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Isozyme Discrimination between *Paragonimus heterotremus* and *P. miyazakii*

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Abstract

The lung flukes, Thai *Paragonimus heterotremus* and Japanese *P. miyazakii* were assayed using polyacrylamide gel electrophoresis to distinguish between the isolates. Twelve loci were obtained from ten enzymes recovered from two species of *Paragonimus* populations. Nine enzymes tested showed only a single band, whereas one enzyme, malic dehydrogenase (Mdh), showed three banding patterns. Three of twelve loci in *P. heterotremus* and one locus in *P. miyazakii* were polymorphic, whereas nine loci were a single monomorphic band, which was seen in both species. Isozyme patterns for acid phosphatase (Acph), adenylate kinase (Ak), hexokinase (Hk) and malic dehydrogenase (Mdh) were identical in all *Paragonimus*. Esterase (Est), phosphoglucomutase (Pgm) and 6-phosphogluconate dehydrogenase (6Pgd) showed slightly different patterns, while glucose phosphate isomerase (Gpi), glucose-6-phosphate dehydrogenase (G6pdh) and malic enzyme (Me) showed remarkable differentiations, indicating a specie-specific marker between the two different populations.

Keywords: *Paragonimus heterotremus*, *P. miyazakii*, isozyme, electrophoresis, Thailand, Japan

Introduction

Lung fluke infection or paragonimiasis, is a typical food-borne parasitic disease, mainly endemic in Asia, the Far East, Africa, and America. *Paragonimus heterotremus* has been considered the most important causative agent of paragonimiasis in Southeast Asia, especially in Thailand, whereas in Japan, *P. miyazakii* is known as the medically important pathogen causing human paragonimiasis [1]. Studies on the phylogenetic relationship between Thai (*P. heterotremus* and *P. siamensis*) and Japanese (*P. miyazakii*, *P. ohirai* and *P. westermani*) lung flukes using protein and isozyme electrophoresis have indicated that Thai *P. heterotremus* was closer to Japanese *P. miyazakii* than other species [2-3]. The morphological characteristics of these two species adult flukes are very similar [1]. However, species identification is reliable only when experienced laboratory personnel carry out the investigation using specialized laboratory techniques. Therefore, isozyme electrophoresis was performed to determine the isozyme marker distinguishing two morphologically similar parasites.

Materials and methods

Parasites

Metacercariae of *P. heterotremus* were obtained from waterfall crabs, *Larnaudia beusekomae* Bott 1970, collected from Amphoe Pakphli, Nakhon Nayok Province, Thailand. Thirty-two adult *P. heterotremus* were harvested from experimental cats, 70 days after oral inoculation with their metacercariae. *P. miyazakii* metacercariae were obtained from the crabs, *Geothelphusa dehaani* which were purchased from Sukiji market, Tokyo, Japan. These crabs were caught in Shizuoka Prefecture. Thirty-five adult worms were harvested

from albino rats 60 days after oral inoculation with metacercariae. All samples of adult worms were washed several times with physiological saline solution and the individual worms were then kept at -80˚C until used.

Preparation of lung fluke extracts for electrophoresis

The extract was prepared by homogenizing the specimens individually in 20% sucrose solution with Teflon homogenizer in an ice water bath. The homogenized worms were centrifuged at 10,000 g for 3 minutes at 4˚C, and then the clear supernatant was used immediately for all studies.

Electrophoresis procedure

Electrophoresis was performed in polyacrylamide slab gel according to the method described by Takai [4]. The gel consisted of two layers, lower and upper, laminated vertically in the slit of the gel mold. Sample constituents were concentrated in the upper or stacking gel, and then separated in the lower or separating gel. Electrophoresis was carried out at a constant voltage of 200 V at 6˚C. The isozymes examined with reference to other electrophoretic conditions are summarized in Table 1.

Loci and allele designation

Isozymes were numbered in order of decreasing mobility from the most anodal, and allozymes were named numerically according to their relative mobility to the commonest allele of *P. heterotremus* 100 (mm).

