86 individual worms from 6 isolates of *O. viverrini* from Thailand, Lao PDR and Cambodia [18]. Their DNA sequencing-based study found that there was no correlation of *O. viverrini* haplotypes with river wetlands. However, Thaenkham et al. do point out that their data is based on the analysis of a single gene and indicate that to further clarify whether an *O.viverrini* species complex exists in the region, more extensive studies are required, using other molecular markers and greater numbers of samples from different geographical areas. This has in part been carried out by Laoprom et al. [45] who confirmed substantial population genetic variation between geographical regions.

4. Source of genetic variations

4.1. Bithynia snail

Snail intermediate hosts, *Bithynia* species, belong to the phylum Molluska, class Gastropoda, subclass Prosobranchia, order Mesogastropoda, family Bithyniidae. In Thailand, members of the family Bithyniidae occur in three genera, *Bithynia*, *Hydrobioides* and *Wattebledia*, while the genus *Bithynia* can be divided into 2 subgenera, namely *Digoniostoma* and *Gabbia* [19,20]. Although a number of species of Bithyniidae are present in Thailand, only 2 species are known to serve as the intermediate hosts of *O. viverrini*, namely *B. funiculata* and *B. siamensis*. Within *B. siamensis* there are 2 subspecies, *B. siamensis siamensis* and *B. s. goniomphalos*. To date there have been no comprehensive studies of the distributions of these species. It is currently thought that *B. funiculata* is found in the north (from 5 of 9 provinces), *B. s. goniomphalos* is found in the northeast (from 12 of 19 provinces), while *B. s. siamensis* is considered to be in central Thailand (3 of 22 provinces including Bangkok) [19–29].

Accurate and up to date information of the systematics and distributional ranges of these *Bithynia* species is essential because they are medically very important in that they act as the critical amplifying host of *O. viverrini*. Typically, rates of infection of *O. viverrini* in different species of *Bithynia* sampled from different regions of Thailand vary between 0.03 and 1.6% [22,25,26,29,30]. Laboratory evidence has indicated that both *B. funiculata* and *B. s.*

siamensis are more susceptible to *O. viverrini* infection than *B. s. goniomphalos* [31]. The level of genetic variability within and between species and subspecies has been examined by MEE analyses and has provided preliminary evidence of genetic variation within *B. s. goniomphalos* in northeast Thailand [14]. Results obtained suggest an association with river wetlands and a possibility of co-evolution between *O. viverrini* and snail hosts. Our current investigations have shown that *B. s. goniomphalos* collected from the Songkram River wetland, shed 8 times more *O. viverrini* cercariae/day/snail than those from the Nam Ngum River wetland, Lao PDR (Kiatsopit, unpublished) reflecting significant biological differences between the 2 currently proposed cryptic species of *O. viverrini*.

A recent MEE study provides genetic evidence that suggests the systematics of *Bithynia* snails requires urgent re-examination since fixed genetic differences within the subspecies of *B. s. saimensis* and *B. s. goniomphalos* are almost the same as between currently morphologically defined species of *B. siamensis* and *B. funiculata* [15]. Utilization of DNA-based genetic markers such as RAPD has also suggested wetland associated genetic variability of *B. s. goniomphalos* in northeast Thailand [23]. The observed low rate of infection in *Bithynia* snails in contrast to high transmission in fish intermediate hosts and humans may depend on specific parasite genotypes and other ecological factors. The role of genetic variation in snails and hence susceptibility to *O. viverrini* infection cannot be ruled out and requires further investigation. The areas of further investigation suggested above need urgent attention as they directly influence the transmission of this liver fluke and the diseases it causes, including CCA.

