ALLOZYME ANALYSIS OF THE TEMPORAL POPULATIONS OF *ECHINOSTOMA REVOLUTUM* COLLECTED FROM DOMESTIC DUCKS IN KHON KAEN PROVINCE, THAILAND

Weerachai Saijuntha[1], Paiboon Sithithaworn[2], Chairat Tantrawatpan[3], Sumonta Tapdara[4] and Ross H Andrews[2]

[1]Walai Rukhavej Botanical Research Institute (WRBRI), Mahasarakham University, Maha Sarakham; [2]Department of Parasitology, Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen; [3]Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathum Thani; [4]Clinical Pathology Laboratory, Amnatcharoen Hospital, Amnat Charoen, Thailand

Abstract. Four temporal populations of *Echinostoma revolutum* (ER1, ER2, ER3, ER4) were collected from domestic ducks in Khon Kaen Province, Thailand during February - October 2008. The ER1, ER2, ER3 and ER4 were collected in February, April, June and October, respectively. The 12 enzymes encoding 15 loci were examined. Two loci were found in each of 3 enzymes, namely glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME) and peptidase valine-leucine (PEPA). Of these, three loci, namely, *G6pd-1*, *Me-1* and *PepA-2*, were polymorphic. Genotypes were assigned for the specific allelic profiles detected at these three polymorphic loci. Twenty-eight genotypes were observed in the 4 temporal populations of *E. revolutum*. Three genotypes, Er_{22}, Er_{23} and Er_{25}, were found in all populations. The Er_6 genotype occurred had the highest frequency of all the populations. These 28 genotypes were clustered into 3 groups with genetic differences of 2-12% among the loci. A cluster of genotypes (Er_1, Er_3, Er_9 and Er_{12}) showed the greatest genetic difference among the genotypes (12% difference). These results show intraspecific variation exists in *E. revolutum* populations in domestic ducks from Khon Kaen Province, Thailand.

Keywords: *Echinostoma revolutum*, allozyme, temporal population, domestic duck, Thailand

INTRODUCTION

The echinostomatidae trematodes are foodborne, zoonotic and intestinal parasites. Human echinostomiasis, is attributed to at least 16 species and is endemic in Southeast Asia and the Far East (Chai, 2009). The number of collar spines that define a particular genus varies from 21 to 51. The largest genus is *Echinostoma*; *Echinostoma revolutum* complex consists of a number of 37-collar-spined species of

Correspondence: Weerachai Saijuntha, Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham 44150, Thailand.
Tel: +66 (0) 4375 4407; Fax: +66 (0) 4375 4407
E-mail: weerachai.s@msu.ac.th

Echinostoma or the "revolutum" group (Fried and Graczyk, 2004). Nine species of this group are recognized: *Echinostoma caproni*, *E. trivolvis*, *E. paraensei*, *E. revolutum*, *E. friedi*, *E. miyagawai*, *E. echinatum*, *E. parvocirrus*, and *E. jurini* (Fried and Graczyk, 2004). These echinostomes infect various species of snails, such as lymnaeid and planorbid snails, as first and second intermediate hosts and several avian and mammalian species as definitive hosts (Kanev, 1994). Of the 37-collar-spined species, a European-Asian species, referred to as *E. revolutum,* infects lymnaeid as their first intermediate host, various pulmonate and prosobranch snails, mussels, tadpoles and freshwater turtles as second intermediate hosts, and only avians as their definitive hosts (Fried and Graczyk, 2004).

The life cycle of *E. revolutum* is as follows: eggs are passed with host feces into the water. Embryonation depends on environmental temperature, and takes about 2-3 weeks at 22ºC to reach the fully developed miracidial stage. The miracidia hatch, seek out and penetrate their snail hosts. Miracidia transform into sporocysts, radiae and cercariae, which either encyst in the original host snail or emerge to seek one of several different species of snails or tadpoles as second intermediate hosts. Cercariae begin to emerge from infected snails at 4-6 weeks post-infection. Definitive host infection occurs when the intermediate hosts are eaten and metacercarial cysts excyst in the small intestine. The juvenile worms mature in 6-8 days when egg production begins and continues until the worms are expelled (Huffman and Fried, 1990). Interestingly, the life span of *E. revolutum* in duckling hosts is relatively short, with adult worms usually expelled a few weeks post-infection (Davis, 2005). This may affect the population dynamics of *E. revolutum* in duck hosts, which fluctuate over short periods (*ie,* a few months). Determining genetic patterns (genotypes) of *E. revolutum* populations over time (temporal) may provide evidence for fluctuations in genotype frequencies. For example, in the liver fluke *Opisthorchis viverrini* an allozyme electrophoresis study by Saijuntha *et al* (2009a) showed the genotype frequencies of *O. viverrini* at 3 polymorphic loci fluctuated in different populations during different years.

