GENETIC VARIABILITY IN ISOZYMES OF *ANGIOSTRONGYLUS MALAYSIENSIS*

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Abstract. The genetic difference between *Angiostrongylus malaysiensis* and *A. cantonensis* was assayed by electrophoretic analysis of isozymes. Six enzymes were analyzed using 5% polyacrylamide gel electrophoresis. Seven of 10 loci, namely GPI-1, GPI-2, HK-1, HK-2, MDH-1, MDH-2 and PGM-2, were shown to be polymorphic, but the remaining 3 loci, LDH, ME and PGM-1, were not. Both *A. malaysiensis* and *A. cantonensis* were polymorphic at 6 of the loci ($p = 0.600$) with heterozygosity H of 0.286 and 0.151, respectively. The Nei's genetic distance (D) between *A. malaysiensis* and *A. cantonensis* was 0.27470. This value indicates the level of interspecific variation within a genus. Through isozyme analysis, the present study demonstrated that *A. malaysiensis* of Japan is a valid species, separate from *A. cantonensis*.

INTRODUCTION

Angiostrongylus malaysiensis is known to be distributed throughout Malaysia, Indonesia and Thailand but had never been found in Japan until our reports (Makiya, 1991; Makiya and Sawabe, 1992). *Angiostrongylus malaysiensis* was described as a new species by Bhaibulaya and Cross (1971). Its validity as a species was confirmed by cross-breeding experiments with *A. cantonensis* (Cross and Bhaibulaya, 1974), however, the level of differentiation between the 2 species is still unknown.

In our previous study (Sawabe and Makiya, 1992), we described some of the physiological characteristics of *A. malaysiensis* based on its infectivity to a snail, *Biomphalaria glabrata,* and the survival capacity of first-stage larvae under various conditions: water temperature, desiccation of rat feces, pH and protease. The morphological features and measurement data of adult worms (Makiya, 1991; Makiya and Sawabe, 1992), as well as their physiological characteristics (Sawabe and Makiya, 1992) strongly suggested that *A. malaysiensis* of Japan is a separate species from *A. cantonensis*.

Differentiation between species is determined mainly by morphological and physiological characteristics, and by the level of reproductive isolation. However, validity as a species is not easily demonstrated by this information alone. The genetic distance between populations, thus, can be helpful in distinguishing them from closely allied species. Among the species of the genus *Angiostrongylus,* this kind of genetic analysis using protein electrophoresis has not previously been performed.

In the present study, we shall first determined the genetic variability in isozymes of *A. malaysiensis* and *A. cantonensis*. To compare the electrophoretic band patterns among *Angiostrongylus* species, a few samples of *A. costaricensis,* which is a member of this genus known to be similar to both *A. malaysiensis* and *A. cantonensis* in its morphology (Kamiya and Fukumoto, 1988), were also used in this analysis. We investigated the level of genetic differentiation between *A. malaysiensis* and *A. cantonensis*.

MATERIALS AND METHODS

Parasites

Angiostrongylus malaysiensis was obtained from natural intermediate host *Rumina decollata* collected in Kitakyushu City in 1991 (Makiya, 1991; Makiya and Sawabe, 1992). *Angiostrongylus cantonensis* was collected in Nagoya City in 1982 (Makiya and Onitake, 1982). Both species were maintained and colonized in our laboratory using albino rats *Rattus scorvegicus* var. *albus* and *B. glabrata* snail. First-stage larvae obtained from feces of rats infected by the Baermann technique were exposed to 20 snails in a plastic cup (8 cm in diameter, 12 cm in height) containing about 50 ml of water for 1 day for *A.*

cantonensis and 4 to 6 days for *A. malaysiensis* at 25 ± 1°C. During exposure, the snails were exposed to newly collected first-stage larvae every day. Thirty-six days after infection, third-stage larvae were retrieved from the snails after 2 hours of artificial digestion with pepsin-0.1% HCI. Five-week-old male Wistar rats obtained from Kyudo Co Ltd, Fukuoka, Japan, were used as definitive hosts in all the experimental infection. Each rat was intubated orally with 100 or 50 infective third-stage larvae of *A. malaysiensis* and *A. cantonensis*, respectively, using a syringe attached to a stomach tube.