4.2. Cyprinid fish intermediate host

Freshwater fish are the second intermediate host of the liver fluke and are the most important sources of transmission to definitive hosts including humans as they harbor infective metacercariae. It has been recorded that more than 100 species of freshwater native fish are infected with a related liver fluke *Clonorchis sinensis* mainly in Korea, China as well as Russia and more than 35 with *Opisthorchis* species. [3]. In Thailand and Lao PDR, 18 species of fish from seven genera have been reported to serve as the intermediate hosts of *O. viverrini*. Of these, *Cyclocheilichthys apogon*, *C. armatus*, *C. repasson*, *Puntius leiacanthus*, and *Hampala dispar* are considered to be the most important [32]. The parasite burden in fish is highly aggregated and the majority of infected fish harbor 1–2 metacercariae, so that a repeated, low dose of infection and/or exposure is likely (Sithithaworn et al., unpublished). Natural infections of these cyprinid fish differ markedly in their relative infection with *O. viverrini*, with *C. armatus* having the highest intensity of infection followed by *H. dispar* [33,34].

Our recent report on genetic variation of *O. viverrini* based on fish host species using allozyme markers revealed that there was a significant heterozygote deficiency in one out of 4 species of cyprinid fish examined [33]. Obviously, given the extensive diversity of cyprinid fish species, the differences in the intensity of infection and differences in the biology and ecology of these intermediate hosts, could strongly influence the population genetic structure (i.e. allele and genotype frequencies; presence/absence specific genotypes) of *O. viverrini*. The mechanism by which this could be achieved may possibly be via host selection as in the case of *Schistosoma japonicum* [35,36]. Moreover, increasing use of fish aquaculture, with increasingly high usage of cyprinid species [37], provides ample opportunities for host species switching by parasites, including those with complex life cycles [38].

5. Population genetic structure of *O. viverrini*

The population genetics of *O. viverrini* from 5 different geographical isolates, 4 from Thailand (Khon Kaen, Mahasarakham, Buri Ram, Sakon Nakhon) and one from Lao PDR, (Vientiane), has been examined by MEE at 5 polymorphic enzyme loci [39] namely enolase (*Enol*), fructose-1,6-diphosphatase (*Fdp*), phosphoglycerate mutase (*Pgam*), phosphoglucomutase (*Pgm*) and triose phosphate isomerase (*Tpi*). Comparatively high levels of genetic variability (3–4 alleles/locus) were detected between the 5 populations of *O. viverrini*. Heterozygote deficiency ($F_{IS}>0$) which was detected conformed to Hardy–Weinberg equilibrium (HWE) in 4 of the 5 locations. The only exception was an isolate from Buri Ram which showed significant heterozygote excess ($F_{IS}<0$). It is important to note that the level of genetic differentiation (F_{ST} value) between populations from Thailand and Lao PDR was very high, whereas the comparison among the Thai populations was low [39].

An analysis of an *O. viverrini* population (231 adult worms) collected from Lawa Reservoir, Ban Phai District, Khon Kaen Province was examined by MEE using 3 polymorphic enzymes, Enol, Pgm and Tpi. Deviations from HWE, heterozygote deficiencies, were observed within this population [40]. To ascertain the impact of temporal changes and fish host factors on genetic structure, *O. viverrini* isolates from Ban Phai District, were examined [33]. Adult *O. viverrini* were collected in 4 different years (2003–2006) together with 4 different cyprinid fish populations. These were analyzed at 3 polymorphic enzyme loci, namely *Enol*, *Pgm* and *Tpi*. The allele and genotype frequencies of *O. viverrini* isolates collected in different years as well as different species of cyprinid fish were relatively consistent. Significant heterozygote deficiencies compared to HWE were detected in some populations at a particular locus. So far, we have not found significant differences in genetic differentiation (pairwise F_{ST} values) between *O. virerrini* collected from different years or different host species but more extensive studies in other wetlands are still needed.

Population genetic analyses using microsatellite DNA, also known as simple sequence repeats (SSRs) or short tamdem repeats (STRs), are tandemly repeated tracts of DNA composed of 1–6 base pair long units [41] and have been increasingly used in many organism including parasite [42]. Microsatellite DNA molecular markers are well established for genetic analyses because of their abundance and high level of polymorphism [36,43,44].

