Analysis of population genetics of *Opisthorchis viverrini* sensu lato in the Nam Ngum River wetland, Lao PDR, by multilocus enzyme electrophoresis

Nadda Kiatsopit[a, b], Paiboon Sithithaworn[a, b, *], Weerachai Saijuntha[c], Opal Pitaksakulrat[a, b], Trevor N. Petney[d], Joanne P. Webster [e], Ross H. Andrews[a,b,e]

[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] *Walai Rukhavej Botanical Research Institute (WRBRI), Mahasarakham University, Mahasarakham 44150, Thailand*

[d] *Department of Ecology and Parasitology, University of Karlsruhe, Kornblumenstrasse 13, Karlsruhe 76131, Germany*

[e] *Imperial College London, Faculty of Medicine, St Mary's Campus, South Wharf Street, London W2 1NY. United Kingdom*

* Corresponding author: Paiboon Sithithaworn, 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: paibsit@gmail.com

Abstract

A previous population genetic study of *Opisthorchis viverrini* from a locality in an endemic area in Thailand found little genetic variation over time and second intermediate fish host species. Since a similar comparative analysis is not available for Lao PDR, we conducted a study of *O. viverrini* from different endemic foci in Vientiane Province, Lao PDR based on spatial, temporal and fish host species. A total of 620 adult *O. viverrini* originating from the Nam Ngum River wetland were analyzed at five previously defined polymorphic enzyme loci. Of these worms, 252 were from six different localities (spatial samples), 162 worms from different years (temporal samples) and 206 worms from four different cyprinid fish species. Significant heterozygote deficiency was found in most *O. viverrini* populations with levels of genetic differentiation ranging between F_{ST} 0.0000 – 0.0197 suggesting that gene flow occurred at a variable rate. The role of temporal factors and fish host species had little influence on the level of genetic differentiation. As for *O. viverrini* from Thailand, these findings indicate that self-fertilization and/or a clonal distribution of *O. viverrini* occurs in Lao PDR. Unlike the results for *O. viverrini* from Thailand spatial population substructuring may be the underlying population processes for *O. viverrini* in Lao PDR. These findings indicate that geographical variation may contribute to the transmission dynamics of the parasite with implications for parasite control. However, other host factors, such as snail intermediate hosts and mammal reservoir hosts, as well as human beings, may also play significant roles.

Key words: Population genetics, *Opisthorchis viverrini*, Lao PDR, multilocus enzyme electrophoresis

1. Introduction

Opisthorchis viverrini sensu lato is a carcinogenic food-borne trematode endemic in the Mekong area of continental Southeast Asia, especially in Thailand and Lao PDR. More than 10 million people are estimated to be infected with an estimated eight million in Thailand and two million in Lao PDR (WHO 1995; Andrews et al. 2008). To complete its life cycle it requires two intermediate hosts, a freshwater snail as the first and a cyprinid fish as the second, as well as a third definitive human or carnivore (cat and dog) host (Petney et al. 2013). Infection is caused by eating raw or partially cooked cyprinid fish containing viable metacercariae (Andrews et al. 2008). The infection is known as opisthorchiasis and it causes hepatobiliary diseases including cholangiocarcinoma (CCA) (Sripa et al. 2007). In northeast Thailand, *O. viverrini* is a major medical problem with prevalences commonly reaching 30 – 80% or more in rural populations (Saowakontha et al. 1993; Sripa et al. 2011; Sithithaworn et al. 2012). A similarly high prevalence (60.7 – 86.2%) of *O. viverrini* infection also occurs in Lao PDR (Kobayashi et al. 2000; Sayasone et al. 2009)

