Preliminary genetic evidence of two different populations of Opisthorchis viverrini *in Lao PDR*

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Abstract

Opisthorchis viverrini is a major public health concern in Southeast Asia. Various reports have suggested that this parasite may represent a species complex, with genetic structure in the region perhaps being dictated by geographical factors and different species of intermediate hosts. We used four microsatellite loci to analyze *O. viverrini* adult worms originating from six species of cyprinid fish in Thailand and Lao PDR. Two distinct *O. viverrini* populations were observed. In Ban Phai, Thailand, only one subgroup occurred, hosted by two different fish species. Both subgroups occurred in fish from That Luang, Lao PDR, but were represented to very different degrees among the fish hosts there. Our data suggest that, although geographical separation is more important than fish host specificity in influencing genetic structure, it is possible that two species of *Opisthorchis*, with little interbreeding, are present near Vientiane in Lao PDR.

Keywords Microsatellite DNA, *Opisthorchis viverrini*, Population genetics, Host factors, Cyprinid fish

Introduction

Opisthorchis viverrini is one of the most important food-borne trematodes (Sithithaworn et al. 2014). It is endemic in several Southeast Asian countries. The highest prevalence is seen in Thailand (eight million people infected) followed by the Lao People's Democratic Republic (Lao PDR) (two million people infected) (Andrews et al. 2008; Sithithaworn et al. 2012). The life-cycle involves freshwater *Bithynia* snail species as the first intermediate hosts, cyprinid fish as second intermediate hosts and humans as definitive hosts. Cats and dogs act as reservoir hosts (Saijuntha et al. 2014). Humans are exposed by eating raw or partially cooked fish infected with viable metacercariae of *O. viverrini* (Grundy-Warr et al. 2012; Sithithaworn and Haswell-Elkins 2003). Early human infection is commonly asymptomatic but chronic infection can lead to hepatobiliary disease and subsequent cholangiocarcinoma (CCA) (Chamadol et al. 2014; Sithithaworn et al. 2014). CCA has a very poor prognosis with death typically occurring within a few months of diagnosis (Sripa et al. 2007). Based on human and experimental studies, *O. viverrini* is classified as a group 1 carcinogen along with *Clonorchis sinensis* and *Schistosoma haematobium* (IARC 2012).

Studies using multilocus enzyme electrophoresis (MEE) have shown that *O. viverrini* is a species-complex comprising of at least two cryptic sibling species, one in Thailand and the other in Lao PDR, with subgroups associated with major river wetlands in those countries (Kiatsopit et al. 2011; Saijuntha et al. 2007). Additionally, independent biological evidence has revealed significant differences in body size, fecundity and infectivity of *O. viverrini* that occur in different wetlands in Thailand and 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. A high level of genetic diversity, population-genetic differentiation between geographical regions and frequent deviations from Hardy-Weinberg equilibrium were also found using microsatellite markers (Laoprom et al. 2010). Suggestions put forward to explain these findings involve various factors such as geography, influence of different species of first and second intermediate hosts, founder effects and mating system of the parasite (Criscione et al. 2005; Prugnolle et al. 2005) .

Further work using MEE of individual worms from naturally infected fish found no population-genetic differentiation of parasites among four species of fish at one locality in Thailand, nor between years (n = 4) in one of these fish species (Saijuntha et al. 2009). A very similar study in Vientiane Province, Lao PDR reached similar conclusions (Kiatsopit et al. 2014). However, numerous species of cyprinid fish can act as second intermediate hosts for *O. viverrini*, and there has been no subsequent study, using more sensitive markers such

as microsatellites, to confirm these observations. The original intention of this study was to use microsatellite markers to test the hypothesis that different fish species affect the population genetics of *O. viverrini* in endemic areas across Thailand and Lao PDR. However, the data indicated something unexpected, which is now the focus of this paper: that two distinct genetic groups of *O. viverrini* occur in Vientiane Province, Lao PDR, and one of these is the one found in Khon Kaen, Thailand.