Microsatellite markers have been recently established and used to explore genetic variation of *O. viverrini* in northeast Thailand and Lao PDR [45]. Within the total of 41 microsatellite DNA that were isolated, sequences detected had no significant homology with known DNA sequence in GenBank suggesting that they are type II (non-coding) markers and the most frequent repeats in decreasing order were 13 CA/TG — 13/41 (31.7%), GT/AC — 9/41 (21.9%) and CAT/ATG — 6/41 (14.6%). The number of repeated motifs ranged from 4 to 44. Fifteen out of 41 (36.5%) were perfect microsatellite repeats, 25 out of 41 (60.9%) were interrupted or non-perfect repeats and 1 out of 41 (2.4%) was a compound repeat. Within the perfect microsatellites, 60% (9/15) were dinucleotide, 33.3% (5/15) were trinucleotide and 6.6% (1/15) were tetranucleotide. Imperfect microsatellites, 60% (15/25) were dinucleotide, 20% (5/25) were trinucleotide and 20% were tetranucleotide. One compound microsatellite consisted of a dinucleotide repeat (1/1–100%).

Twelve microsatellite markers were used for genetic analysis of 150 individual worms from 5 different geographical localities from Thailand and Lao PDR. Among the 5 localities examined, the range of polymorphic loci per locality varied from 8 to 11. Positive F_{IS} values and highly significant deviations from HWE expectations due to homozygote excess were found between the majority of localities and loci. The deviations from HWE may be the result of inbreeding as a consequence of self-fertilization and/or founder effects in *O. viverrini* populations. The findings of heterozygote deficiency at a number of loci support previous results by Saijuntha et al. [40] and the hypothesis that selfing (inbreeding) rather than cross-fertilization (outbreeding) is a predominant mode of reproduction in *O. viverrini*.

Additionally, significant geographic heterogeneity was found between different geographical isolates in Thailand and Laos. This

result suggests that these *O. viverrini* populations were not a single panmictic population but rather had been differentiated genetically into different gene pools, indicating the existence of intraspecific population structures of *O. viverrini* as previously reported from allozyme population genetic analyses [40]. This may be due to the geographical location of distinct wetlands and the flow and flooding patterns of the different river systems. For example, worms from Lampang in the Wang River wetland which does not flow or connect with other wetlands exhibit greater degrees of genetic differentiation than among worms from within Thailand and Lao PDR (Fig. 1) from river wetlands that eventually flow into the Mekong River.

Recently, Laoprom et al. have provided preliminary evidence that worms from Nakhon Phanom may represent a biologically different species based on significant differences in infectivity, fecundity and body size of *O. viverrini* from worms from other localities in Thailand [46]. This correlates with genetic evidence that *O.viverrini* from Nakhon Phanom is genetically distinct from other populations throughout Thailand and Lao PDR [14]. The existence of geographic differentiation in *O. viverrini* allows for studies on genotype-specific prevalence of infection in different hosts (intermediate vs. human host) as well as in different environments which need to be evaluated in the future. Current data lend more support to this hypothesis and add further independent evidence to support the hypothesis that *O. viverrini* is indeed a species complex [14].

To date, microsatellite DNA analyses have shown and supported previous evidence of the existence of considerable genetic diversity and population sub structuring of *O. viverrini* within and between geographical localities. The observed inbreeding parameter (positive F_{IS}) suggested that self-fertilization and/or clonal distribution of the parasite may be the underlying population processes. Based on F_{ST} the lack of gene flow between populations from Thailand and Lao PDR supported the previous hypothesis of the existence of cryptic species and that *O. viverrini* is a species complex. In spite of these new and informative findings, caution in interpretation is needed to assure that there is no sampling bias, particularly as adult worms are obtained from experimentally infected animals and are used for molecular, genetic and other analyses. A more direct way of sampling of other life stages of the parasite is needed to confirm current findings.

It is important to note that in a preliminary analyses of isolates *O. viverrini* from the same localities in Thailand and Lao PDR, there was concordance between the results from microsatellite DNA and allozyme markers (unpublished). For example, highly significant concordance between both methodologies was obtained in gene diversity, trends of heterozygote deficiency suggesting self-fertilization or inbreeding as a major mode of reproduction as well as the patterns of genetic differentiation between geographical localities.