Multilocus enzyme electrophoresis (MEE) analyses by Saijuntha et al. (2007) and Kiatsopit et al. (2011) found that *O. viverrini* is a species complex, potentially consisting of genetically very distinct but morphologically similar (hence cryptic) species which are associated with specific wetlands in Thailand and Lao PDR. Additionally, independent biological evidence has revealed significant differences in body size, fecundity and infectivity of *O. viverrini* that occur in different wetlands, namely the Chi and Songkram in Thailand and the Nam Ngum in Lao PDR. From this biological evidence, in conjunction with molecular genetic data, Laoprom et al. (2009) suggested that *O. viverrini* from the Songkram wetland (Sakon Nakhon and Nakhon Phanom) is a morphologically, genetically, and biologically distinct species. Furthermore, Saijuntha et al. (2007) and Kiatsopit et al. (2013) have shown that the first intermediate snail host, *Bithynia siamensis goniomphalos* also

represents a species complex consisting of cryptic species that occur in the same wetlands as the cryptic species of *O. viverrini* in Thailand and Lao PDR.

A preliminary MEE study of the population genetics of *O. viverrini* from Thailand from one locality within the Chi wetland suggested the possibility that population sub-structuring may be associated with inbreeding (Saijuntha et al. 2008). Subsequently, Saijuntha et al. (2009) found no significant differences in the population genetic structure temporally or in different species of second intermediate fish hosts. This suggests a high rate of gene flow within this parasite population and that there is no population sub-structuring occurring at this locality in Thailand (Saijuntha et al. 2008).

Information regarding the levels of population genetic variation and the effect of temporal factors and second fish intermediate hosts for *O. viverrini* from Lao PDR are not currently available. For comparative purposes we conducted an MEE analysis of the population genetics of *O. viverrini* from the Nam Ngum River wetland, Vientiane Province, Lao PDR based on geographical localities, temporal sampling and fish host species.

2. Materials and methods

2.1. Opisthorchis viverrini *population samples*

The population samples of *O. viverrini* were divided into three sets. In the first set for the spatial study, 252 individual worms were obtained from metacercariae collected from cyprinid fish from six different geographical localities along the Nam Ngum River wetland (Fig. 1). In the second set for the temporal study, 162 individual samples were obtained from *Cyclocheilichthys armatus* (Ca; Sai Tan Ta Khao) in That Luang District, Vientiane Province, in different years. In the last set for the host study, 206 worms were isolated from metacercariae from four different species of cyprinid fish: *C. armatus*, *Henicorhynchus siamensis* (HS; Soi Khao), *Barbonymus gonionotus* (Bg; Taphian Khao) and *Puntius brevis*

(Pb; Taphian Jut) (Table 1). Information on the number of fish sampled and the distribution of *O. viverrini* metacercariae from Nam Ngum River wetlands in Lao PDR are shown in Table 2.

2.2 Preparation of O. viverrini *adult worms*

The cyprinid fish were sorted according to time and place of capture and species. The recovery of metacercariae was carried out using the pepsin digestion method (0.3% pepsin) as described previously (Sithithaworn et al. 1997). The *O. viverrini* metacercariae were sorted and identified under a stereomicroscope (Kaewkes 2003). Fifty to 100 live metacercariae from each locality were given orally to each of a group of male golden Syrian hamster, aged 6 – 8 weeks, by intragastric intubation. Four months after infection, the hamsters were sacrificed and the worms recovered from the bile duct. The worms were washed several times in 0.85% normal saline and stored at –80°C until use. All animal experimentation was approved and controlled under the guidelines of the Animal Ethics Committee, Khon Kaen University.

2.3. Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE) has been used very effectively over many years to study the population genetics and systematics of protozoan, arthropod and helminth parasites (Andrews and Chilton 1999), including *O. viverrini* (Saijuntha et al. 2007; Saijuntha et al. 2008; Saijuntha et al. 2009; Kiatsopit et al. 2011). Frozen homogenates of *O. viverrini* from Loa PDR were prepared for electrophoretic analyses by placing an individual worm into each well of a U-plate and adding 5 μL of lysing solution (100 mL distilled water, 100 μL β-mercaptoethanol, 10 mg nicotinamide adenine dinucleotide phosphate) to the thawed sample. This was then ground using a grinder tube. Supernatants were removed directly by loading