Adult worms were collected from the heart, pulmonary artery and lungs of the rat about 40 days after infection with third-stage larvae. The worms were washed thoroughly with 0.75% physiological saline, and stored at -80°C until electrophoresis. Adult *A. costaricensis* were provided by Dr Akira I Ishii of Hamamatsu University School of Medicine, and maintained in the laboratory according to the method of Terada *et al* (1982, 1984).

Sample preparation for electrophoresis

Individual adult worms were homogenized usually in 60 µl of 5 mM 2-mercaptoethanol using a Teflon homogenizer in an ice-bath. Ten µl of 5 mM 2-mercaptoethanol was used for detection of malic enzyme (ME) in both sexes of worms and for phosphoglucomutase (PGM) in male worms, respectively. For PGM in female worms, 100 µl of 5 mM 2-mercaptoethanol was used. Homogenates were then centrifuged at 10,000*g* for 10 minutes at 4°C, and 5 µl of the supernatant fluid was loaded into the sample slot.

The adult worm ingested blood in the internal environment of the rat. To avoid any possibility of the ingested blood being affected, serum and red blood cells (RBC) of the rat were loaded as controls. Blood was collected directly from the heart of a rat anesthetized by an injection of 50 mg/kg (rat weight) of nembutal (Dainabot Co Ltd). To separate the serum, the collected blood was left for 30-40 minutes at room temperature, and then centrifuged at 3,000*g* for 15 minutes without cooling. The supernatant was collected as serum. To separate RBC, the collected blood was immediately poured into a phosphate buffer saline (PBS) (135 mM NaCl, 3 mM $Na_2HPO_4 \cdot 12H_2O$, 13 mM $NaH_2PO_4 \cdot 2H_2O$). The blood and PBS mixture was agitated, and centrifuged at 2,000*g* for 10 minutes without cooling. The supernatant was removed and new PBS was added to the precipitation. This procedure, from the centrifugation to the adding of PBS, was carried out at least 3 times to clean the RBC. Both serum and RBC were stored at -80°C until electrophoresis.

Electrophoresis

Electrophoresis was performed in a 5% horizontal polyacrylamide gel at 4°C. Electrophoretic techniques usually followed the method of Tsukamoto (1984, 1989). Buffer systems for the enzymes examined are shown in Table 1. Six enzymes from the 3 *Angiostrongylus* species provided discrete bands and were selected for characterization. Forty to 50 adult *A. malaysiensis* and *A. cantonensis* worms, and 2 to 3 *A. costaricensis* were used in each enzyme electrophoresis.

Table 1

Enzymes examined in electrophoretic analysis of the *Angiostrongylus* species.

Enzymes	Abbreviation	Enzyme commission no.	Gel buffer
Glucosephosphate isomerase	GPI	EC 5.3.1.9	0.1M Tris-HCl, pH8.5
Hexokinase	HK	EC 2.7.1.1	0.1M Tris-HCl, pH7.4
Lactate dehydrogenase	LDH	EC 1.1.1.27	0.1M Tris-HCl, pH7.4
Malate dehydrogenase	MDH	EC 1.1.1.37	0.2M phosphate, pH7.4
Malic enzyme	ME	EC 1.1.1.40	0.1M Tris-HCl, pH7.4
Phosphoglucomutase	PGM	EC 2.7.5.1	0.1M Tris-HCl, pH8.0

Calculations of genetic diversity

After the gel film had dried, the distance migrated by each band was measured and the relative mobility was calculated as the R_f value (× 100).

The genetic variability within populations of each species was quantified by measuring the proportion of polymorphic loci (P) and the average heterozygosity per individual ($\bar{H}$). A locus was defined as polymorphic if the frequency of the most common allele was less than or equal to 0.99. This definition is clearly arbitrary and therefore is not as good a measure of the heterogeneity of a population as the average heterozygosity. The average heterozygosity is calculated as

$$\bar{H} = 1 - \bar{\Sigma} q_i^2,$$

where q_i is the frequency of the i-th allele at a locus, and the average is calculated for all the loci examined.