Twenty-two allozyme markers have been used previously to differentiate *E. revolutum* from *Hypoderaeum conoideum* isolates from Khon Kaen Province, Thailand (Saijuntha *et al*, 2009b). In the present study 12 enzymes, which gave sufficient staining intensity and resolution to enable reliable genetic interpretation, were used as genetic markers to assess genetic variation among temporal populations of *E. revolutum*. Of these, only the polymorphic loci detected were used to assess temporal population variation of *E. revolutum* isolates from Khon Kaen Province, Thailand.

MATERIALS AND METHODS

Sample collection

One hundred six adult *E. revolutum* worms were collected from the intestines of infected domestic ducks from a slaughterhouse in Khon Kaen Province during February-October 2008. Four populations were collected at different times: 20 worms from the ER1 population were collected from 7 infected ducks in February, 50 worms from the ER2 population were collected from 15 ducks in April, 25 worms from the ER3 population were collected from 5 ducks in June and 11 worms from the ER4 population were collected from 4 ducks in October. These adult worms were

morphologically identified to species-level by size of the circumoral disc, testes morphology and number of collar spines (Miliotis and Bier, 2003) using a stereomicroscope. The worms were then washed in physiological saline (0.85% NaCl) and frozen for subsequent electrophoretic analysis. Enzyme homogenate from each worm was prepared as described by Saijuntha *et al* (2009b).

Multilocus enzyme electrophoresis (MEE)

MEE was performed according to the methods of Saijuntha *et al* (2009b). The electrophoretic patterns of 12 enzymes established when individual worms were compared are as follows (abbreviation, enzyme commission no.): aldolase (ALD, 4.1.2.13), enolase (ENOL, 4.2.1.11), glucose-6-phosphate dehydrogenase (G6PD, 1.1.1.49), glucose-phosphate isomerase (GPI, 5.3.1.9), hexokinase (HK, 2.7.1.1), isocitrate dehydrogenase (IDH, 1.1.1.42), malate dehydrogenase (MDH, 1.1.1.37), malic enzyme (ME, 1.1.1.40), peptidase valine-leucine (PEPA, 3.4.13.11), peptidase phenylalanine-proline (PEPD, 3.4.13.9), phosphoglucomutase (PGM, 2.7.5.1), and pyruvate kinase (PK, 2.7.1.40). Electrophoretic banding patterns were interpreted allozymically (Saijuntha *et al*, 2009b). For each enzyme, bands detected in each sample were scored alphabetically, the allozyme with the least mobility from the cathode was designated as allele *a*.

Data analysis

The multiple banding patterns of an individual worm at a particular locus were consistent with the expectations of heterozygous individuals for enzymes with a quaternary structure, (*eg*, double-banded and triple-banded patterns for heterozygous individuals for monomeric and dimeric enzymes, respectively; Richardson *et al*, 1986). For example, 3 banded heterozygotes were observed with G6PD, which is a dimeric enzyme, and 2 banded heterozygotes were observed in ME and PEPA, which are monomeric enzymes (Saijuntha *et al*, 2009b). In the case of *G6pd-1*, the hybrid band (middle band) of the heterozygote was not scored. In this study, the genotype pattern was interpreted by combining the allelic patterns of the 3 polymorphic loci: *G6pd-1*/*Me1*/*PepA*-2. A

