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Genetic variation at three enzyme loci within a Thailand population of *Opisthorchis viverrini*

Weerachai Saijuntha

Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand

Paiboon Sithithaworn

Department of Parasitology and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Sopit Wongkham

Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Thewarach Laha

Department of Parasitology and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Rojchai Satrawaha

Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand

Neil B. Chilton

Department of Biology, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E2

Trevor N. Petney

Institute of Zoology 1: Ecology and Parasitology, University of Karlsruhe, Kornblumen Strasse 13, Karlsruhe, Germany

Ross H. Andrews

School of Pharmacy and Medical Sciences, University of South Australia, GPO Box 2471, Adelaide, SA 5001, Australia

Abstract

Genetic variation among 231 individuals of *Opisthorchis viverrini* from the Ban Phai District, Khon Kaen Province (Thailand) was examined at three polymorphic enzymes: enolase (ENOL), phosphoglucosmutase (PGM), and triose phosphate isomerase (TPI). Four alleles were detected for TPI and PGM, whereas only two alleles were detected for ENOL. The inferred genotype frequencies for both TPI and ENOL were not significantly different from Hardy–Weinberg equilibrium. In contrast, the inferred genotype frequencies for PGM showed a significant

departure from Hardy–Weinberg equilibrium, with a lack of heterozygous individuals. This heterozygote deficiency suggests non-random mating and/or potentially high self fertilization.

Introduction

The food-borne trematode, *Opisthorchis viverrini* sensu lato, is a medically important parasite endemic in Southeast Asian countries, including Thailand, Lao PDR, Cambodia, and Vietnam. An estimated nine million people are infected with *O. viverrini*, a majority of which live in Thailand (WHO 1995). Endemic areas of opisthorchiasis in Thailand are mainly in the northeast and north, but the parasite is absent in the south (Sithithaworn and Haswell-Elkins 2003). *O. viverrini* is also classified as a type 1 carcinogen because of its role as an initiator of chronic inflammation and the subsequent development of cholangiocarcinoma. Understanding the genetic variability within and among populations of *O. viverrini* is of importance with respect to public health in Southeast Asia.

Analysis of the genetic variability and population genetic structure of parasitic trematodes is now widely reported (e.g., Andrews and Chilton 1999; Gasser 2001; Vilas et al. 2004; Saijuntha et al. 2007). In general, the population genetic structure of digenetic trematodes appears to depend on a number of factors including the relative mobility of different hosts, the non-random distribution of parasites, the geographical and ecological characteristics of the area of transmission, and the reproductive structure of the population (Chilton et al. 1999; Park et al. 2000; Sire et al. 2001; Criscione and Blouin 2004; Saijuntha et al. 2007). However, most population genetic studies on trematodes have been carried out on species that utilize terrestrial hosts. Hence, there is only limited information about population genetics and spatial distribution of food-borne trematodes, which includes *O. viverrini*.

Multilocus enzyme electrophoresis (MEE) has been used very effectively over many years to study the population genetics and systematics of a variety of parasitic helminths (see review by Andrews and Chilton 1999), including *O. viverrini*. For example, Saijuntha et al. (2006b) established 33 enzyme loci to genetically compare *O. viverrini* from different populations. Of these, 17 loci were polymorphic in pooled samples of *O. viverrini* (Saijuntha et al. 2006b). Subsequently, the genetic variation detected among different populations was indicative that *O. viverrini* represented a species complex, consisting of at least two sibling species, one in Thailand, the other in Lao PDR (Saijuntha et al. 2007). However, most MEE studies on *O. viverrini* have been conducted on analyses of pooled samples of adult worms. The aim of the present study was to explore the level of genetic variation (i.e., allele and genotype frequencies) within a single population of one species of *O. viverrini* sensu lato based on MEE analyses of individual adult worms at three polymorphic enzyme loci.

Materials and methods

Metacercariae of *O. viverrini* were obtained from infected cyprinid fish-collected from Kang Lawa Reservoir in the Ban Phai District of Khon Kaen Province (Thailand). To ensure a high recovery of metacercariae, several hundred freshwater cyprinid fish, from five species, *Puntius orphoides*, *Cyclocheilichthys armatus*, *Puntioplites protozoon*, *Hampala dispar*, and *Cirrhina jullien*, were pooled and their combined tissues digested with 0.3% pepsin solution. The metacercariae were pooled from the different fish species because it was determined previously (Sithithaworn unpublished) that there is no evidence of cryptic species of the parasite within the Kang Lawa Reservoir. Metacercariae were identified using a light microscope, 50–100 meta-cercariae were used to infect hamsters. After 4–6 months, adult worms were collected from the bile duct and identified using morphological characters (Kaewkes 2003). Adult *O. viverrini* were then washed extensively in physiological saline,

placed individually into separate microcentrifuge tubes, frozen live, and stored at -80°C for subsequent MEE analyses.