Materials and methods

Parasite samples

Six species of cyprinid fish known to act as second intermediate hosts of *O. viverrini* were sampled for this study during the peak transmission season between December 2012 and February 2013. Four species, *Cyclocheilichthys armatus*, *Henicorhynchus siamensis*, *Barbonymus gonionotus* and *Puntius brevis* were from That Luang Lake, Vientiane, Lao PDR, and two species, *Cyclocheilichthys apogon* and *Hampala dispar*, were from Kang Lawa Lake, Ban Phai, Khon Kaen, Thailand (Fig. 1). That Luang Lake is a part of the Nam Ngum River wetland (Kiatsopit et al. 2014) and is located approximately 240 km from Kang Lawa Lake of the Chi River wetland (Saijuntha et al. 2007).

The fish were caught by local fishermen in nets left overnight at several locations in the lakes. Between 48 and 587 fish were sampled depending on species. The fish samples were kept in ice and brought to the laboratory for determination of metacercarial infection. Screening for *O. viverrini* infection and determination of prevalence and intensity of infection in each species of fish were accomplished using pepsin digestion of a subsample (15−20 randomly selected fish) of specimens as previously described (Sithithaworn et al. 1997). For each fish species, prevalence and intensity of *O. viverrini* infection were determined using these randomly selected individuals (Pitaksakulrat et al. 2013) (Table 1).

Since the metacercariae did not have a sufficient quantity of DNA, adult worms were used for microsatellite analysis (Laoprom et al. 2012). The *O. viverrini* metacercariae were carefully sorted and removed from the residue of fish tissue after pepsin digestion, identified, counted, pooled by host fish species, and fed to 3−5 hamsters/fish species via gastric intubation (50 metacercariae/animal). This yielded sufficient adult worms for analysis, but not so many as to injure the hamsters: all animal procedures were classified as ‘mild’. The hamsters were maintained in group-cages with food and water *ad lib*. Four months after infection, when the worms were fully developed, the hamsters were humanely sacrificed using a standard euthanasia protocol approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 74/2555). After the hamsters were killed, the livers were dissected to recover adult worms from within the biliary system. The worms were

washed three times with sterile 0.85% NaCl, pooled per fish species and subsequently frozen at −80 °C until used.

Preparation of genomic DNA

Adult worms for analysis (about 30 per fish species) were randomly selected from those recovered from hamsters. Genomic DNA (gDNA) was extracted from individual worms using the DNeasy blood and tissue kit (QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturer's instructions. The gDNA, eluted in a total of 100 µl elution buffer, was then used as a template for PCR.

Microsatellite genotyping

Four loci (Ovms1, Ovms6, Ovms10 and Ovms15-Table 2) from Laoprom et al. (2010) were selected based on their known polymorphism, and DNA was amplified using previously described methods (Schuelke 2000). We used three primers per reaction, a sequence-specific forward primer with M13 (−21) sequence tail, a sequence-specific reverse primer and a fluorescently labeled M13 (−21) primer (HEX and NED, Applied Biosystems) to avoid the requirement for individual dye-labeling of each set of primers. All loci were individually amplified in 25 µl reactions containing 1 µl of template DNA (approximately 20−30 ng), 0.5 µM of each primer and 0.125 µM of forward primer with an M13 tail in 2.5 µl of PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl, 2 mM Mg^{2+}), 0.25 mM each deoxynucleoside triphosphate, 0.06 U Taq DNA polymerase (Intron Biotechnology Inc.). PCR conditions were an initial denaturing step at 94 °C for 1 min, followed by 29 cycles (94 °C 1 min, 55 °C 1 min, 72 °C 3 min), then by 8 cycles (94 °C 30 s, 53 °C 45 s, 72 °C 45 s), and a final extension at 72 °C for 10 min. PCR products were run on an Applied Biosystems Genetic Analyzer and loci analyzed using the GeneMapper® version 4.0 analysis software

Data analysis

The number of alleles per locus and the observed and expected heterozygosity were calculated (Nei 1987). Each microsatellite locus, both by fish host and overall, was examined for departure from the Hardy–Weinberg equilibrium (HWE) using the exact test (Rousset and Raymond 1995). F_{IS} statistics (Wright 1978) were calculated to assess whether deviations from HWE were due to deficient or excessive heterozygosity. Genetic differentiation between populations (defined by fish host-species) was determined using F_{ST} statistics (Weir and Cockerham 1984). All analyses were performed using Genepop Version 3.4 software (Rousset and Raymond 1995) and GDA version 1.0 (Lewis and Zaykin 2001).