6. Relationship between genetic diversity and disease epidemiology

The possibility that particular genotypes of the parasite may be associated with disease presentation and/or pathology has been investigated in *S. haematobium* [47]. Khon Kaen Province is an area with a high incidence of CCA and in a smaller geographical area there are considerable variations in epidemiology of CCA as well as opisthorchiasis among districts within the Province. Cancer registration

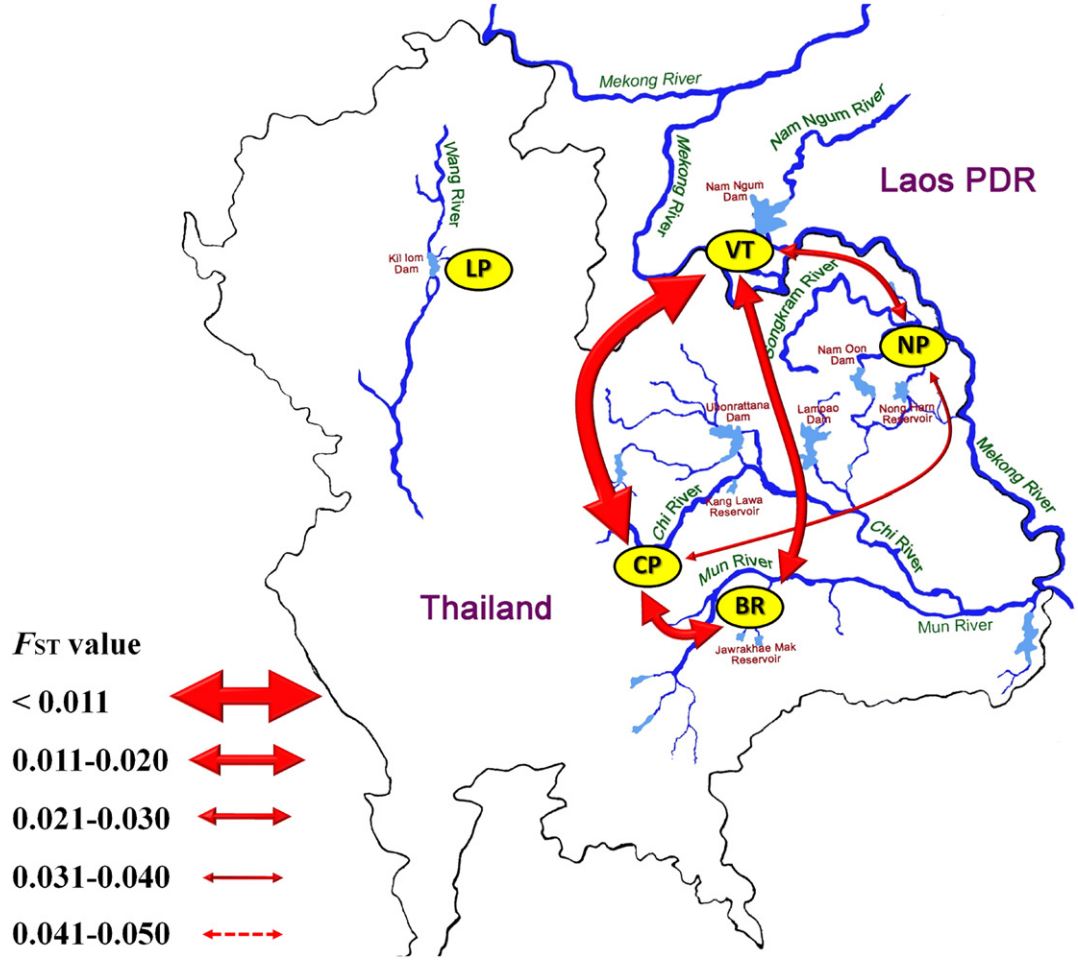

Fig. 1. Comparative levels of gene flow between isolates of *O. viverrini* from different wetland systems in Thailand and Lao PDR based on genetic differentiation (pairwise F_{ST} value). Thickness of the arrows represents level of gene flow and a lack of connecting arrow indicates low or no gene flow (data from Laoprom et al.) [44].

Table 1
The relationship between the prevalence of *O. viverrini*, the incidence of cholangiocarcinoma and distribution of *O. viverrini* genotypes in a micro-geographical area of Khon Kaen Province based on microsatellite DNA analyses and published data.