pen from each well and loaded onto the cellulose acetate gel (Cellogel; Milan) as the support medium. Each gel was stained histochemically for a specific enzyme as described for the five enzymes that were previously shown to be polymorphic (Saijuntha et al. 2006; Saijuntha et al. 2007). The polymorphic enzymes used for the population genetic study in Thailand were used for in the present study for *O. viverrini* from Lao PDR, namely: enolase (ENOL, EC 4.2.1.11), fructose-1,6-diphosphatase (FDP, 3.1.3.11), phosphoglycerate mutase (PGAM, 2.7.5.3), phosphoglucomutase (PGM, EC 2.7.5.1), and triose phosphate isomerase (TPI, EC 5.3.1.1). The electrophoretic banding patterns were scored alphabetically in order of increasing anodal migration (a, b, c, etc.). Heterozygote and homozygote banding patterns were scored and analysed as indicated in Andrews and Chilton (1999).

2.4. Population genetic analysis

Allele frequencies and genotype frequencies for each locus were calculated, as were the observed and expected heterozygosity (Nei 1987). Hardy-Weinberg equilibrium (HWE) for each locus was examined using the exact test (Rousset and Raymond 1995). To detect whether significant departure from HWE resulted from a deficiency (positive value F_{IS}) or excess (negative value F_{IS}) heterozygosity F_{IS} statistics (Wright 1943) were used (Weir and Cockerham 1984). Genetic differentiation (F_{ST}) between populations and between geographical areas and the significance of pairwise F_{ST} values was also evaluated (Weir and Cockerham 1984). The relationship between genetic isolation among localities was assessed by testing for independence between F_{ST} and geographical distances by a Mantel test. The majority of the population genetic analyses were performed using Genepop software version 4.1 (Rousset and Raymond 1995).

3. Results

Of the five polymorphic enzymes examined in this study, following histochemical staining three loci, *Enol*, *Pgam* and *Tpi* provided a high degree of staining intensity and resolution to enable accurate scoring of the electrophoretic bands for population genetic analysis.

Allele and genotype frequencies of *O. viverrini* for the spatial and temporal subsets, as well as for the species of fish hosts are shown in Tables 3 and 4, respectively. The number of total alleles ranged from 2 to 4 per locus in each particular population (Table 3). Two alleles were detected for *Enol* and *Pgam*, with allele *a* being the most common in all populations (0.690 – 0.875 for *Enol* and 0.719 – 0.960 for *Pgam*). In addition, the frequencies of all genotypes belonging to *Enol* and *Pgam* were also consistent. Four alleles were detected at the *Tpi* locus with the frequency of allele *b* being the highest in all populations (0.552 – 0.680). The allele occurring at the lowest frequency (*d*) was detected in *O. viverrini* individuals collected from TL and PK, temporally in 2009 from *C. armatus*, and two fish species, *C. armatus* (2009) and *H. siamensis* (2010). Seven genotypes of *Tpi* were found with relatively consistent frequencies in all spatial, temporal and host species populations.

For the six spatially sampled populations, significant heterozygote deficiency (positive F_{IS} values, $P < 0.05$) compared with the predictions under HWE were detected in all populations for *Enol* except for the TH population. The F_{IS} values observed for this locus ranged from 0.179 to 0.795 (Table 5). The three populations, VV, NG, and TL significantly deviated from HWE ($P < 0.05$) for *Pgam*. The positive F_{IS} values in all spatial populations ranged from 0.197 in PK to 0.837 in NG populations for *Pgam*. For the *Tpi* locus, no significant heterozygote deficiency was observed in any population compared to HWE ($P > 0.05$), and observed F_{IS} values ranged from 0.038 to 0.262. Interestingly, a low level of genetic differentiation was found among all populations (F_{ST} ranging from 0.0000 to 0.0197;

$P > 0.05$) (Table 6). The correlation between F_{ST} and geographical distance between the spatial populations showed no significant correlation ($P > 0.05$; data not show) independently of whether the distances were calculated in straight lines or along river courses.