The interspecific gene difference between *A. malaysiensis* and *A. cantonensis* was estimated by calculating Nei's genetic distance (D) and genetic identity (I) (Nei, 1972).

RESULTS

Description of enzyme variants

Fig 1 shows band morph patterns of the 3 *Angiostrongylus* species at the 10 enzyme loci. No bands from the serum or RBC having the same relative mobility were detected. All of the detectable bands for the 3 species, therefore, could be considered specific bands of *Angiostrongylus*. Of the 6 enzymes examined, 5 (GPI, HK, MDH, LDH and PGM) showed electrophoretic variations among the 3 species. No variation was observed in the remaining enzyme, ME.

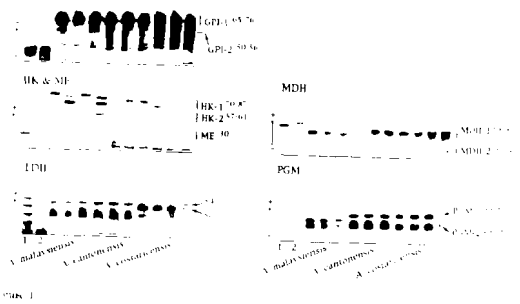

Fig 1–Band morph patterns of the 3 *Angiostrongylus* species at 6 enzymes. A single adult worm was loaded on each lane. The 2 lanes at the left (1 and 2) are controls for serum and red blood cells of the rat *Rattus norvegicus*, respectively.

The GPI-1 locus appeared in all species. *Angiostrongylus malaysiensis* and *A. cantonensis* had 2 bands, *Gpi-1*^{67} and *Gpi-1*^{73}; but *A. costaricensis* had a third band, *Gpi-1*^{76}. The GPI-2 locus had 2 bands in *A. malaysiensis* and *A. cantonensis*, but none in *A. costaricensis*. The HK-1 locus was found in *A. malaysiensis* and *A. cantonensis*, but not in *A. costaricensis*. This locus showed high polymorphism in *A. malaysiensis*. In the HK-2 locus, both *A. malaysiensis* and *A. cantonensis* had 2 bands, *Hk-2*^{57} and *Hk-2*^{61}; but *A. costaricensis* had only the *HK-2*^{61} band. The ME locus showed a single band with the same mobility, *Me*^{30}, in all the species examined. Both the MDH-1 and MDH-2 loci appeared as a single band in each species, *Mdh-1*^{34} or *Mdh-1*^{40} in *A. malaysiensis*, and *Mdh-2*^{10} or *Mdh-2*^{12} in *A. cantonensis*, respectively. The *Ldh*^{54} band appeared in both *A. malaysiensis* and *A. cantonensis*, while *Ldh*^{50} was found only in *A. costaricensis*. The PGM-1 locus had 2 bands, *Pgm-1*^{44} and *Pgm-1*^{50}. Both *A. malaysiensis* and *A. cantonensis* had the *Pgm-1*^{44}, while *A. costaricensis* had 2 bands; *Pgm-1*^{44} and *Pgm-1*^{50}. The PGM-2 locus showed high polymorphism in both *A. malaysiensis* and *A. cantonensis*. Three, *Pgm-2*^{24}, *Pgm-2*^{33} and *Pgm-2*^{36} and 2 bands, *Pgm-2*^{24} and *Pgm-2*^{33} were found in *A. malaysiensis* and in *A. cantonensis*, respectively, while *A. costaricensis* had only the *Pgm-2*^{27} band.

As only a few samples of *A. costaricensis* were examined for electrophoresis, the following genetic analysis was carried out using the data of *A. malaysiensis* and *A. cantonensis*.