The life-cycle of trematodes includes a phase of asexual reproduction in the snail host, yielding many identical or near-identical cercariae. Several sibling cercariae may enter the same individual fish and become genetically identical metacercariae. The discovery of worms with identical genotypes might indicate that clonal siblings had been sampled. For analysis, only a single representative of each clone should be included. Identical genotypes may also occur in unrelated individuals purely by chance. Given that we only had available four microsatellite loci, the possibility of chance identity might be quite high. GenAlEx v6.5 (Peakall and Smouse 2012) was used to look for identical genotypes and to assess the probability of unrelated individual worms sharing the same genotype.

Assignment tests are an excellent way to assess whether discrete genetic clusters occur. Two approaches were used. GENALEX 6.5 was used to create principal coordinate analysis (PCoA) for all populations using the covariance-standardized method. This multivariate technique uses distance estimates (Nei et al. 1983) and F_{ST} to discover patterns of genetic variation in multiple samples across loci, where patterns are proportioned to different axes based on their variation. Groups who share similar genetic patterns will thus group more or less together along the axes. The first axis has the highest explanatory power, with successive axes explaining proportionally less. The second was the Bayesian approach implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000). This analysis uses a model-based clustering algorithm that identifies subgroups with distinctive allele frequencies and places individuals into *K* series or clusters (where *K* must be specified by the user *a priori*). Identifying the true value of *K* is not a trivial task (Breunig et al. 2000; Papadimitriou et al. 2003). Ten replicates were run for each value of *K* from 1 to 10. The locprior model was used. The first 100,000 steps were discarded as burn in, and a further 10^6 steps run thereafter. Results were analyzed using Structure Harvester (Earl and Vonholdt 2012) and Clumpak (Jakobsson and Rosenberg 2007). The method of Evanno et al. (2005) was used to find the best-supported value of *K*.

Results

Prevalences and intensities of infection with *O. viverrini* in each fish species are given in Table 1. Both were highest in *C. armatus* from Lao PDR and lowest in *H. siamensis*, also from Lao PDR. Within the worms sampled from *C. armatus*, there were three pairs of identical genotypes. One example of each was removed prior to further analysis. In two cases, genotypes were shared between a fish from Thailand and a fish from Lao PDR. Both pairs were left in the analyses. One genotype was shared between a worm from *C. armatus* and one from *H. siamensis*, both from Lao PDR. Again, both were left in the analyses.

Allele frequencies of *O. viverrini* by locus and host are shown in Table 3. The allele distribution patterns at the four polymorphic microsatellite loci varied greatly among worms from the six different species of fish. Analyses in Structure and Structure Harvester using the Evanno method, suggest that the optimal value of K is two (Fig. 2). A bar-plot of the data based on $K = 2$ is shown in Fig. 3. The two subpopulations seem to be strongly differentiated. Assignment tests in GenAlEx (Table 4) always failed to assign more than 50% of individuals back to the fish-host of origin. With one exception, all worms from the two Thai fish hosts were assigned back to one of the Thai hosts, or to Pb from Lao PDR. The majority of worms from the remaining three Lao fish hosts were assigned to one of these hosts, and a minority to the Thai fish hosts or to Pb.

The assignment tests indicate two different subpopulations (here termed A and B) of *O. viverrini* in Vientiane Province, Lao PDR, only one of which (A) occurs at Ban Phai, Thailand, some 240 km distant. The two subpopulations are not equally represented in fish species in Lao PDR. In three species, subpopulation B is numerically dominant, but in one, only A is represented. A common measure of population differentiation, F_{ST}, found no significant difference between two subpopulations in Thailand and one in Laos.

Significant departures from HWE due to homozygote excess were seen in the worms from *C. armatus*, (at loci Ovms10 and 15), *H. siamensis* (loci Ovms6 and 15) and *B. gonionotus* (loci Ovms1, 6 and 15), *P. brevis* (locus Ovms15), *C. apogon* (locus Ovms15) and *H. dispar* (locus Ovms10) (Table 5). Significant departure due to heterozygote excess was found for *O. viverrini* from *C. armatus* (loci Ovms1 and 6), *H. siamensis* (locus Ovms10) and *B. gonionotus* (locus Ovms10). Across the data set, estimates of F_{IS} showed heterozygote deficiency in 17 cases and heterozygote excess in 7 cases. Heterozygote deficiency is less apparent in worms from the three fish species harbouring only subpopulation A.