Frozen homogenates of *O. viverrini* were prepared for electrophoretic analyses by placing an individual worm into each well of U-plate, adding 5 μl of lysing solution (100 ml distilled water, 100 μl β -mercaptoethanol, 10 mg nicotinamide adenine dinucleotide phosphate) to the thawed sample and grinding using a grinder tube. Supernatants were removed directly by loading pen from each well and loaded onto the gel. MEE was conducted on 231 individual *O. viverrini* using cellulose acetate (Cellologel; Milan) as the support medium. Each gel was stained histochemically (Richardson et al. 1986) for three enzymes that were shown previously to be polymorphic (i.e., more than one electromorph; Saijuntha et al. 2006b, 2007). These three enzymes and their Enzyme Commission (EC) numbers were: enolase (ENOL, EC 4.2.1.11), phosphoglucosmutase (PGM, EC 2.7.5.1), and triose phosphate isomerase (TPI, EC 5.3.1.1). The banding patterns of samples at each enzyme locus were interpreted allozymically (Andrews and Chilton 1999), with allele “a” designated as the allele migrating the least distance from the cathode.

The GENEPOP population genetics software version 3.2 (Raymond and Rousset 1995) was used to calculate allele frequencies, genotype frequencies, and fixation index within subpopulations (F_{IS}) and to test compliance with Hardy–Weinberg equilibrium (HWE) expectations in the sample populations using Fisher's exact test. Estimation without bias of exact p values was made using the Markov-chain method, following the algorithm of Guo and Thompson (1992). Values of F_{IS} range from -1 to 1 ; with negative values indicating an excess of heterozygotes, positive values indicating heterozygote deficiency compared with values predicted under the HWE.

Results and discussion

For each enzyme (ENOL, PGM, and TPI), a single locus (i.e., *Enol*, *Pgm*, and *Tpi*) was observed among parasite samples (Figs. 1, 2, and 3). Genetic variation was detected among individual *O. viverrini* adults for all three enzyme loci. Relatively high levels of allelic variability were detected among individual worms from the Ban Phai population, with four alleles detected for *Tpi* and *Pgm* and two alleles for *Enol*. The electrophoretic banding patterns were consistent with a dimeric quaternary structure for both TPI and ENOL; that is, those individuals heterozygous at either or both of these two loci had a triple-banded pattern. In the case of PGM, heterozygous individuals had a double-banded pattern consistent with a monomeric quaternary structure for the enzyme.

The allele frequencies of *O. viverrini* at each enzyme locus are shown in Table 1. For *Tpi*, there were four alleles. This differs to previous studies (Saijuntha et al. 2006a, 2006b, 2007) where only three alleles for this locus were detected. However, in the present study, allele *d* was detected in only one individual (=allele frequency of 0.2%) with the genotype *ad* (Fig. 1). The extremely low frequency of allele *d* may account for the inability to detect it within samples of 20 individual worms or pooled samples of 20 individuals (Saijuntha et al. 2006b, 2007). This finding highlights the need for population-based studies (as opposed to taxonomic studies) to genetically compare a large number of individuals in order to decrease the probability that rare alleles are not overlooked.

For *Enol*, two alleles (*a* and *b*) were detected in the samples (Fig. 3), but they occurred at different frequencies (87% and 13%, respectively; Table 1). Twenty-one percent of the individuals were heterozygous for *Enol*. The observed frequencies for the three genotypes were in HWE.

Four alleles for *Pgm* were detected among the 231 individual worms, whereas only three alleles for this locus were reported in previous studies (Saijuntha et al. 2006b, 2007). However, in this case, this discrepancy in the number of alleles between studies may relate to differences in the electrophoretic mobilities on gels. In the present study, there was very limited separation between alleles *a* and *b* (Fig. 2); hence, allele *b* may not have been distinguished from allele *a* in previous studies (e.g., Saijuntha et al. 2007). Nonetheless, allele *b* occurred at a relatively low frequency (5%) compared to the other three alleles (Table 1). The frequencies of alleles *c* and *d* were also low (<10%); hence, a low level of genotype frequencies of these alleles (0–3%) was detected. It is interesting to note that no individuals with the genotype *bc* were detected (Table 1). More samples need to be examined electrophoretically to detect individuals with this genotype, as sample size depends on the level of allele frequencies (Richardson et al. 1986).

A population of *O. viverrini* from Kang Lawa Reservoir showed a significant heterozygous deficiency for *Pgm* locus, but not for the other two loci (Table 1). A lower than expected number of heterozygous individuals at the *Pgm* locus may relate to the mating behavior of this parasite. *O. viverrini* is hermaphroditic, but it can self-fertilize and/or cross-fertilize (Trouvé et al. 1996; Vilas et al. 2004) depending upon the presence of conspecifics. It is known that this parasite species has an uneven (clumped) distribution in its definitive host (Sithithaworn et al. 1991), which may result in unequal probabilities of individual adults to cross fertilize (i.e., low cross-fertilization in persons with a low worm burden). Deviation from HWE may also indicate the existence of two or more subpopulations that are reproductively isolated spatially and/or temporally or that the sample included representatives of more than one species occurring in sympatry (i.e., presence of a cryptic species). The latter explanation is highly unlikely, as there was neither a total absence of heterozygous individuals for *Pgm*, nor any other evidence of fixed genetic differences at any other enzyme loci among *O. viverrini* samples collected in sympatry (see Saijuntha et al. 2006b). Thus, it is more likely that the reduction of heterozygosity in this *O. viverrini* population may be the consequence of population substructuring (i.e., a Wahlund effect). In such a case, two or more subpopulations would be present, each with different allele frequencies (but each of their genotype frequencies may be in HWE). Although the samples used in the present study were collected from the same reservoir, they were derived from several species of fish. Different fish species may act as selective agents against some *O. viverrini* genotypes (at the cercarial/metacercarial stage). This would be important if the definitive host individuals (e.g., humans) preferentially selected different fish species in their diets. However, this hypothesis needs to be explored in further detail.