Results

The isozyme patterns of Thai *P. heterotremus* and Japanese *P. miyazakii* were studied and compared by polyacrylamide gel electrophoresis. Twelve loci were obtained from ten enzymes recovered from two species of *Paragonimus* populations. The zymograms and allele frequencies of those species are shown in Fig 1 and Table 2. Nine of ten enzymes tested showed only a single band of activity, while Mdh showed three banding patterns. Three of twelve loci found in *P. heterotremus* (Acph, Est and G6pdh) and one locus in *P. miyazakii* (G6pdh) were polymorphic, whereas nine loci (Ak, Gpi, Hk, Mdh-I, Mdh-II, Mdh-III, Me, Pgm and 6Pgd) showed a single monomorphic band, which was seen in both species (Fig 1).

The isozyme mobility of Acph, Ak, Hk, Mdh-I, Mdh-II, Mdh-III was the same for both species, except for Est, Pgm and 6Pgd which were slightly faster than the others. Moreover, the fast-

Table 1 Enzyme assayed in this study.

Proteins (abbreviation)	Enzyme commission numbers	Buffer system *	Staining method referred **
Acid phosphatase (Acph)	3.1.2.3	I-10	(B)
Adenylate kinase (Ak)	2.7.4.3	I-10	(B)
Esterase (Est)	3.1.1.1	I-10	(B)
Glucose phosphate isomerase (Gpi)	5.3.1.9	I-10	(A)
Glucose-6-phosphate dehydrogenase (G6pdh)	1.1.1.49	I-7	(B)
Hexokinase (Hk)	2.7.1.1	I-10	(A)
Malic dehydrogenase (Mdh)	1.1.1.37	I-7	(B)
Malic enzyme (Me)	1.1.1.40	I-10	(B)
Phosphoglucomutase (Pgm)	2.7.5.1	I-10	(B)
6-phosphogluconate dehydrogenase (6Pgd)	1.1.1.44	I-10	(B)

*Takai [4]; **A = Agatsuma and Suzuki [5], B = Steiner and Joslyn [6]

migrating band of Me, and the slowest-migrating bands of Gpi and G6pdh were clearly shown in *P. heterotremus* when compared with *P. miyazakii* indicating a remarkable differentiation of the two species (Figs 1 and 2).

Discussion

The lung fluke, *Paragonimus* is very complex genus in the class Trematoda. It is very hard to distinguish adult parasites by relying only on morphological features. Attempts have been made to discriminate among them. Electrophoretic analysis of isozymes is one useful tool for the classification and characterization of closely related species, and the assessment of inter- and intraspecific variations in various organisms [7-16]. Agatsuma and Suzuki studied the electrophoretic properties of six enzymes in the oriental lung flukes, *P. ohirai* and *P. miyazakii* in Japan. They reported that the two *Paragonimus* species were distinguishable from each other by the electrophoretic profiles of their enzymes [5]. The characterization of two morphologically similar species, *P. peruvianus* and *P. ecuadoriensis*, by isoenzyme electrophoresis of four enzymes: peptidase, alanine aminotransferase, glucosephosphate isomerase and glucose-6-phosphate dehydrogenase, revealed that they could only be distinguished by the peptidase [17]. The isozyme patterns of *P. iloktsuenesis* and *P. westermani* were studied by isoelectric focusing on polyacrylamide gels for six enzymes, which showed interspecific differences. The result showed that the two species could be distinguished from one another by such species-specific isozyme patterns [18].

In the present study, there were remarkably different banding patterns of Gpi, G6pdh and Me between two species of *Paragonimus*, and Gpi and G6pdh presented especially dense bands in both