For the temporally separated populations, significant heterozygote deficiency (positive F_{IS} values) compared with the predictions under HWE were detected in all populations at *Enol* (F_{IS} values ranging from 0.474 in 2010 to 0.635 in 2009). For the *Pgam* locus, two populations from the years 2008 and 2009 significantly deviated from HWE ($P < 0.05$) with positive F_{IS} values in 2008 (1.000) and 2009 (0.353). Only the population in 2010 showed a negative F_{IS} value (heterozygote excess) but this was not significant. No significant heterozygote deficiency was detected for *Tpi* in any temporal population as determined by F_{IS} values (F_{IS} ranging from 0.076 to 0.121, $P > 0.05$) (Table 5). Low levels of genetic differentiation were recorded between these populations with F_{ST} ranging from 0.0020 to 0.0061 (Table 6).

For the four samples isolated from the four different species of fish, all populations deviated from HWE ($P < 0.05$) except for a population isolated from *H. siamensis* at *Pgam* and all populations at *Tpi*. Significant heterozygote deficiency ($P < 0.05$) compared with the predictions under HWE were detected in all populations at the *Enol* locus with F_{IS} ranging between 0.377 – 0.734. F_{IS} values ranging from 0.197 to 0.779 were observed at the *Pgam* locus with significant heterozygote deficiency except for a population isolated from *H. siamensis*. F_{IS} values ranging from 0.053 to 0.121 were detected for the *Tpi* locus with no significant heterozygote deficiency (Table 5). Low levels of genetic differentiation were recorded between these differentiated populations with F_{ST} ranging from 0.0000 to 0.0098 (Table 6).

4. Discussion

Five polymorphic enzymes, ENOL, FDP, PGAM, PGM and TPI, all of which are known to be polymorphic in *O. viverrini* from Thailand (Saijuntha et al. 2006; Saijuntha et al. 2007), were examined in samples from populations of the *O. viverrini* species from Lao PDR. Only three of these polymorphic enzymes, namely ENOL, PGM and TPI, were suitable and selected for genetic analysis of the Thai populations as they showed typical heterozygote patterns and were reliably scorable, whereas FDP and PAGM did not (Saijuntha et al. 2008; Saijuntha et al. 2009). The differences between countries may reflect differences between the populations of *O. viverrini* from Thailand and Lao PDR, which would support previous systematic analyses using MEE which indicate that different, cryptic species are involved.

For direct comparative purposes, our results are concordant with previous temporal and fish host species MEE findings by Saijuntha et al. (2009) in a population *O. viverrini* from Khon Kaen Province in Thailand. Our results indicate that populations of *O. viverrini* from Lao PDR which are separated spatially, and which were sampled at different times and from different fish host species, predominantly showed heterozygote deficiency and were not in HWE. Inbreeding or the Wahlund effect could be responsible for this finding. In particular, self-fertilization usually occurs in *O. viverrini* because of a low parasite burden in an infected host, including humans (Sithithaworn et al. 1991b; Trouve et al. 1999; Criscione et al. 2005). In Lao PDR and Thailand heterozygote deficiency was found in *O. viverrini* populations for different years and different species of fish host. The only exceptions being temporal in which no heterozygote deficiency was found for *Enol* in the population from Thailand (over 4 years) (Saijuntha et al., 2009) and for *Tpi* in the Lao PDR populations (over 3 years). For fish host species significant heterozygote deficiencies were detected at *Pgm* and *Tpi* for *O. viverrini* infecting *Puntioplites protozsron* in Thailand, while no significant heterozygote deficiencies were detected among *O. viverrini* in *H. siamensis* and for *Pgam* or for *Tpi* in