Genotype and allele frequencies for *A. malaysiensis* and *A. cantonensis*

Genotype frequencies at each of the 7 polymorphic enzyme loci, GPI-1, GPI-2, HK-1, HK-2, MDH-1, MDH-2 and PGM-2, and allele frequencies at 10 loci, including the monomorphic locus, LDH, ME and PGM-1, are shown in Tables 2 and 3, respectively. Detailed interpretations of enzyme variation are as follows:

Glucosephosphate isomerase (GPI): In the GPI-1 locus, both *A. malaysiensis* and *A. cantonensis*

Table 2

Genotype frequencies at 7 polymorphic enzyme loci, GPI-1, GPI-2, HK-1, HK-2, MDH-1, MDH-2 and PGM-2, for adult *A. malaysiensis* and *A. cantonensis* worms; 3 other loci, LDH, ME and PGM-1, were monomorphic.

Locus and genotype	*A. malaysiensis*	*A. cantonensis*
GPI-1		
Gpi-1^{67}/Gpi-1^{67}	13* (14.0)**	0 (0.5)
Gpi-1^{67}/Gpi-1^{73}	23 (21.0)	10 (9.0)
Gpi-1^{73}/Gpi-1^{73}	7 (8.0)	41 (41.5)
n	43	51
GPI-2		
Gpi-2^{50}/Gpi-2^{50}	33 (33.1)	25 (25.5)
Gpi-2^{50}/Gpi-2^{56}	5 (4.7)	13 (12.1)
Gpi-2^{56}/Gpi-2^{56}	0 (0.2)	1 (1.4)
n	38	39
HK-1		
Hk-1^{75}/Hk-1^{75}	2 (1.5)	12 (12.1)
Hk-1^{70}/Hk-1^{77}	0 (0.6)	0 (0.0)
Hk-1^{77}/Hk-1^{77}	2 (1.1)	0 (0.0)
Hk-1^{70}/Hk-1^{87}	3 (1.2)	0 (0.0)
Hk-1^{87}/Hk-1^{87}	5 (4.3)	0 (0.0)
Hk-1^{75}/Hk-1^{84}	7 (1.6)[a]	16 (15.8)
Hk-1^{84}/Hk-1^{84}	0 (0.4)	5 (5.1)
Hk-1^{75}/Hk-1^{87}	2 (5.1)	0 (0.0)
Hk-1^{77}/Hk-1^{87}	7 (4.3)	0 (0.0)
n	28	33
HK-2		
Hk-2^{57}/Hk-2^{57}	3 (5.4)	1 (0.0)
Hk-2^{57}/Hk-2^{61}	22 (17.1)	0 (1.9)
Hk-2^{61}/Hk-2^{61}	11 (13.4)	51 (50.0)
n	36	52
MDH-1		
Mdh-1^{34}/Mdh-1^{34}	18 (11.3)	0 (0.3)
Mdh-1^{34}/Mdh-1^{40}	0 (19.4)[c]	8 (7.4)
Mdh-1^{40}/Mdh-1^{40}	21 (8.3)[b]	41 (41.3)
n	39	49
MDH-2		
Mdh-2^{10}/Mdh-2^{10}	0	49
Mdh-2^{12}/Mdh-2^{12}	32	0
n	32	49
PGM-2		
Pgm-2^{24}/Pgm-2^{24}	7 (13.3)	25 (27.6)
Pgm-2^{24}/Pgm-2^{33}	7 (4.1)	22 (16.8)
Pgm-2^{24}/Pgm-2^{36}	24 (14.2)[a]	0 (0.0)
n	38	47

*Observed number.

**Expected number, assuming of random mating.

a, b and c: Significantly different (χ^2 -test, $p < 0.01$ and $p < 0.001$, respectively).

Table 3

Allele frequencies at 10 loci, including a monomorphic locus, for adult *A. malaysiensis* and *A. cantonensis* worms.