Parameter	Locality in Khon Kaen Province[a]			
	KBs	KLp	KBp	KPv
CCA incidence[b](cases/100,000/year)	92.4	92.4	123.5	208.9
O. viverrini prevalence (%)[b]	26.4	26.4	36.2	37.8
Unique allele[c]	—[d]	+	+	+
mt DNA(*nad*1)[e]	NA[f]	OVN3[g]	OVN4	OVN1
Enzyme locus[h]				
Ald	*b*[i]	*b*	*c*	*b*
G6pd	*b*	*b*	*a*	*a*
Pk	*b*	*c*	*b*	*b*

[a] KBs (Ban Sa-ard), KLp (Ban Lerngpleuy), KBp (Ban Phai), KPv (Phu Wiang).
[b] Sriamporn et al. [6].
[c] Microsatellite unique/private allele (unpublished).
[d] No unique alleles.
[e] Mitochondrial DNA; the subunit 1 of NADH dehydrogenase gene (*nad*1).
[f] No data available.
[g] Haplotype (Saijuntha et al., 2008) [17].
[h] Saijuntha et al., 2007 [14].
[i] Allele.

records indicate that high incidence levels of CCA are aggregated in certain communities within Khon Kaen Province [6,48]. For instance, within an area of approximately 100 km in radius among Khon Kaen, Ban Phai and Phu Wiang districts of Khon Kaen Province, high levels of CCA incidence (cases/100,000/year) have been recorded in Phu Wiang (208.9) and Ban Phai (123.5). Lower but still high levels have also been recorded for the Muang district (92.4) where Lerngpleuy and Ban Sa-ard are located. Additionally, the highest levels of *O. viverrini* prevalence have been recorded from this area, for instance, highest prevalence was found in Phu Wiang (37%) and Ban Phai (36.2%). Lower prevalence (26.4%) was found in Muang District [6,48]. From the parasite genetic variation perspective, locality-specific alleles detected allozymically and mitochondrial DNA (mtDNA) haplotype do exist and are positively correlated (Table 1).

This preliminary data suggests potential roles of parasite genotype on observed CCA incidence. It is therefore highly desirable to examine the relationship between population genetic variation of *O. viverrini* and the recorded local and regional differences in epidemiology, CCA incidence and host morbidity. Currently, this question is being addressed by using microsatellite DNA as genetic markers in an area with high incidence of CCA in Khon Kaen, Thailand.

7. Conclusion

The discovery that *O. viverrini* is a species complex raises fundamental questions and lays the foundation for cutting edge research about the role that each cryptic species and the levels of genetic variation between populations within each species play on local and regional variability in parasite epidemiology and human disease, including CCA. Currently, there are at least 2 cryptic species of *O. viverrini* in Thailand and Lao PDR. Based on fixed genetic differences inferred from MEE, the 2 cryptic species contain 6 genetically very distinct groups that are associated with major river wetland systems in Thailand and Laos PDR, including the Chi, Mun, Songkram and Wang Rivers in Thailand and the Nam Ngum River in Laos. Further independent evidence that supports the existence of a species complex has been provided by data from microsatellite DNA, mtDNA, and RAPD analyses as well as from infectivity, fecundity and morphological studies.

Even though currently a relatively limited number of investigations have used allozyme and microsatellite DNA markers they have indicated similar different levels of population genetic variation at both a macro- and micro-geographical scale. Although based on a limited number of polymorphic loci, there are similar trends of significant heterozygote deficiency which may suggest inbreeding or selfing as the predominant reproductive mode of *O. viverrini*. The significance of these preliminary data needs to be evaluated by determining and defining how many species make up the *O. viverrini* species complex, determining their distributional ranges and utilizing finer scale population genetic analyses within each sibling/cryptic species.

Once this is achieved, it will then enable us to determine whether there is any correlation between parasite genetics and clinical outcome (i.e. incidence of CCA). These studies are essential because a potential outcome of the on-going control program with praziquantel and other anthelminthics directed at *O. viverrini* could result in changes in heterozygosity, as appears to be occurring with *S. mansoni* following the recent implementation of mass praziquantel drug control programs [49–51].