each of the four species of fish from Lao PDR. Overall, no significant differences in genetic differentiation (F_{ST}) between *O. viverrini* populations from Lao PDR, or in the population from Thailand (Saijuntha et al. 2009) were detected over times and different species of fish. There were, however, varying levels of *O. viverrini* genetic differentiation between *H. siamensis* and *B. gonionotus*, and *H. siamensis* and *P. brevis* in Lao PDR and between all species of fish in Thailand (*C. armatus*, *Hampala dispar*, *P. protozsron* and *Puntius orphoides*) but this was not statistically significant. Interestingly, in Lao PDR there was no significant genetic differentiation when *C. armatus* was compared to the other three species, but in Thailand, where *C. armatus* also occurs, there were varying levels of genetic differentiation in *O. viverrini* from the different species of fish examined, although this was not statistically significant. Nonetheless, it seems that there may be differences associated with different fish host species in Lao PDR and in Thailand. This requires examination by more sensitive population genetic methods such as microsatellite DNA. It should be kept in mind, however, that a bottleneck could be occurring at the first, second and definitive stages of the life cycle to preferentially select specific genotypes of *O. viverrini*, including laboratory animal passage to obtain adult worms (Shrivastava et al. 2005).

Almost all trematodes, including *O. viverrini*, have complex life cycles with an obligate alternation of asexual and sexual reproduction during their lifespan. The asexual phase occurs within the mollusc intermediate host in which usually genetically identical cercariae are produced; whereas the sexual phase occurs in the vertebrate definitive host. This suggests that the severe deficit of heterozygotes observed in monoecious trematode populations might be produced by the aggregation of clones among hosts. Vilas et al. (2004) have suggested that the most likely cause of these deviations from Hardy-Weinberg expectations is the mode of reproduction of these hermaphroditic helminths, and that inbreeding generated by selfing or crossing between genetically identical individuals is a

phenomenon that affects the whole trematode genome. Is it likely that a fish can be infected by aggregated cercariae from a single snail or infected by different cercariae from more than one snail which in turn may lead to preferential selection of different genotypes in species of fish hosts (Lo and Lee 1996; Mitchell et al. 2002).

The high rate of population turnover between three consecutive years and four fish host species, indicated by a lack of or very low genetic differentiation or a high rate of gene flow (with F_{ST} value < 0.050) among the temporally differentiated and fish host populations, suggests that no major events have influenced the evolutionary process for these variables, e.g. no genetic drift and bottlenecks have occurred during this time period or between different fish host species. The absence of large genetic differences between *O. viverrini* populations and high levels of gene flow suggest that this is probably due to the mobility of the fish and potentially human hosts (Blouin et al. 1995; Schlotterer 2000; Li et al. 2002; McCoy et al. 2003; Criscione and Blouin 2004; Petney et al. 2013). It is likely that the *Bithynia* snails have a low capacity for dispersal as well. In this case, temporal and fish host species factors appear not to have a major influence on the genetic variation of *O. viverrini* in Lao PDR.

Interestingly, however, higher genetic differentiation (F_{ST} ranging between 0.0024 – 0.0197) was detected between the VV, TH and NG populations and the three populations in VT, which may be related to the Nam Song diversion downstream from VV into the Nam Ngum dam (Fig. 1). Moreover, the allopatric populations of VV, TH and NG are separated from the VT populations by a dam wall at NG (Fig. 1), which may serve as a block, restricting gene flow between the *O. viverrini* populations located in the upper (VV, TH and NG) and lower (TL, PK and NK) areas of Nam Ngum dam. However, the level of genetic differentiation between the upper and lower areas of Nam Ngum dam was relatively low, perhaps due to the early period of differentiation among those populations, because the Nam

Ngum dam was built only 42 years ago (1971 to 2013). Laoprom et al. (2012) found genetic differentiation among *O. viverrini* populations from Thailand at a micro-scale (10 – 60 km) similar to geographical distances between the populations which we examined here using microsatellite markers which provided a finer population genetic resolution than MEE (Laoprom et al. 2012). It would therefore be useful to examine the genetic differentiation detected here using MEE for *O. viverrini* populations from the upper (VV, TH and NG) and lower (TL, PK and NK) areas of Nam Ngum dam using microsatellite analyses.