Locus and allele	*A. malaysiensis*	*A. cantonensis*
GPI-1		
67	0.570	0.098
73	0.430	0.902
*H**	0.490	0.177
GPI-2		
50	0.934	0.808
56	0.066	0.192
H	0.123	0.311
HK-1		
70	0.054	
75	0.232	0.606
77	0.196	
84	0.125	0.394
87	0.393	
H	0.735	0.478
HK-2		
57	0.389	0.019
61	0.611	0.981
H	0.475	0.037
LDH		
54	1.000	1.000
MDH-1		
34	0.538	0.082
40	0.462	0.918
H	0.497	0.151
MDH-2		
10		1.000
12	1.000	
ME		
30	1.000	1.000
PGM-1		
44	1.000	1.000
PGM-2		
24	0.592	0.766
33	0.092	0.234
36	0.316	
H	0.541	0.358

*Heterozygosity

indicated heterozygosity; the genotype was named as *Gpi-1*^{67}/*Gpi-1*^{73}. These 2 alleles appeared equally in frequency in *A. malaysiensis*, while *Gpi-1*^{73} was dominant in *A. cantonensis* (Table 3). Two alleles, *Gpi-2*^{50} and *Gpi-2*^{56}, were found in GPI-2 locus; the frequency of *Gpi-2*^{50} was considerably higher in both *A. malaysiensis* and *A. cantonensis*, being 0.934 and 0.808, respectively (Table 3).

Hexokinase (HK): The HK-1 locus showed high polymorphism in *A. malaysiensis*. As shown in Table 2, 8 genotypes were found to have been constructed by 5 alleles, *Hk-1*^{70}, *Hk-1*^{75}, *Hk-1*^{77}, *Hk-1*^{84} and *Hk-1*^{87} (Table 3). In contrast, *A. cantonensis* had 2 alleles, *Hk-1*^{77} and *Hk-1*^{84}; but *Hk-1*^{77} was dominant. In the HK-2 locus, 2 alleles, *Hk-2*^{57} and *Hk-2*^{61} (Table 2), were found, and the frequency of *Hk-2*^{61} allele was high in both *A. malaysiensis* and *A. cantonensis* (Table 3).

Malate dehydrogenase (MDH): In the MDH-1 locus, the *Mdh-1*^{34} and *Mdh-1*^{40} alleles appeared in equal frequency in *A. malaysiensis*, while *Mdh-1*^{40} was dominant in *A. cantonensis* (Table 3). In the MDH-2 locus, the *Mdh-2*^{12} allele was dominant in *A. malaysiensis*, but the *Mdh-2*^{10} allele dominated in *A. cantonensis* (Table 3).

Phosphoglucomutase (PGM): The PGM-2 locus showed high polymorphism in both *A. malaysiensis* and *A. cantonensis* (Table 3). *Angiostrongylus malaysiensis* had 3 alleles, *Pgm-2*^{24}, *Pgm-2*^{33} and *Pgm-2*^{36}, and there were 2 genotypes identified which indicated heterozygosity, *Pgm-2*^{24}/*Pgm-2*^{27} and *Pgm-2*^{24} / *Pgm-2*^{36} (Table 2), while *A. cantonensis* indicated heterozygosity with *Pgm-2*^{24}/ *Pgm-2*^{33}, constructed by 2 alleles, *Pgm-2*^{24} and *Pgm-2*^{24}, (Table 2).

Each allele of the *Ldh*^{54}, *Me*^{30} and *Pgm-1*^{44} appeared in each locus, LDH, ME and PGM-1 of *A. malaysiensis* and *A. cantonensis*. Therefore, these 3 enzyme loci are regarded as the monomorphic loci, at least in the present analysis.

Hardy-Weinberg equilibrium

As a test of the genetic hypothesis for each locus, electrophoretic patterns were examined for their goodness-of-fit with regard to expectations based on the Hardy-Weinberg equilibrium (HWE) model. As shown in Table 2, chi-square (χ^2)-tests to fit the HWE revealed the distribution of the genotypes of almost all the polymorphic enzymes examined, except for 3 loci of *A. malaysiensis*. In the HK-1, MDH-1 and PGM-2 loci, there was a significant difference between the observed and the expected distributions of genotypes calculated based on HWE (χ^2) *tests*, $p < 0.001$-0.05).