However precaution in interpretation is needed since existing data on genetic diversity of *O. viverini sensu lato* is based on analyses of adult stages obtained from experimental animals. Whether this creates sampling bias or not is currently not known. This warrants detailed examination and direct analyses using other life stages and different types of host species, similar to those studies reported for Schistosomes [49].

Acknowledgements

We wish to acknowledge support from the Thailand Research Fund Basic Research Grant and the on-going Faculty of Medicine, Khon Kaen University, Resident International Professor Program. This work was also supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, *through the Health Cluster* (*SHeP-GMS*), Khon Kaen University.

References

[1] Andrews RH, Sithithaworn P, Petney TN. *Opisthorchis viverrini*: an underestimated parasite in world health. Trends Parasitol 2008;24:497–501.
[2] Sithithaworn P, Haswell-Elkins M. Epidemiology of *Opisthorchis viverrini*. Acta Trop 2003;88:187–94.
[3] WHO. Control of food borne trematode infections. WHO Technical Report Series, 849; 1995.
[4] Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, et al. Liver fluke induces cholangiocarcinoma. PLoS Med 2007;4:e201.
[5] Jongsuksuntigul P, Chaeychomsri W, Techamontrikul P, Jeradit P, Suratanavanit P. Study on Prevalence and intensity of intestinal helminthiasis and opisthochiasis in Thailand. J Trop Med Parasitol 1992;15:80–95.
[6] Sriamporn S, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S, Parkin DM. Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. Trop Med Int Health 2004;9:588–94.
[7] Cancer Unit. Hospital-based tumor registry 2009: statistical report. Khon Kaen: Cancer Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University; 2009.
[8] Sueblinvong T, Piyamputra A, Priyanont S, Thaithong S. Isoenzyme characterization of *Opisthorchis viverrini* from man. Chulal Med J 1993;37:375–85.
[9] Ando K, Sithithaworn P, Nuchjungreed C, Tesana S, Srisawangwong T, Limviroj W, et al. Nucleotide sequence of mitochondrial CO I and ribosomal ITS II genes of *Opisthorchis viverrini* in northeast Thailand. Southeast Asian J Trop Med Public Health 2001;32(Suppl 2):17–22.
[10] Nuchjungreed C. A study on genetic variations of the liver fluke (*Opisthorchis viverrini*) in Northeast Thailand by Random Amplified Polymorphic DNA. [M.Sc Thesis]. Khon Kaen: Khon Kaen University; 2001.
[11] Sithithaworn P, Nuchjungreed C, Srisawangwong T, Ando K, Petney TN, Chilton NB, et al. Genetic variation in *Opisthorchis viverrini* (Trematoda: Opisthorchiidae) from northeast Thailand and Laos PDR based on random amplified polymorphic DNA analyses. Parasitol Res 2007;100:613–7.
[12] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Pipitgool V, Petney TN, et al. Enzyme markers to identify and characterize *Opisthorchis viverrini* in Thailand and Lao PDR. Southeast Asian J Trop Med Public Health 2006;37(Suppl 3):43–7.
[13] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Pipitgool V, Petney TN, et al. Genetic markers for the identification and characterization of *Opisthorchis viverrini*, a medically important food borne trematode in Southeast Asia. Acta Trop 2006;100:246–51.
[14] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Pipitgool V, Tesana S, et al. Evidence of a species complex within the food-borne trematode *Opisthorchis viverrini* and possible co-evolution with their first intermediate hosts. Int J Parasitol 2007;37:695–703.