Self-fertilization of the parasite may be the underlying population processes occurring at Nam Ngum Dam (Laoprom et al. 2010). For the population genetics of *O. viverrini* from Thailand and Lao PDR, there is no genetic differentiation based on different times and different fish host as with *O. viverrini* from Thailand. An alternative explanation might be stability and persistence of infectious stages over long periods, however there are no data currently for *O. viverrini*, but isolated metacercariae of *O. viverrini* and *Clonorchis sinensis* survive only for few weeks (Sithithaworn et al. 1991a; Li et al. 2006). The role of temporal factors and fish host species appears to have little influence on the levels of genetic differentiation. Interestingly, spatially related genetic differentiation may be occurring between *O. viverrini* located in the upper (VV, TH and NG) and lower (TL, PK and NK) areas of Nam Ngum dam. This hypothesis requires further investigation.

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Table 1 Details of the *Opisthorchis viverrini* sampling localities including six sample localities from Nam Ngum River wetlands in Lao PDR.

Wetland	Reservoir	Collecting locality (village/district, Province)	Code	No. of *O. viverri* isolated
Nam Ngum River	Nam Song River	Vang Vieng, Vientiane	VV	50
	Nam Ngum River	Tha Heur, Vientiane	TH	28
	Nam Ngum River	Nam Ngum, Vientiane	NG	23
	Nam Ngum River	That Luang, Vientiane	TL	369
	Nam Ngum River	Phonthan Neua, Vientiane	PK	100
	Nam Ngum River	Ban Na Khouay, Vientiane	NK	50

Table 2 Information of the numbers of fish and distribution of *Opisthorchis viverrini* metacercariae from Nam Ngum River wetlands in Lao PDR.

Factor	Locality[1]	Fish	Years	No. of fish	Weight of fish (kg)	Abun
Locality						
	VV	*Cyclocheilichthys armatus*	2008	599	13	7.50
	TH	*Cyclocheilichthys armatus*	2006	257	3.2	3.09
	NG	*Hampala macrolepidota*	2006	4	0.7	125
	TL	*Cyclocheilichthys armatus*	2009	1,057	4.8	49.95
	PK	*Henicorhynchus siamensis*	2010	217	0.7	6.91
	NK	*Cyclocheilichthys armatus*	2009	255	1.05	17.25
Temporal						
	TL	*Cyclocheilichthys armatus*	2008	255	3.6	15.68
	TL	*Cyclocheilichthys armatus*	2009	1,057	4.8	49.95
	TL	*Cyclocheilichthys armatus*	2010	692	2.75	36.13
Fish host						
	TL	*Cyclocheilichthys armatus*	2009	1,057	4.8	49.95
	PK	*Henicorhynchus siamensis*	2010	217	0.7	6.91
	TL	*Barbonymus gonionotus*	2010	587	2.0	0.50
	TL	*Puntius brevis*	2010	107	1.3	1.25

[1] Details of the *O. viverrini* sampling localities are given in Table 1.

Table 3 Allele frequencies of *Opisthorchis viverrini* samples collected in different localities, different years from *Cyclocheilichthys armatus* and from different fish host species at three loci.

Locus	Allele	Geographical localities[1]						Year			Fish host[2]			
		VV	TH	NG	TL	PK	NK	2008	2009	2010	Ca	Hs	Bg	Pb
	N	(n=50)	(n=28)	(n=23)	(n=51)	(n=50)	(n=50)	(n=55)	(n=51)	(n=56)	(n=51)	(n=50)	(n=55)	(n=50)
Enol	*a*	0.796	0.848	0.875	0.755	0.690	0.740	0.750	0.755	0.833	0.755	0.690	0.824	0.792
	b	0.204	0.152	0.125	0.245	0.310	0.260	0.250	0.245	0.167	0.245	0.310	0.176	0.208
Pgam	*a*	0.838	0.846	0.841	0.807	0.765	0.857	0.960	0.807	0.896	0.807	0.765	0.861	0.719
	b	0.163	0.154	0.159	0.193	0.235	0.143	0.040	0.193	0.104	0.193	0.235	0.139	0.281
Tpi	*a*	0.281	0.146	0.310	0.255	0.190	0.256	0.314	0.255	0.219	0.255	0.190	0.240	0.240
	b	0.552	0.604	0.571	0.627	0.680	0.622	0.588	0.627	0.583	0.627	0.680	0.615	0.560
	c	0.167	0.250	0.119	0.108	0.120	0.122	0.098	0.108	0.198	0.108	0.120	0.144	0.200
	d	0.000	0.000	0.000	0.010	0.010	0.000	0.000	0.010	0.000	0.010	0.010	0.000	0.000

[1] Details of the *O. viverrini* sampling localities are given in Table 1.