Genetic variability within populations

Table 4 shows p and $\bar{H}$ values used to quantify the genetic variability within the population of each *A. malaysiensis* and *A. cantonensis*. A high value of p (0.600) was obtained from both of these *Angiostrongylus* species. Six among the 10 loci showed high polymorphism. A range of p values has been described for other taxonomically different organisms, but values usually were found to range within 0.2-0.4 (Nei, 1975). This p value, 0.600, seems to be a little higher than that from other organisms. The $\bar{H}$ value obtained from *A. malaysiensis* (0.286) was higher than that from *A. cantonensis* (0.151). The average $\bar{H}$ for the 2, *Angiostrongylus* species was 0.219. These p and $\bar{H}$ values suggested that the level of genetic polymorphism of *A. malaysiensis* is higher than that of *A. cantonensis*.

Genetic distance between *A. malaysiensis* and *A. cantonensis*

Table 5 shows the genetic diversity between *A. malaysiensis* and *A. cantonensis* in Nei's genetic distance (*D*) and identity (*I*) calculated from the allele frequencies of each species at the 10 respective loci. The *D* value was calculated as 0.27470. This value

Table 4

Proportion of polymorphic loci (*P*) and average heterozygosity (*H*) calculated for the two *Angiostrongylus* species.

Species	Proportion of polymorphic loci P (10 loci)	Average heterozygosity *H* (10 loci)
A. malaysiensis	0.600	0.286
A. cantonensis	0.600	0.151
Average	0.600	0.219

Table 5

Values of Nei's genetic distance (*D*) and genetic identity (*I*) between *A. malaysiensis* and *A. cantonensis* based on 10 enzyme loci.

D	*I*	
	A. malaysiensis	*A. cantonensis*
A. malaysiensis		0.75980
A. cantonensis	0.27470	

indicates that about 27% of the total genetic diversity of this species is attributable to genetic differences between conspecific populations. This value also suggests that each species indicates a level of interspecific variation within the same genus based on the reliable criteria established by both Nei (1975) and Thorpe (1982).

DISCUSSION

This is the first report to attempt to classify the isozyme pattern 2 closely related *Angiostrongylus* species. Based on 10 loci, the level of genetic differentiation between *A. malaysiensis* and *A. cantonensis* has been discussed in this study. From the result of the allele frequencies of respective species, genetic distance (*D*) between *A. malaysiensis* and *A. cantonensis* was calculated as 0.27470 (Table 5). This value suggests that *A. malaysiensis* is a genetically separated species from *A. cantonensis*, supporting the findings from morphological studies (Bhaiburaya, 1979; Kamiya and Fukumoto, 1988; Makiya, 1991; Makiya and Sawabe, 1992).

Nei (1975) described that *D* between local races within a same species is less than 0.0058, between subspecies within a same species 0.1-0.2, and between species within a same genus in the range of 0.5-2.5. According to Thorpe (1982), a *D* range of 0.02-3.9 (mainly 0.22-1.6) indicates an interspecific variation within a genus. He then concluded that between the populations of 2 species if *D* is more than 0.16, they cannot be regarded as the same species, even if the 2 are identified as the same species by morphological and physiological characteristics, and by the level of reproductive isolation. If *D* is less than 0.16 and other factors are not available between the populations of the 2 species which are considered to be separate species, they should not be regarded as separate species. Our estimation of *D* value between *A. malaysiensis* and *A. cantonensis* (0.27470) was close to 0.2, and 0.22 from the differentiation points of Nei (1975) and Thorpe (1982). Based on the above-mentioned criteria by Thorpe (1982), however, it appears that the differentiation between the 2 species indicates a level of interspecific variation within a same genus, but is close to the level of a subspecies. In addition, *A. costaricensis* is suggested to be far from those 2 *Angiostrongylus* groups in their isozyme patterns. However, because only a few samples of *A. costaricensis* were used for the analysis in this study, a larger sample size should be used in the future to confirm this point.