Significant genetic differentiation (pairwise F_{ST} values) was observed in *O. viverrini* between four fish species ($p < 0.05$) (Table 6) with the two Thai samples significantly different to three of the four Lao PDR samples, but not significantly different to *P. brevis* or to each other.

Discussion

The main findings are the apparent presence of two strongly divergent subpopulations (A and B) in Lao PDR (only one of which – A – occurred in Thailand), and a tendency towards heterozygote deficiency, especially in three of the Lao hosts. In addition, there appeared to be no significant restriction to gene flow between Pb in Lao PDR and the two fish species from Ban Phai in Thailand (Table 6). This latter point suggests that geography alone is not an explanation for the findings: different fish species on opposite sides of the Mekong contained the same subpopulation of worms. Nor is it clear that different fish hosts may be more or less susceptible to

different subpopulations of *O. viverrini*, unless the differences in proportions of these subpopulations between *Ca*, *Hs* and *Bg* on one hand and *Pb* on the other, can be taken as evidence for this. The heterozygote deficiency, more marked among worms from Lao fish hosts than in Thai ones, may be evidence of a Wahlund effect. This is seen when data from at least two different, non-interbreeding, (sub) populations are mistakenly analyzed under the assumption that a single population is present. The two fish species from Thailand (and *Pb* from Lao PDR) contained only members of a single subpopulation. These exhibited fewer heterozygote deficits. By contrast, a recent study on *Schistosoma japonicum* in China reported no evidence of a Wahlund effect and clonal expansion of small or fragmented population as a result of control programs may counteract heterozyote deficiency (Huo et al. 2016).

Important questions for future work are raised here. If indeed genetically and biologically different subpopulations/cryptic species of *O. viverrini* exist, this is of considerable epidemiological importance. This study was initially conceived with a different question in mind, and used only a small number of loci, locations and fish species. More systematic sampling, investigating areas thought to be inhabited by different cryptic liver fluke species (see introduction), should be undertaken. Earlier studies that raised the possibility of cryptic species assumed a single cryptic species at any given locality. It might be that two such species, with little interbreeding, are present near Vientiane in Lao PDR. This would indicate that geography alone is not a sufficient explanation for the findings.

Previous studies utilizing the same four polymorphic loci (Laoprom et al. 2010, 2012) showed that the majority of *O. viverrini* populations (60–65%) examined had significant deviations (positive F_{IS}) from HWE. Similar results were found in our study. Highly significant deviations from HWE due to *O. viverrini* homozygote excess were found in 11 cases (71%) across all species of fish and loci. We also found similar levels of heterozygote deficiency to that reported previously for spatially separated *O. viverrini* populations from Thailand and Lao PDR, and which were sampled at different times and from different fish host species (Kiatsopit et al. 2014; Saijuntha et al. 2009). Our F_{IS} results trended towards heterozygote deficiency, supporting previous studies that the predominant mode of reproduction in *O. viverrini* is selfing rather than cross-fertilization. However, with our sample sizes we cannot be definitely sure whether this is due to lack of partner, or due to positive worm-driven selfing.

Despite our parasite genetic diversity measures being potentially affected by sibling infection, our results revealed high levels of genetic differentiation of *O. viverrini* (F_{ST} ranging between 0.002–0.134) from *B. gonionotus*, *H. dispar*, *C. armatus* and *H. siamensis*, as well as high levels of polymorphism. STRUCTURE

analysis revealed two main genetic clusters, one containing *O. viverrini* from *C. armatus*, *H. siamensis* and *B. gonionotus* and the other containing *O. viverrini* from *P. brevis*, *C. apogon* and *H. dispar*. With the two fish sampling locations being from two distinct watersheds, this potential mix of parasite genotypes between these locations could be due to human as well as fish movement and an introduction of a specific genotype from one country to another. Further studies, drawing a larger sample from both study regions, would indicate whether this is likely to be a founder effect from Lao PDR to Thailand, or a new introduction from Thailand into Lao PDR.