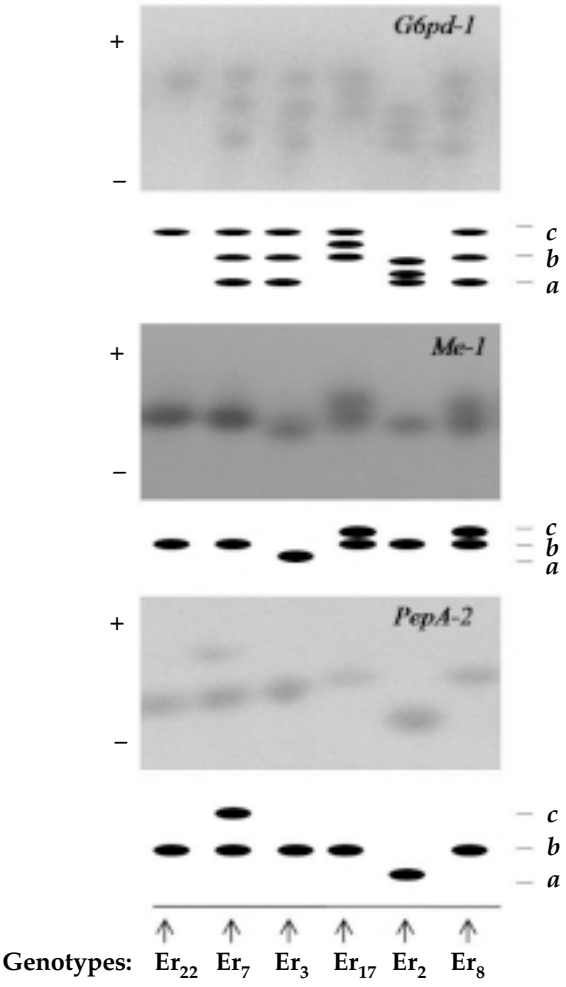

Fig 1–Example of electrophoretic patterns of 3 polymorphic loci of each genotype combining *G6pd-1*/*Me-1*/*PepA-2*; Er_{22} = *cc*/*bb*/*bb*; Er_7 = *ac*/*bb*/*bc*; Er_3 = *ac*/*aa*/*bb*; Er_{17} = *bc*/*bc*/*bb*; Er_2 = *ab*/*bb*/*aa*; Er_8 = *ac*/*bc*/*bb*.