[15] Kiatsopit N, Sithithaworn P, Sithithaworn J, Boonmars T, Tesana S, Pitaksakulrat O, et al. Genetic relationships within the *Opisthorchis viverrini* species complex with specific analysis of *O. viverrini* from Savannakhet, Lao PDR by multilocus enzyme electrophoresis. Parasitol Res 2011;108:211–7.
[16] Andrews RH. Chilton N.B. Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. Int J Parasitol 1999;29:213–53.
[17] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Chilton NB, Petney TN, et al. Mitochondrial DNA sequence variation among geographical isolates of *Opisthorchis viverrini* in Thailand and Lao PDR, and phylogenetic relationships with other trematodes. Parasitology 2008;135:1479–86.
[18] Thaenkham U, Nuamtanong S, Sa-nguankiat S, Yoonuan T, Touch S, Manivong K, et al. Monophyly of *Opisthorchis viverrini* populations in the lower Mekong Basin, using mitochondrial DNA nad1 gene as the marker. Parasitol Int 2010;59:242–7.
[19] Brandt RAM. The non-marine aquatic Mollusca of Thailand. Arch Mollusk 1974;105:1–423.
[20] Chitramvong YP. The Bithyniidae (Gastropoda: Prosobranchia) of Thailand: comparative external morphology. Malacol Review 1992;25:21–38.
[21] Adam R, Arnold H, Pipitgool V, Sithithaworn P, Hinz E, Storch V. Studies on lophocercous cercariae from *Bithynia siamensis goniomphalus* (Prosobranchia: Bithyniidae). Southeast Asian J Trop Med Public Health 1993;24:697–700.
[22] Brockelman WY, Upatham ES, Viyanant V, Ardsungnoen S, Chantanawat R. Field studies on the transmission of the human liver fluke, *Opisthorchis viverrini*, in northeast Thailand: population changes of the snail intermediate host. Int J Parasitol 1986;16:545–52.
[23] Duangprompo W. Genetic variation of snails in the family Bithyniidae in Thailand and identification of Bithynia siamensis goniomphalos by PCR. [M.Sc Thesis]. Khon Kaen: Khon Kaen University; 2007.
[24] Kodcharin P. Genetic variation of *Bithynia siamensis goniomphalos*, first intermediate host of *Opisthorchis viverrini* in the basin of Mun and Chi Rivers, Thailand by RAPD. [M.Sc Thesis]. Khon Kaen: Khon Kaen University; 2005.
[25] Ngern-klun R, Sukontason KL, Tesana S, Sripakdee D, Irvine KN, Sukontason K. Field investigation of *Bithynia funiculata*, intermediate host of *Opisthorchis viverrini* in northern Thailand. Southeast Asian J Trop Med Public Health 2006;37:662–72.
[26] Sri-Aroon P, Butraporn P, Limsomboon J, Kerdpuech Y, Kaewpoolsri M, Kiatsiri S. Freshwater mollusks of medical importance in Kalasin Province, northeast Thailand. Southeast Asian J Trop Med Public Health 2005;36:653–7.
[27] Sri-Aroon P, Butraporn P, Limsoomboon J, Kaewpoolsri M, Chusongsang Y, Charoenjai P, et al. Freshwater mollusks at designated areas in eleven provinces of Thailand according to the water resource development projects. Southeast Asian J Trop Med Public Health 2007;38:294–301.
[28] Tesana S. Diversity of mollusks in the Lam Ta Khong reservoir, Nakhon Ratchasima, Thailand. Southeast Asian J Trop Med Public Health 2002;33:733–8.
[29] Upatham ES, Sukhapanth N. Field studies on the bionomics of *Bithynia siamensis siamensis* and the transmission of *Opisthorchis viverrini* in Bangna, Bangkok, Thailand. Southeast Asian J Trop Med Public Health 1980;11:355–8.
[30] Lohachit C. Ecological studies of *Bithynia siamensis goniomphalos* a snail intermediate host of *Opisthorchis viverrini* in Khon Kaen Province, Northeast Thailand. [PhD Thesis]. Bangkok: Mahidol University; 2001.
[31] Chanawong A, Waikagul J. Laboratory studies on host–parasite relationship of *Bithynia* snails and the liver fluke, *Opisthorchis viverrini*. Southeast Asian J Trop Med Public Health 1991;22:235–9.
[32] Sithithaworn P, Yongvanit P, Tesana S, Pairojkul C. Liver Flukes. In: Murrell D, Fried B, editors. Parasitic Foodborne Zoonoses: Springer Verlag; 2008. p. 1–51.