[2] Details of the species of cyprinid fish are given in Materials and Methods.

Table 4 Genotype frequencies of *Opisthorchis viverrini* samples collected in different localities, different years from *Cyclocheilichthys armatus* and from different fish host species at three loci.

Locus	Genotype	Geographical localities[1]						Year			Fish host[2]			
		VV	TH	NG	TL	PK	NK	2008	2009	2010	Ca	Hs	Bg	Pb
	N	(n=50)	(n=28)	(n=23)	(n=51)	(n=50)	(n=50)	(n=55)	(n=51)	(n=56)	(n=51)	(n=50)	(n=55)	(n=50)
Enol	*aa*	0.74	0.74	0.85	0.68	0.58	0.70	0.67	0.69	0.76	0.68	0.58	0.78	0.69
	ab	0.12	0.22	0.05	0.14	0.22	0.08	0.17	0.14	0.15	0.14	0.22	0.08	0.21
	bb	0.14	0.04	0.10	0.18	0.20	0.22	0.17	0.17	0.09	0.18	0.20	0.14	0.10
Pgam	*aa*	0.75	0.77	0.82	0.70	0.62	0.77	0.96	0.70	0.79	0.70	0.62	0.83	0.62
	ab	0.17	0.15	0.04	0.20	0.29	0.17	0.00	0.20	0.21	0.20	0.29	0.06	0.19
	bb	0.08	0.08	0.14	0.10	0.09	0.06	0.04	0.10	0.00	0.10	0.09	0.11	0.19
Tpi	*aa*	0.06	0.04	0.14	0.08	0.06	0.11	0.10	0.08	0.04	0.08	0.06	0.06	0.08
	ab	0.33	0.13	0.19	0.27	0.20	0.22	0.31	0.27	0.25	0.27	0. 20	0.31	0.24
	bb	0.33	0.38	0.43	0.43	0.48	0.45	0.39	0.43	0.38	0.43	0.48	0.38	0.32
	ac	0.11	0.08	0.14	0.08	0.06	0.07	0.12	0.08	0.10	0.08	0.06	0.06	0.08
	bc	0.11	0.33	0.10	0.10	0.18	0.13	0.08	0.10	0.17	0.10	0.18	0.15	0.24
	cc	0.06	0.04	0.00	0.02	0.00	0.02	0.00	0.02	0.06	0.02	0.00	0.04	0.04
	bd	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.02	0.00	0.02	0.02	0.00	0.00

[1] Details of the *O. viverrini* sampling localities are given in Table 1.

[2] Details of the species of cyprinid fish are given in Materials and Methods.

Table 5 The expected (*He*) and observed heterozygosity (*Ho*) at three polymorphic loci for *Opisthorchis viverrini* collected in different localities, years and fish hosts from Vientiane Province, Lao PDR. A *P*-value of <0.05 indicates deviation from Hardy-Weinberg equilibrium (HWE). *P*-values considered significant are in bold. N: Sample size; H_e: expected heterozygosity; H_o: observed heterozygosity; F_{IS}: inbreeding coefficient; *P*: probability of significant deviation from HWE.