Host preference has been reported in other trematodes, for example, for *S. japonicum* similarly high levels of polymorphisms were detected identifying two main genetic clusters, one in water buffalo, cattle and humans and the other in goats, pigs, dogs and cats (Wang et al. 2006). *O viverrini* has three hosts: the snail intermediate host, the fish intermediate hosts and the definitive mammalian hosts, and host-parasite compatibility at each of these life stages may play significant roles in the population genetics of *O. viverrini*. For example, the first intermediate snail host, *Bithynia siamensis goniomphalos*, has recently been shown to consist of a species complex of at least 11 cryptic species that occur in the same wetlands as the cryptic species of *O. viverrini* in Thailand and Lao PDR (Kiatsopit et al. 2013; Saijuntha et al. 2007). In particular, self-fertilization usually occurs in *O. viverrini* because of a low parasite burden in an infected definitive host, including humans (Gorton et al. 2012). This is likely to influence and enhance the complexity of the host selection process within each wetland. Since the six species of fish have distributions throughout the region, *O. viverrini* from the same species of fish in Thailand and Lao PDR could have different or the same population structure, which is a key limitation of this study and remains to be determined in future work (e.g. *O. viverrini* in *P. brevis* from Lao PDR compared with *O. viverrini* in *P. brevis* from Thailand).

In this study, the use of adult worms from experimentally infected animal may create host-selection bias. We are currently pursuing methods that will allow direct analysis of life stages such as metacercariae or cercariae, without the need for laboratory passage. Another limitation is that an existence and effect of genetic cluster as a result of clonal structure as observed in another trematode *(Lecithochirium fusiforme)* hence creating Wahlund effect (Criscione et al. 2011) was not examined. This is because adult worm analyzed were pooled from several fish of the same species. Future analysis using metacercaria directly should help to solve this limitation as well as allowing us to evaluate *O. viverrini* infrapopulations in individual fish host.

In conclusion, the main findings of this study are the presence of two divergent subpopulations of *O. viverrini* in Lao PDR and only one of which occurred in Thailand. There is a tendency towards heterozygote deficiency, particularly in three fish host species from Lao PDR which may due to Wahlund effect. The high gene flow between parasite in *Pb* in Lao PDR and the two fish species from Ban Phai in Thailand suggests that geography alone is not an explanation for the findings since the same subpopulation of worms occurred in two distant localities. Whether host factors i.e. fish compatibility, snail intermediate hosts, mammal reservoir hosts and human contribute in the occurrence of subpopulation of *O. viverrini* remain to be investigated.

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Conflict of interest The authors declare that they have no conflict of interest.

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Table 1 The details of sampling localities of six naturally infected cyprinid fish species, and prevalence and intensity of infection by *O. viverrini* metacercariae

Fish host species	Locality	Total no. of fish collected	No. used for estimating prevalence	Prevalence	Intensity[a] (Cysts/infected fish)
Cyclocheilichthys armatus (*Ca*)	That Luang, Vientiane, Lao PDR	255	20	90%	111.1 ± 112.9
Henicorhynchus siamensis (*Hs*)	That Luang, Vientiane, Lao PDR	217	20	5%	1
Barbonymus gonionotus (*Bg*)	That Luang, Vientiane, Lao PDR	587	20	10%	5 ± 0
Puntius brevis (*Pb*)	That Luang, Vientiane, Lao PDR	107	20	25%	5 ± 0
Cyclocheilichthys apogon (*Cap*)	Ban Phai, Khon Kaen, Thailand	167	15	29%	1 ± 1.27
Hampala dispar (*Hd*)	Ban Phai, Khon Kaen, Thailand	48	15	22%	2.8 ± 4.9

[a] Mean and SD of metacercariae

Table 2 Primer sequences and characteristics of *O. viverrini* sensu lato microsatellite loci