The range of p values found for different groups of organisms is very large, but usually within a range of 0.2-0.4 (average 0.3; Nei, 1975). The p value of 0.600 obtained from both *A. malaysiensis* and *A. cantonensis* (Table 4) was much larger than the average p value. The $\bar{H}$ values of 0.286 and 0.151, for *A. malaysiensis* and *A. cantonensis*, respectively (Table 4), were also larger than the average $\bar{H}$, 0.1 (Nei, 1975). In the isozyme analysis, natural populations are usually used as much as possible. However, the 3 *Angiostrongylus* species used in this study were adult worms obtained from a laboratory colony. *Angiostrongylus malaysiensis* has been maintained for about 1 year in the laboratory, but *A. cantonensis* has been laboratory reared for more than 10 years. Laboratory colonies may lose a large amount of initial electrophoretic variation due to inbreeding and inadvertent selection (Berlocher, 1984), the genetic differentiation between 2 of the populations could have undergone intensive acceleration due to random genetic drift (Mukai, 1977; Mukai *et al*, 1974, 1980) and the bottleneck effect (Chakraborty and Nei, 1977). Nevertheless, the high values of p and $\bar{H}$ obtained from *A. malaysiensis* were clearly shown to be a higher polymorphic species than *A. cantonensis*. In comparison with *A. cantonensis*, *A. malaysiensis* can be considered nearly a natural population.

Each genotype was interpreted at each locus from the electrophoretic band patterns (Fig 1) based on the previous electrophoretic study (data not shown) and other studies (Østergaard *et al*, 1985; Agatsuma and Habe, 1986). Almost all of the observed distributions of genotypes agreed with

the expected ones according to the HWE, but 4 of the *A. malaysiensis* genotypes did not (Table 2). In a small number of observed individuals, genotypes were grouped as if they were produced by a pair of alleles. And it is known that inbreeding and assortative mating induce an increase in number of heterozygote. Cross-breeding experiments should also be performed to verify F_1 hybrid band patterns at all enzyme loci. Further examinations are needed in the future.

We determined that the ingested rat blood does not affect the isozyme band of adult worms of any species (Fig 1). To analyze the genetic variability more precisely, however, an analysis should be made at the larval stage to avoid any possible bad effect by ingested blood. When approximately 1,000 first stage larvae were used in this electrophoretic system, no protein band was detected on the gel, suggesting that the protein content of first stage larvae was too small for our electrophoretic conditions. Each pre-adult worm of the 3 *Angiostrongylus* species used in this study can be found on the surface of the rat brain from 10 days after infection (Alicata, 1970). *Angiostrongylus malaysiensis*, after infecting Balb/c mice, stops growing and dies at a pre-adult stage (Ratanaponglakha and Ambu, 1989). If it were possible for pre-adult stage worms to be collected easily and abundantly from the brain, we would like to use them in our electrophoretic system.

Further investigation on other aspects, such as DNA analysis and karyological studies on both the euchromatin and heterochromatin fractions are necessary to determine more reliably the phylogenic relationship among the *Angiostrongylus* species. The mitochondrial DNA (mtDNA) can be a sensitive marker of phylogenic relationship between related members at the genetic level for many kinds of organisms (Nei, 1983). An analysis of mtDNA of *A. cantonensis* was performed and a restriction cleavage map of mtDNA was drawn by lida (1987), but differentiation among *Angiostrongylus* species was not determined in her study. Therefore, this type of study should be carried out in detail.

Human cases of angiostrongyliasis by *A. malaysiensis* have not been reported as yet. However, 3 cases of eosinophilic meningitis and/or meningoencephalitis in Sarawak, Malaysia (Lim and Ramachandran, 1979) might have been caused by this species. To differentiate of *A. malaysiensis* from *A. cantonensis* only by their morphological features and measurements of worms is very difficult. However, the present study suggests that when the isozyme technique is used, it is easy to classify *Angiostrongylus* even within its same genus. Based on these results of genetic analysis using isozymes and DNA, it should be possible to describe infection of humans in more detail.

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