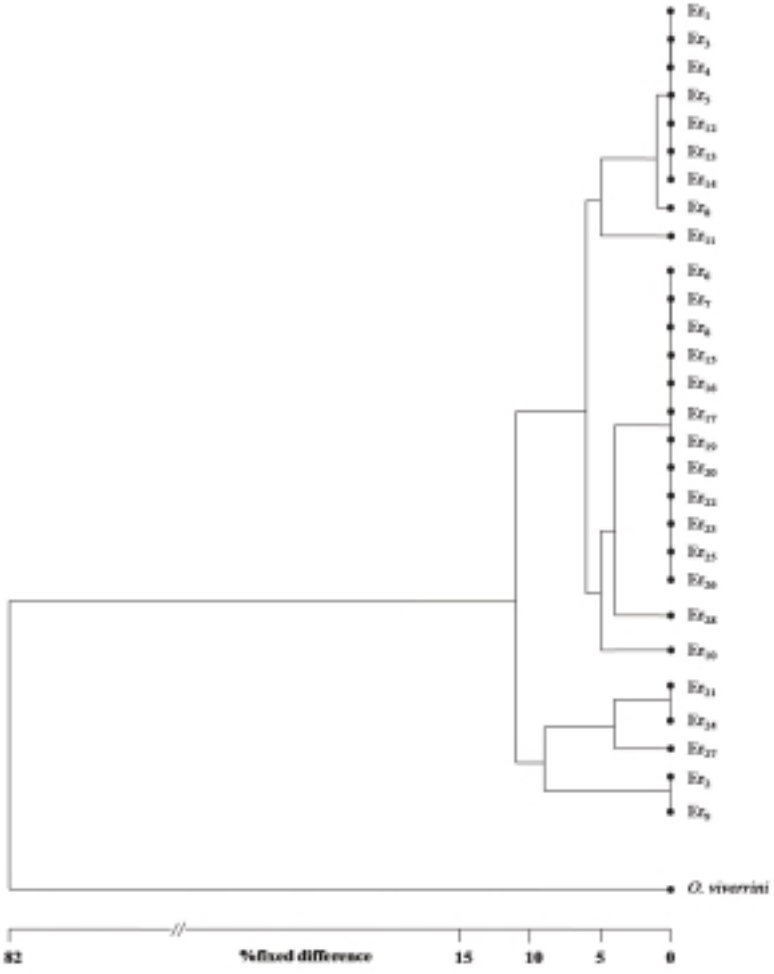

Fig 2–Phenogram constructed from the allelic profile of 15 loci depicts the intra-specific variation among 28 genotypes of *E. revolutum*. Electrophoretic patterns of each genotype as listed in Table 1 and exampled in Fig 1.

these, 12 loci were monomorphic: *Ald, Enol, G6pd-2, Gpi, Hk, Idh, Mdh, Me-2, PepA-1, PepD, Pgm* and *Pk*, and 3 loci were polymorphic: *G6pd-1*, *Me-1* and *PepA-2* among the *E. revolutum* individuals. Three alleles were detected at all 3 polymorphic loci. Twenty-eight genotypes were observed by combining the allelic patterns of these 3 polymorphic loci in each individual sample (Table 1 and Fig 1). Twelve genotypes were observed with ER1, 19 genotypes with ER2, 12 genotypes with ER3 and 6 genotypes with ER4. Three genotypes were observed in all population: Er_{22}, Er_{23} and Er_{25}. The genotype Er_6 (*G6pd-1/Me-1/PepA-2*; *ac/bb/bb*) and Er_{22} (*G6pd-1/Me-1/PepA-2*; *cc/bb/bb*) were the most common genotypes detected in 21 and 20 individual worms (~20%), respectively, in all four populations (Table 1).

phenogram of all the genotypes was constructed from allelic data from all 15 loci examined by using the GWbasic program based on UPGMA (Unweighted Pair Group Method of Analysis; Sneath and Sokal, 1978).

RESULTS

The 12 enzymes encoding 15 presumptive loci were used in this study. Of

A phenogram constructed using the allelic profiles of all 15 loci of the three polymorphic loci in each genotype. Twenty-eight genotypes were clustered into 3 major groups with genetic differences ranging between 2 and 12% (Fig 2). A cluster of genotypes (Er_2, Er_9, Er_{21}, Er_{24}, and Er_{27}) had the greatest number of genetic differences from the other genotypes (12% difference).

Table 1
Allelic patterns of 3 polymorphic loci and observed numbers of individual worms in each genotype for each *E. revolutum* population.

Genotypes	Allelic patterns			Number of individuals observed[a] (%)			
	G6pd-1	*Me-1*	*PepA-2*	ER1 (*n*=23)	ER2 (*n*=50)	ER3 (*n*=22)	ER4 (*n*=11)
Er_1	*ab*	*aa*	*bb*	0	1(2.0)	0	0
Er_2	*ab*	*bb*	*aa*	0	1(2.0)	0	0
Er_3	*ac*	*aa*	*bb*	1(4.3)	1(2.0)	0	0
Er_4	*ac*	*ab*	*bb*	2(8.7)	0	0	0
Er_5	*ac*	*ac*	*bb*	1(4.3)	1(2.0)	0	0
Er_6	*ac*	*bb*	*bb*	2(8.7)	14(28.0)	5(22.7)	0
Er_7	*ac*	*bb*	*bc*	3(13.0)	1(2.0)	0	1(9.1)
Er_8	*ac*	*bc*	*bb*	2(8.7)	6(12.0)	1(4.5)	0
Er_9	*bb*	*bb*	*aa*	0	2(4.0)	0	0
Er_{10}	*bb*	*bc*	*bb*	0	0	1(4.5)	0
Er_{11}	*bc*	*aa*	*ac*	0	1(2.0)	0	0
Er_{12}	*bc*	*aa*	*bb*	0	2(4.0)	0	0
Er_{13}	*bc*	*aa*	*bc*	0	1(2.0)	0	0
Er_{14}	*bc*	*ac*	*bb*	0	2(4.0)	0	0
Er_{15}	*bc*	*bb*	*bb*	0	0	2(9.1)	0
Er_{16}	*bc*	*bb*	*bc*	0	2(4.0)	0	0
Er_{17}	*bc*	*bc*	*bb*	0	1(2.0)	1(4.5)	0
Er_{18}	*cc*	*aa*	*bb*	0	3(6.0)	0	0
Er_{19}	*cc*	*ab*	*bb*	1(4.3)	0	2(9.1)	2(18.2)
Er_{20}	*cc*	*ab*	*bc*	0	0	0	1(9.1)
Er_{21}	*cc*	*bb*	*aa*	1(4.3)	0	1(4.5)	0
Er_{22}	*cc*	*bb*	*bb*	7(30.4)	3(6.0)	5(22.7)	5(45.5)
Er_{23}	*cc*	*bb*	*bc*	1(4.3)	3(6.0)	1(4.5)	1(9.1)
Er_{24}	*cc*	*bc*	*aa*	0	0	1(4.5)	0
Er_{25}	*cc*	*bc*	*bb*	1(4.3)	4(8.0)	1(4.5)	1(9.1)
Er_{26}	*cc*	*bc*	*bc*	0	1(2.0)	0	0
Er_{27}	*cc*	*cc*	*aa*	0	0	1(4.5)	0
Er_{28}	*cc*	*cc*	*bb*	1(4.3)	0	0	0