[33] Saijuntha W, Sithithaworn P, Chilton NB, Petney TN, Klinbunga S, Satrawaha R, et al. Impact of temporal changes and host factors on the genetic structure of a population of *Opisthorchis viverrini sensu lato* in Khon Kaen Province (Thailand). Parasitology 2009;136:1057–63.
[34] Sithithaworn P, Pipitgool V, Srisawangwong T, Elkins DB, Haswell-Elkins MR. Seasonal variation of *Opisthorchis viverrini* infection in cyprinoid fish in north-east Thailand: implications for parasite control and food safety. Bull World Health Organ 1997;75:125–31.
[35] Rudge JW, Carabin H, Balolong E, Tallo V, Shrivastava J, Lu DB, et al. Population genetics of *Schistosoma japonicum* within the Philippines suggest high levels of transmission between humans and dogs. PLoS Negl Trop Dis 2008;2:e340.
[36] Shrivastava J, Gower CM, Balolong Jr E, Wang TP, Qian BZ, Webster JP. Population genetics of multi-host parasites — the case for molecular epidemiological studies of *Schistosoma japonicum* using larval stages from naturally infected hosts. Parasitology 2005;131:617–26.
[37] Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, et al. Effect of aquaculture on world fish supplies. Nature 2000;405:1017–24.
[38] Taraschewski H. Hosts and parasites as aliens. J Helminthol 2006;80:99–128.
[39] Saijuntha W. A comprehensive investigation of genetic variation among natural populations of *Opisthorchis viverrini* in Thailand and Lao PDR. [Ph.D Thesis]. Khon Kaen: Khon Kaen University; 2007.
[40] Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Satrawaha R, Chilton NB, et al. Genetic variation at three enzyme loci within a Thailand population of *Opisthorchis viverrini*. Parasitol Res 2008;103:1283–7.
[41] Schlotterer C. Evolutionary dynamics of microsatellite DNA. Chromosoma 2000;109:365–71.
[42] Shrivastava J, Qian BZ, McVean G, Webster JP. An insight into the genetic variation of *Schistosoma japonicum* in mainland China using DNA microsatellite markers. Mol Ecol 2005;14:839–49.
[43] Tang S, Popongviwat A, Klinbunga S, Tassanakajon A, Jarayabhand P, Menasveta P. Genetic heterogeneity of the tropical abalone (*Haliotis asinina*) revealed by RAPD and microsatellite analyses. J Biochem Mol Biol 2005;38:182–90.
[44] Taylor AC, Sherwin WB, Wayne RK. Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. Mol Ecol 1994;3:277–90.
[45] Laoprom N, Sithithaworn P, Ando K, Sithithaworn J, Wongkham S, Laha T, et al. Microsatellite loci in the carcinogenic liver fluke, *Opisthorchis viverrini* and their application as population genetic markers. Infect Genet Evol 2010;10:146–53.
[46] Laoprom N, Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Ando K, et al. Biological variation within *Opisthorchis viverrini sensu lato* in Thailand and Lao PDR. J Parasitol 2009;95:1307–13.
[47] Brouwer KC, Ndhlovu PD, Wagatsuma Y, Munatsi A, Shiff CJ. Urinary tract pathology attributed to *Schistosoma haematobium*: does parasite genetics play a role? Am J Trop Med Hyg 2003;68:456–62.
[48] Vatanasapt V, Uttaravichien T, Mairiang E, Pairojkul C, Chartbanchachai V, Haswell-Elkins MR. Northeast Thailand: a region with high incidence of cholangiocarcinoma. Lancet 1990;1:116–7.
[49] Gower CM, Shrivastava J, Lamberton PH, Rollinson D, Webster BL, Emery A, et al. Development and application of an ethically and epidemiologically advantageous assay for the multi-locus microsatellite analysis of *Schistosoma mansoni*. Parasitology 2007;134:523–36.
[50] Lamberton PH, Hogan SC, Kabatereine NB, Fenwick A, Webster JP. *In vitro* praziquantel test capable of detecting reduced *in vivo* efficacy in *Schistosoma mansoni* human infections. Am J Trop Med Hyg 2010;83:1340–7.
[51] Toure S, Zhang Y, Bosque-Oliva E, Ky C, Ouedraogo A, Koukounari A, et al. Two-year impact of single praziquantel treatment on infection in the national control programme on schistosomiasis in Burkina Faso. Bull World Health Organ 2008;86:780–7.