Factor	N	*Enol*				*Pgam*				*Tpi*			
		He	*Ho*	F_{IS}	*P*	*He*	*Ho*	F_{IS}	*P*	*He*	*Ho*	F_{IS}	*P*
Localities[1]													
VV	50	0.164	0.061	0.629	**0.000**	0.137	0.087	0.368	**0.044**	0.297	0.270	0.089	0.183
TH	28	0.131	0.108	0.179	0.414	0.132	0.076	0.425	0.077	0.281	0.270	0.038	0.655
NG	23	0.112	0.025	0.781	**0.010**	0.136	0.022	0.837	**0.001**	0.288	0.214	0.262	0.131
TL	51	0.186	0.068	0.635	**0.000**	0.157	0.102	0.353	**0.033**	0.267	0.235	0.121	0.267
PK	50	0.216	0.110	0.493	**0.000**	0.182	0.147	0.197	0.340	0.245	0.230	0.065	0.648
NK	50	0.194	0.040	0.795	**0.000**	0.124	0.085	0.313	0.116	0.269	0.211	0.218	0.202
Year													
2008	55	0.189	0.083	0.562	**0.000**	0.039	0.000	1.000	**0.019**	0.275	0.254	0.076	0.260
2009	51	0.186	0.068	0.635	**0.000**	0.157	0.102	0.353	**0.036**	0.267	0.252	0.121	0.542
2010	56	0.140	0.074	0.474	**0.002**	0.095	0.104	-0.095	1.000	0.289	0.260	0.101	0.520
Type of fish[2]													
Ca	51	0.186	0.068	0.635	**0.000**	0.157	0.102	0.354	**0.033**	0.267	0.235	0.121	0.548
Hs	50	0.216	0.110	0.493	**0.000**	0.182	0.147	0.197	0.340	0.245	0.230	0.065	0.648
Bg	55	0.146	0.039	0.734	**0.000**	0.123	0.027	0.779	**0.013**	0.273	0.259	0.053	0.674
Pb	50	0.166	0.104	0.377	**0.017**	0.205	0.093	0.547	**0.004**	0.297	0.280	0.059	0.797

[1] Details of the *O. viverrini* sampling localities are given in Table 1.

[2] Details of the species of cyprinid fish are given in Materials and Methods.

Table 6 Pairwise F_{ST} values and correlations with geographical distances of *Opisthorchis viverrini* from six different localities, different years and four different fish hosts in Lao PDR at three loci.

Pairwise comparison	F_{ST}	*P*-value	Distance (km)	
			Straight line	Along river
Localities[1]				
VV-TH	0.0000	0.5970	16.53	33.06
VV-NG	0.0000	0.9224	44.35	110.09
VV-TL	0.0000	0.7766	106.49	223.16
VV-PK	0.0106	0.1955	107.77	224.54
VV-NK	0.0000	0.8570	110.25	226.07
TH-NG	0.0000	0.6064	28.20	91.72
TH-TL	0.0024	0.2479	90.53	204.37
TH-PK	0.0162	0.1441	91.72	205.70
TH-NK	0.0010	0.3709	94.00	207.36
NG-TL	0.0000	0.6947	63.31	120.26
NG-PK	0.0197	0.2302	63.49	121.80
NG-NK	0.0000	0.7780	65.87	126.70
VTTL-PK	0.0000	0.8019	1.51	2.13
VTTL-NK	0.0000	0.9816	6.47	6.84
VTPK-NK	0.0000	0.5772	6.83	8.95
Year				
2008-2009	0.0020	0.2867	-	-
2008-2010	0.0061	0.1504	-	-
2009-2010	0.0022	0.2408	-	-
Type of fish[2]				
Ca-Hs	0.0000	0.8006	-	-
Ca-Bg	0.0000	0.8104	-	-
Ca-Pb	0.0000	0.4308	-	-
Hs-Bg	0.0098	0.2550	-	-
Hs-Pb	0.0055	0.2937	-	-
Bg-Pb	0.0000	0.5848	-	-

[1] Details of the *O. viverrini* sampling localities are given in Table 1.

[2] Details of the species of cyprinid fish are given in Materials and Methods.

Fig. 1 Map of sampling localities of *Opisthorchis viverrini* from six localities in Nam Ngum River wetland, Lao PDR. Details of the *O. viverrini* sampling localities are given in Table 1