[a]Four temporal populations of *E. revolutum* collected during 4 different months in 2008: ER1, February; ER2, April; ER3, June and ER4, October.

DISCUSSION

The 37-collar-spined echinostomes have long been recognized as consisting of a species complex. Moreover, morphology, biology and genetic variation have been detected with *E. revolutum* and have been shown to be present at intra- and inter species specific levels (*eg*, Sorensen *et al*, 1998; Kostadinova *et al*, 2000, 2003; Saijuntha *et al*, 2009b). In the present study, genetic variations among 106 individual *E. revolutum* worms from 4 temporal populations were examined for allelic patterns

observed at 3 polymorphic loci, which yielded 28 genotypes. The distribution of these genotypes in the 4 temporal populations fluctuated. The number of worms in each genotype and the number genotypes in each population varied from 1-21 worms and 6-19 genotypes, respectively. However, the number of genotypes was greatest in the ER2 population (19 genotypes) and lowest in the ER4 population (6 genotypes). Several unique genotypes in each temporal population were observed, which may affect the population dynamics of this parasite, because the life span of *E. revolutum* in duckling hosts was relatively short (Davis, 2005). The sample size may also have affected the fluctuation in the number of observed genotypes in this study. The highest and lowest numbers of genotypes observed were found in the ER2 and ER4 populations, which had the highest and lowest sample sizes, respectively. To better understanding the population dynamics of this parasite, a comprehensive analysis of greater numbers of worms and greater numbers of temporal populations are needed.

The 3 polymorphic loci *G6pd-1*, *Me-1* and *PepA-2* had the greatest diversity among the *E. revolutum* specimens. These polymorphic markers provide a basis to comprehensively examine the population genetic structure of this intestinal trematode at intra- and inter-specific levels has previously been examined in the carcinogenic parasitic trematode, *O. viverrini sensu lato* (Saijuntha *et al*, 2006, 2007, 2008). Allozyme analysis has also been used to explore the genetic structure of the parasitic trematode based on different host species (Saijuntha *et al*, 2009a). Various species of snails and avians have been used as intermediate and definitive hosts for *E. revolutum*. The allozyme markers can be used to examine the genetic structure of *E. revolutum* colonizing different host species. Large numbers of morphologically similar species in the "revolutum" group have been described. Many methods have been used to differentiate these similar species, such as morphology, biology and genetic markers (*eg*, Sloss *et al*, 1995; Petrie *et al*, 1996; Sorensen *et al*, 1998; Kostadinova *et al*, 2000; 2003). The allozyme markers used in this study may be useful for species differentiation of the echinostome. Allozyme markers have been successfully used to discriminate 3 echinostome species of the 37-collar-spined group (Sloss *et al*, 1995). They have also been used to discriminate between two morphologically similar echinostome species in domestic ducks from Thailand (Saijuntha *et al*, 2009b).

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