Locus name	Repeat	Primer sequence 5′–3′	*Ta*[b] (°C)
Ovms1	(GT)11	F: M13[a](−21) +GGTCTGATGCAAGTAGACATCC R: GGCACATGAACGCGCATTGGTAAG	55
Ovms6	(GT)5GA(GT)4	F: M13(-21) +TTTATGGATTCAACGGAAC R:CCCCAAGAAACCTGATTCAA	55
Ovms10	(GT)5GC(GT)8	F: M13(−21) +TTGCTTTACTGCTGTTTTTCG R: GCTTCGGTCACAGTTCCTAA	60
Ovms15	(TG)10	F: M13(-21) +GGAGGAGTTTCCCTGAAAGG R: TACGGGGTGTGCACAAATAAA	60

[a] M13(−21) sequence: 5′-TGT AAA ACG ACG GCC AGT-3′ (18 bp)

[b] PCR annealing temperature

Table 3 Allele frequencies at four loci in *O. viverrini* sensu lato from six species of cyprinid fish

Locus	Allele N (base pair length)	Host[a]					
		Lao PDR			Thailand		
		Ca (32)	*Hs* (31)	*Bg* (31)	*Pb* (30)	*Cap* (30)	*Hd* (30)
Ovms1	1 (261)	0.00	0.00	0.04	0.00	0.00	0.00
	2 (265)	0.20	0.32	0.13	0.17	0.20	0.22
	3 (267)	0.11	0.07	0.04	0.14	0.07	0.07
	4 (269)	0.36	0.35	0.46	0.31	0.28	0.22
	5 (271)	0.14	0.05	0.04	0.10	0.10	0.20
	6 (273)	0.09	0.10	0.20	0.12	0.22	0.20
	7 (275)	0.05	0.05	0.04	0.10	0.12	0.08
	8 (277)	0.05	0.07	0.04	0.05	0.02	0.02
Ovms6	1 (334)	0.00	0.00	0.00	0.02	0.00	0.00
	2 (340)	0.02	0.00	0.00	0.02	0.00	0.05
	3 (342)	0.03	0.05	0.00	0.04	0.12	0.08
	4 (344)	0.14	0.15	0.16	0.48	0.43	0.35
	5 (346)	0.23	0.27	0.34	0.18	0.31	0.20
	6 (348)	0.23	0.18	0.15	0.25	0.14	0.30
	7 (350)	0.20	0.20	0.19	0.02	0.00	0.00
	8 (352)	0.11	0.12	0.08	0.00	0.00	0.02
	9 (354)	0.02	0.02	0.05	0.00	0.00	0.00
	10 (358)	0.00	0.02	0.02	0.00	0.00	0.00
	11 (364)	0.00	0.00	0.02	0.00	0.00	0.00
	12 (366)	0.02	0.00	0.00	0.00	0.00	0.00
Ovms10	1 (143)	0.00	0.00	0.02	0.00	0.00	0.00
	2 (145)	0.06	0.15	0.11	0.00	0.00	0.00
	3 (147)	0.31	0.38	0.42	0.00	0.00	0.00
	4 (149)	0.23	0.17	0.29	0.00	0.00	0.00
	5 (153)	0.02	0.00	0.00	0.00	0.00	0.00
	6 (155)	0.03	0.00	0.00	0.03	0.07	0.02
	7 (157)	0.05	0.07	0.03	0.05	0.03	0.05
	8 (159)	0.19	0.12	0.08	0.50	0.62	0.60
	9 (161)	0.11	0.12	0.05	0.42	0.28	0.33
Ovms15	1 (172)	0.09	0.00	0.00	0.00	0.00	0.00
	2 (174)	0.28	0.33	0.48	0.37	0.47	0.27
	3 (176)	0.40	0.27	0.18	0.35	0.35	0.40
	4 (178)	0.02	0.07	0.13	0.07	0.08	0.05
	5 (180)	0.09	0.10	0.05	0.04	0.02	0.07
	6 (182)	0.03	0.02	0.04	0.02	0.02	0.13
	7 (186)	0.00	0.05	0.07	0.09	0.03	0.00
	8 (188)	0.10	0.15	0.04	0.02	0.03	0.02
	9 (190)	0.00	0.00	0.00	0.04	0.00	0.05
	10 (192)	0.00	0.02	0.02	0.00	0.00	0.02

[a] abbreviations used for cyprinid fish species are described in Table 2

Table 4 Results of assignment test in GenAlEx. The left-hand column indicates the source population of each worm (total number in parentheses). Subsequent columns show the fish host to which each was assigned by GenAlEx