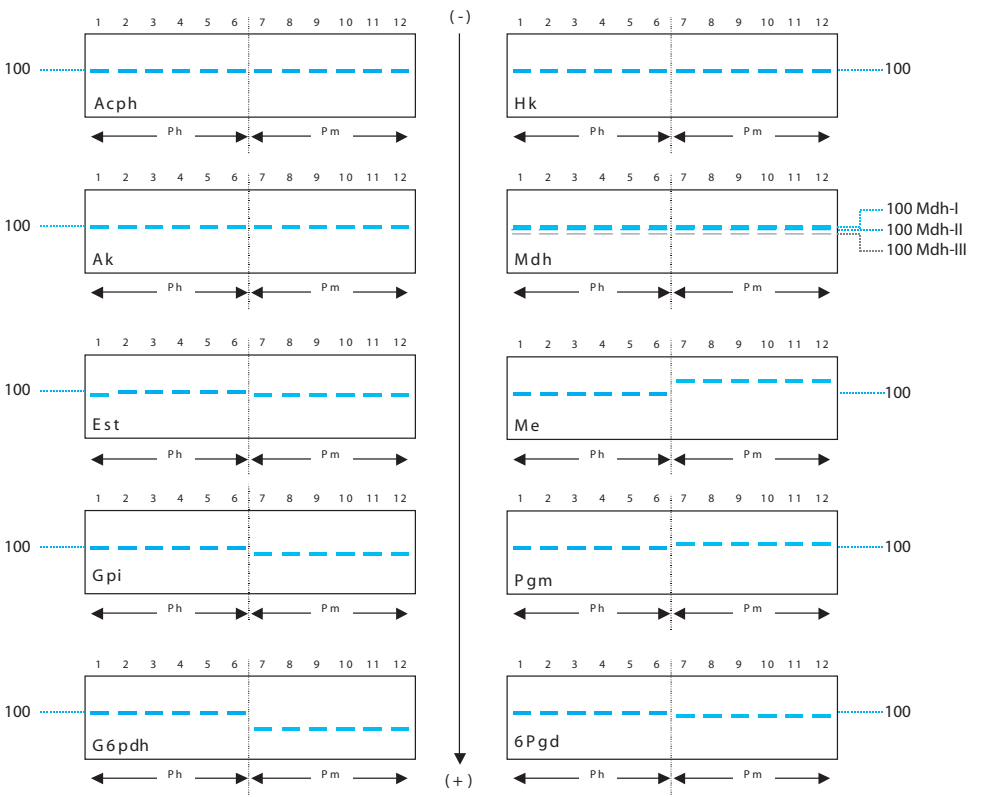

Fig 1 Zymograms of ten enzymes of lung flukes, *P. heterotremus* (Ph) and *P. miyazakii* (Pm).

Table 2 Allele frequencies of twelve loci between two species of *Paragonimus* populations from Japan and Thailand.

Protein loci	Allele	*P. heterotremus*	No. examined	*P. miyazakii*	No. examined
Acph	99	0.06	32	0.00	30
	100	0.94		1.00	
Ak	100	1.00	24	1.00	24
Est	99	0.06	32	0.00	32
	100	0.88		0.00	
	101	0.06		1.00	
Gpi	100	1.00	22	0.00	22
	101	0.00		0.00	
	102	0.00		1.00	
G6pdh	100	0.97	32	0.00	35
	101	0.03		0.00	
	102	0.00		0.00	
	103	0.00		0.00	
	104	0.00		0.00	
	105	0.00		0.97	
	106	0.00		0.03	
Hk	100	1.00	24	1.00	24
Mdh-I	100	1.00	28	1.00	30
Mdh-II	100	1.00	28	1.00	30
Mdh-III	100	1.00	28	1.00	30
Me	97	0.00	28	1.00	31
	98	0.00		0.00	
	99	0.00		0.00	
	100	1.00		0.00	
Pgm	99	0.00	20	1.00	20
	100	1.00		0.00	
6Pgd	100	1.00	29	0.00	25
	101	0.00		1.00	

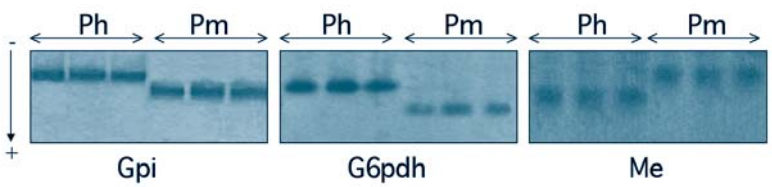

Fig 2 Allele differences of Gpi, G6pdh and Me between *P. heterotremus* (Ph) and *P. miyazakii* (Pm).

species, with faster-migrating bands in *P. miyazakii*. G6pdh also showed the widest interval of isozyme mobility, even though it showed polymorphism in *P. heterotremus* and *P. miyazakii* populations, but no commonly shared alleles, as shown in Table 2. This indicates significant differences in three enzymes from *P. heterotremus* and *P. miyazakii*, which may be partially explained by genetic distinction, geographical distance and their biological differences. Applications of isozyme markers distinguishing two morphologically similar species have been reported in various organisms [19-26] Therefore, Gpi, G6pdh, and Me may be considered species-specific banding patterns of isozymes or species-specific markers, which are important contributions for the identification and discrimination of these two medically important *Paragonimus* species from the two countries.

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