The significant heterozygote deficiency in *O. viverrini* from Kang Lawa Reservoir needs to be explored further, in particular, whether similar deficiencies occur at other polymorphic loci in this population. In addition, detailed studies should be conducted on a large number of individuals from other populations, including those of a putative cryptic species in Laos, to determine if heterozygote deficiencies are of a common occurrence. This may provide further insight into the process of genetic divergence within and among geographically isolated populations of *O. viverrini*. Future studies should also consider the potential influences of worm burden, temporal and spatial variation of the parasite, and the species and subspecies of intermediate host (both snails and fish) on the population genetic structure of *O. viverrini* sensu lato in Southeast Asia using several polymorphic loci, including *Enol*, *Pgm*, and *Tpi*.

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Fig. 1. Examples of electrophoretic patterns obtained for TPI in *O. viverrini*. The resulting genotypes were homozygous aa (lane 13), bb (lanes 4, 5, and 11), and cc (lane 5) and heterozygous of ab (lanes 2, 3, 9, 10, 12, and 14), ac (lanes 1 and 8), and ad (lane 7)

Fig. 2. Examples of electrophoretic patterns obtained for PGM in *O. viverrini*. The resulting genotypes were homozygous aa (lanes 1, 3–5, 8, 9, 11, 13, and 15) and heterozygous of bd (lane 2), ac (lanes 6, 7, and 12), and ab (lanes 10 and 14)

Fig. 3. Examples of electrophoretic patterns obtained for ENOL in *O. viverrini*. The resulting genotypes were homozygous aa (lanes 1, 3, 6–14, and 16) and bb (lane 2) and heterozygous of ab (lanes 4, 5, and 15)

Table 1

The allele and genotype frequencies and number of heterozygous individuals within a population ($n=231$ individual adults) of *O. viverrini* for three polymorphic enzyme loci

Loci	Allele frequencies	Genotype frequencies	Number of individuals	Observed heterozygote	F_{IS}
<i>Tpi</i>	<i>a</i> =0.2922	<i>aa</i> =0.082 (0.085)	9 (19.620)	126 (133.689)	0.036
	<i>b</i> =0.5628	<i>ab</i> =0.316 (0.330)	73 (76.139)		
	<i>c</i> =0.1429	<i>ac</i> =0.100 (0.084)	23 (19.328)		
	<i>d</i> =0.0022	<i>ad</i> =0.004 (0.001)	1 (0.293)		
		<i>bb</i> =0.342 (0.316)	79 (73.037)		
		<i>bc</i> =0.126 (0.161)	29 (37.223)		
		<i>bd</i> =0.000 (0.002)	0 (0.564)		
		<i>cc</i> =0.034 (0.020)	7 (4.653)		
<i>Pgm</i>		<i>cd</i> =0.000 (0.001)	0 (0.143)		
		<i>dd</i> =0.000 (0.000)	0 (0.000)		
	<i>a</i> =0.7597	<i>aa</i> =0.619 (0.577)	143 (133.243)	73 (93.095)	0.216*
	<i>b</i> =0.0498	<i>ab</i> =0.065 (0.076)	15 (17.512)		
	<i>c</i> =0.0996	<i>ac</i> =0.121 (0.152)	28 (35.024)		
	<i>d</i> =0.0909	<i>ad</i> =0.095 (0.138)	22 (31.978)		
		<i>bb</i> =0.009 (0.002)	2 (0.549)		
		<i>bc</i> =0.000 (0.010)	0 (2.295)		
<i>Enol</i>		<i>bd</i> =0.017 (0.009)	4 (2.095)		
		<i>cc</i> =0.030 (0.010)	7 (2.245)		
		<i>cd</i> =0.017 (0.018)	4 (4.191)		
		<i>dd</i> =0.026 (0.008)	6 (1.868)		
	<i>a</i> =0.8723	<i>aa</i> =0.762 (0.761)	176 (175.711)	51 (51.577)	0.002
	<i>b</i> =0.1277	<i>ab</i> =0.221 (0.223)	51 (51.577)		
		<i>bb</i> =0.017 (0.016)	4 (3.711)		

The expected values of each parameter assuming HWE are indicated in parentheses

* $P=0.0000$