	Assigned to:					
	Lao PDR			Thailand		
Source popn[a]	*Ca*	*Hs*	*Bg*	*Pb*	*Cap*	*Hd*
Ca (32)	5	8	5	5	4	2
Hs (31)	2	8	12	3	1	5
Bg (31)	3	10	14	1	2	1
Pb (30)	0	0	0	11	8	11
Cap (30)	1	0	0	8	15	6
Hd (30)	0	0	0	14	7	9

[a] Abbreviations used for cyprinid fish species are described in Table 2

Table 5 Data analyses of worms from six species of cyprinid fish for each polymorphic microsatellite locus examined at 4 microsatellite loci. H_E: expected heterozygosity; H_O: observed heterozygosity; F_{IS}: inbreeding coefficient. Tests of deviation from Hardy–Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4. *p*-values (<0.05) considered significant are bold. "*A*" indicates the number of alleles per locus per population. "A_e" indicates the allelic richness per locus per population. "*N*" indicates the number of individuals successfully typed. FSTAT output agrees well

Factors	Parameter	Locus				All loci	
Fish species[a]		Ovms1	Ovms6	Ovms10	Ovms15	Mean	SD
Ca	H_0	0.803	0.828	0.806	0.753	0.797	0.032
	H_E	0.857	0.969	0.969	0.483	0.819	0.230
	F_{IS}	-0.069	-0.173	0.207	0.363	0.164	-
	p-value	0.033	0.000	0.000	0.000	-	-
	A	7	9	8	7	7.750	0.957
	A_e	6.973	7.854	7.493	6.649	7.242	0.536
	N	28	32	32	29	-	-
Hs	H_o	0.766	0.830	0.784	0.791	0.779	0.043
	H_E	0.633	0.867	0.933	0.733	0.800	0.147
	F_{IS}	0.176	0.045	-0.194	0.074	0.034	-
	p-value	0.174	0.002	0.000	0.023	-	-
	A	7	8	6	8	7.250	0.957
	A_e	6.939	7.376	5.994	7.370	6.920	0.650
	N	30	30	30	30	-	-
Bg	H_o	0.565	0.903	0.968	0.500	0.734	0.236
	H_E	0.743	0.804	0.729	0.722	0.750	0.038
	F_{IS}	0.243	0.019	*-0.335*	0.311	0.021	-
	p-value	0.024	0.000	0.000	0.006	-	-
	A	8	8	7	8	7.750	0.500
	A_e	7.971	7.322	6.544	7.616	7.363	0.607
	N	23	31	31	28	-	-
Pb	H_o	0.831	0.683	0.582	0.735	0.708	0.104
	H_E	0.966	0.643	0.800	0.481	0.722	0.208
	F_{IS}	-0.166	0.060	0.382	0.349	0.208	-
	p-value	0.806	0.741	0.009	0.000	-	-
	A	7	7	4	8	6.500	1.732
	A_e	6.981	6.191	3.89	7.461	6.131	1.583
	N	29	28	30	27	-	-
Cap	H_o	0.867	0.667	0.567	0.300	0.600	0.236
	H_E	0.818	0.703	0.543	0.661	0.681	0.114
	F_{IS}	-0.060	0.052	0.044	0.550	0.121	-
	p-value	0.443	0.225	1	0.000	-	-
	A	7	4	4	7	5.500	1.732
	A_e	6.693	4	3.907	6.226	5.207	1.460
	N	30	21	30	30	-	-
Hd	H_o	0.828	0.750	0.535	0.754	0.717	0.126
	H_E	0.933	0.767	0.800	0.667	0.792	0.110
	F_{IS}	-0.129	0.087	0.508	0.117	0.194	-
	p-value	0.618	0.399	0.003	0.304	-	-
	A	7	6	4	8	6.250	1.708
	A_e	6.692	5.675	3.676	7.346	5.847	1.602
	N	30	30	30	30	-	-

[a] Abbreviations used for cyprinid fish species are described in Table 2

Table 6 Pairwise F_{ST} values (below diagonal) and *p*-values (above diagonal) of *O. viverrini* sensu lato from six different cyprinid fish at four loci. Significant *P-values* are in bold

