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Genetic variation and relationships of four species of medically important echinostomes (Trematoda: Echinostomatidae) in South-East Asia

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ABSTRACT

Multilocus enzyme electrophoresis (MEE) and DNA sequencing of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene were used to genetically compare four species of echinostomes of human health importance. Fixed genetic differences among adults of *Echinostoma revolutum*, *Echinostoma malayanum*, *Echinoparyphium recurvatum* and *Hypoderaeum conoideum* were detected at 51–75% of the enzyme loci examined, while interspecific differences in CO1 sequence were detected at 16–32 (8–16%) of the 205 alignment positions. The results of the MEE analyses also revealed fixed genetic differences between *E. revolutum* from Thailand and Lao PDR at five (19%) of 27 loci, which could either represent genetic variation between geographically separated populations of a single species, or the existence of a cryptic (i.e. genetically distinct but morphologically similar) species. However, there was no support for the existence of cryptic species within *E. revolutum* based on the CO1 sequence between the two geographical areas sampled. Genetic variation in CO1 sequence was also detected among *E. malayanum* from three different species of snail intermediate host. Separate phylogenetic analyses of the MEE and DNA sequence data revealed that the two species of *Echinostoma* (*E. revolutum* and *E. malayanum*) did not form a monophyletic clade. These results, together with the large number of morphologically similar species with inadequate descriptions, poor specific diagnoses and extensive synonymy, suggest that the morphological characters used for species taxonomy of echinostomes in South-East Asia should be reconsidered according to the concordance of biology, morphology and molecular classification.

1. Introduction

Echinostomiasis is a food-borne zoonotic parasitic disease caused by digenean trematodes. Over 60 species of the Echinostomatidae are endemic worldwide (Sorensen et al., 1998). Although most species parasitize poultry, other bird species and mammals other than humans, at least 20 species can cause human disease (Chai, 2009). Eating raw snails, tadpoles and some freshwater fish has been identified as the mode of echinostome transmission (Carney, 1991; Chai et al., 2005). The clinical pathology of echinostomiasis manifests as damage to the intestinal mucosa which can result in extensive duodenal erosion and catarrhal inflammation. Clinical symptoms are typically anemia, headache, dizziness, stomachache, loss of body weight, urinary incontinence, gastric pain and loose stools. The severity of the symptoms depends on the parasite load (Graczyx and Fried, 1998; Chai et al., 2009). These symptoms can be severe compared with those of other intestinal fluke infections (Chai, 2009).

There are four species of echinostomes that commonly infect humans in Thailand; *Echinostoma malayanum*, *Echinostoma revolutum*, *Echinostoma ilocanum* and *Hypoderaeum conoideum* (Graczyx and Fried, 1998). Since 1969, when more than 50% of northern Thai residents were infected with echinostomes (Sornmani, 1969), the prevalence has decreased to 0.7% (Radomyos et al., 1998). Radomyos et al. (1994) reported an infection rate from northeastern Thailand of 15%, and only in Kalasin Province was lower at 6.81%. Echinostomiasis is difficult to control, however, because a wide range of aquatic animals (i.e., snails, bivalves, crustaceans, fish, and amphibians) serve as the second intermediate hosts (Huffman and Fried, 1990; Graczyx and Fried, 1998). A high rate of echinostome infection in domestic animals and wildlife, mainly in poultry and water birds, would be expected as these eat these raw

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aquatic animals in their domestic and native environments. Moreover, various kinds of snails and amphibian larvae (e.g. tadpoles) in Thailand have been reported as first and second intermediate hosts of echinostomes, namely, *Indoplanorbis* spp., *Gyraulus* spp., *Lymnaea* spp., *Pila* spp., *Viviparus* spp. and *Filopaludina* spp. (Pariyanonda and Tesana, 1990; WHO, 1995).

The taxonomic characters used to distinguish between species and genera of the echinostomes include the structure of the circumoral disc, the number and arrangement of rows of uninterrupted crown spines, the number of collar spines as well as testicular characters (Miliotis and Bier, 2003). It is difficult if not impossible to differentiate among the eggs or immature stages of echinostome species using morphological characters. Moreover, a large number of morphologically very similar adult stages from many species exist due to a long history of inadequate descriptions, poor specific diagnoses and extensive synonymy (Kostadinova et al., 2003). As a consequence, a variety of genetic markers have been developed and used effectively to differentiate among echinostome species irrespective of life cycle stage (Voltz et al., 1987; Morgan and Blair, 1995, 1998a,b; Sloss et al., 1995; Petrie et al., 1996; Sorensen et al., 1998; Kostadinova et al., 2003). Multilocus enzyme electrophoresis (MEE) is a technique that has been used successfully over many years to examine the systematics and population genetics of a wide range of parasitic trematodes (e.g. Sloss et al., 1995; Chilton et al., 1999; Park et al., 2000; Saijuntha et al., 2009). In addition, the sequence data of a number of mitochondrial (mt) DNA genes provides a rich source of genetic markers for informative systematic and epidemiological studies, which is enhanced by their large size, the lack of recombination and high variability (Le et al., 2000; Jex et al., 2010). MtDNA sequences have proven useful for analyzing within-species variation of parasitic flatworms (see Le et al., 2000). For example, mtDNA genes were used as genetic markers for investigations of the carcinogenic liver fluke *Opisthorchis viverrini* (Saijuntha et al., 2008). In addition, mitochondrial gene sequences have provided potential markers to elucidate genetic differentiation and phylogenetic relationships among various echinostomes from Europe, America and Australia (Morgan and Blair, 1998a,b; Kostadinova et al., 2003; Detwiler et al., 2010). To date, however, there is insufficient data regarding levels of genetic variation and phylogenetic relationships of Asian, especially Southeast Asian echinostomes.

The aim of the present study was to use two approaches, MEE and DNA sequencing, to determine the extent of the genetic differences and phylogenetic relationships among four species of echinostome in Thailand; *Echinoparyphium recurvatum* (*Ep. recurvatum*), *E. revolutum*, *E. malayanum* and *H. conoideum*. In addition, genetic variation was examined in *E. revolutum* from two geographical locations (Thailand and Lao PDR) as geographical location has been shown to affect genetic variation of this echinostome in Europe, America and Australia (Sorensen et al., 1998; Kostadinova et al., 2003). Additionally, genetic variation in *E. malayanum* was examined between three species of intermediate (snail) host (Pariyanonda and Tesana, 1990; WHO, 1995), as these can affect the levels of genetic variation in echinostomes.

2. Materials and methods

2.1. Sample collection

Adults of three echinostome species, *Ep. recurvatum*, *E. revolutum* and *H. conoideum*, were collected from the intestines of domestic ducks obtained from a slaughterhouse in Khon Kaen Province, Thailand. Morphological identification was performed using a light microscope based on the size of the circumoral disc, testes morphology and the number of collar spines (Miliotis and Bier, 2003). Thirty adult *Ep. recurvatum* were removed from one infected domestic duck, 10 *E. revolutum* were collected from another four ducks, while 10 *H. conoideum* were collected from two different ducks. In addition, 10 *E. revolutum* adults were collected from a domestic duck in Kampang Nakhon district, Vientiane Province, Lao PDR. Metacercarial cysts of a fourth species, *E. malayanum*, were obtained from freshwater snails, *Indoplanorbis exustus*, *Lymnaea rubiginosa*, and *Pomacea canaliculata* collected from a small pond in Muang district, Khon Kaen Province, Thailand. Fifty to 100 cysts from each snail species were then separately fed to hamsters by intragastric intubation. Thirty days after infection, the hamsters were euthanized and the intestine removed to collect adult worms. Five adult *E. malayanum* were also collected from the small intestine of a rice field rat, *Rattus argentiventer* from Khon Kaen Province, Thailand. All adult worms were examined by light microscopy and identified to species based on their morphological characteristics, i.e. small circumoral disc, branched testes and 43 collar spines (Miliotis and Bier, 2003). All specimens were washed thoroughly in physiological saline, frozen and stored at −80 °C until required for electrophoretic or molecular analyses.

2.2. Multilocus enzyme electrophoresis (MEE) analysis

MEE was performed on enzyme homogenate extracts from whole individual worms using cellulose acetate gel (Cellogel; Milan) as the support medium. Each adult worm was homogenized using a glass rod in a microcentrifuge tube to which 10–30 μl of lysing solution (100 ml distilled H_2O, 100 μl β-mercaptoethanol, 10 mg NADP) was added. Homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were transferred into capillary tubes as 5 μl aliquots and stored at −20 °C. Samples were compared electrophoretically using 26 enzymes. The enzymes (abbreviation and enzyme commission number) used were: adenylate kinase (AK, 2.7.4.3), aldolase (ALD, 4.1.2.13), enalase (ENOL, 4.2.1.11), fructose-1,6-diphosphatase (FDP, 3.1.3.11), fumarate hydratase (FUM, 4.2.1.2), glyceraldehyde-3-phosphate dehydrogenase (GAPD, 1.2.1.12), glutamate dehydrogenase (GDH, 1.4.1.3), glucose-6-phosphate dehydrogenase (G6PD, 1.1.1.49), aspartate amino transferase (GOT, 2.6.1.1), glucose-phosphate isomerase (GPI, 5.3.1.9), alanine amino transferase (GPT, 2.6.1.2), 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), nucleotide diphosphate kinase (NDPK, 2.7.4.6), purine nucleotide phosphorylase (NP, 2.4.2.1), peptidase valine-leucine (PEP-A, 3.4.13.11), peptidase leucine-glycine-glycine (PEP-B, 3.4.11.4), peptidase phenylalanine-proline (PEP-D, 3.4.13.9), phosphoglycerate mutase (PGAM, 2.7.5.3), phosphoglycerate kinase (PGK, 2.7.2.3), phosphoglucomutase (PGM, 2.7.5.1), pyruvate kinase (PK, 2.7.1.40), triose phosphate isomerase (TPI, 5.3.1.1), uridine monophosphate kinase (UMPK, 2.7.1.48). MEE was conducted at a constant temperature (4 °C) and voltage (200 V) for 2.5 h (for ENOL, GAPD, MDH and TPI) or 2 h (for all other enzymes). The running buffer used for all enzymes was Phosphate (0.02 M pH 8.0). The methods used for histochemical staining of cellulose acetate gels followed those described in Richardson et al. (1986). Eight enzymes, G6PD, GOT, NDPK, NP, PEP-A, PEP-B, PGK and UMPK, were each encoded by two presumptive loci; the locus with the least electrophoretic mobility from the cathode was referred to as locus-1 (i.e. *G6pd-1*).

Individual worms of each species (except for *Ep. recurvatum*) were compared using MEE. Given the relatively small size of *Ep. recurvatum* adults compared to those of the other three species, MEE was conducted on a pool of 30 worms in order to maximize the number of enzyme markers that could be used for this species. For each enzyme, the electrophoretic banding patterns were scored allozymically (Richardson et al., 1986), with allele *a*