N	Host[a]	*Ca*	*Hs*	*Bg*	*Pb*	*Cap*	*Hd*
32	*Ca*		0.475	**0.009**	**<0.001**	**<0.001**	**<0.001**
31	*Hs*	0.000		0.467	**<0.001**	**<0.001**	**<0.001**
31	*Bg*	0.017	0.005		**<0.001**	**<0.001**	**<0.001**
30	*Pb*	0.075	0.088	0.122		0.554	0.287
30	*Cap*	0.082	0.093	0.119	0.000		0.140
30	*Hd*	0.072	0.093	0.134	0.004	0.006	

[a] Abbreviations used for cyprinid fish species are described in Table 2.

Figure

Fig.1 The localities in Thailand and the Lao PDR where cyprinid fish were collected for determination of *Opisthorchis viverrini*

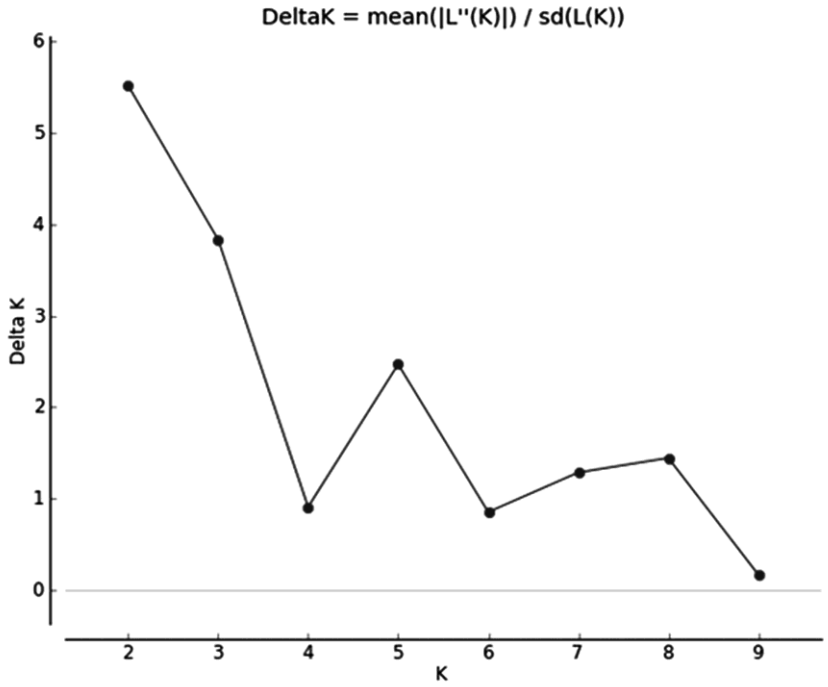

Fig. 2 Analyses of the optimal value of *K* in Structure from Structure Harvester using the Evanno method to indicate that two is the optimal value of *K*

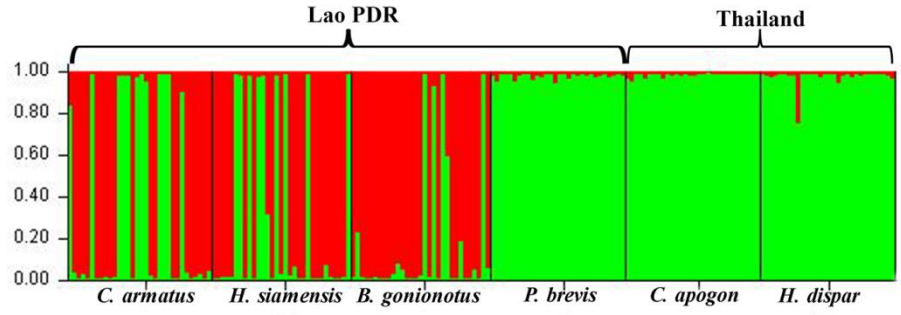

Fig. 3 Structure model-based clustering showing segregation of *Opisthorchis viverrini* samples into two main subgroups (*K* = 2). In Ban Phai, Thailand, only one subgroup occurred, hosted by *C. apogon* and *H. dispar*. Both subgroups occurred in fish from That Luang, Lao PDR, but were represented to very different degrees among the fish hosts there