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Electrophoretic studies on enzymes of diploid and triploid *Paragonimus westermani*

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SUMMARY

An enzyme analysis of diploid and triploid *Paragonimus westermani* was conducted using starch gel electrophoresis. In total, 16 enzymes, probably encoded by 18 loci, were studied for 3 populations of the diploid form sampled from 2 localities, and 4 populations of the triploid form from 4 localities. Comparison of the enzymes of the triploid and the diploid digeneans showed 5 different patterns; diaphorase (EC 1.6.2.2), glutamic-oxaloacetic transaminase (EC 2.6.1.1), hexokinase (EC 2.7.1.1), leucylglycylglycine aminopeptidase (EC 3.4.1.3), and phosphoglucomutase (EC 2.7.5.1). On the basis of the numbers of bands and their patterns, all individuals of the triploid are probably heterozygous at each of these 5 loci and homozygous at the remaining 13 loci. The occurrence of fixed heterozygotes found in triploid populations cannot be easily explained by only a single mutation. It is suggested that the variability may have been introduced by hybridization with a different sub-species or a closely related species and may, thus, have been maintained since the time of the origin of triploids.

INTRODUCTION

Paragonimiasis, caused by *Paragonimus westermani*, is one of the most important parasitic diseases in Asia. Recently, Miyazaki (1978) noticed the existence of two types, diploid and triploid, in *P. westermani*, and regarded them as separate species on the basis of their various morphological, cytological, and ecological differences. In the light of the present taxonomy of *Paragonimus*, Miyazaki (1978) proposed to name the former type *P. westermani* and the latter *P. pulmonalis*, which was the first species found and named in Japan by Baelz in 1880, but later regarded as a synonym of *P. westermani* Kerbert, 1878. Yokogawa (1982), however, pointed out the problems associated with this nomenclature. He stated that the name, *P. ringeri*, should be taken instead, because of its priority to *P. pulmonalis*. Moreover, he drew attention to the difficulties in identifying the two forms, since, apart from the size of adults, no other morphological differences are obvious. Terasaki (1980*a*) analysed karyotypes of both types of *P. westermani*, but found little difference between them except for the chromosome number. From his results, Terasaki thought that *P. pulmonalis* (triploid type) may be simply an autotriploid originating from a diploid form of *P. westermani*.

Enzyme electrophoresis has proved to be a useful tool for species and strain differentiation with various parasites (e.g. Taylor & Müller, 1979: Chance & Walton, 1982; Vrijenhoek, 1978; Miles, Toye, Oswald & Godfrey, 1977). Agatsuma & Suzuki (1981) compared 6 enzymes in 2 species, *P. ohirai* and *P. miyazakii*, using starch gel electrophoresis, and found distinct differences in 4 enzymes. In this study, an enzyme analysis of the two types of *P. westermani* was conducted using gel electrophoresis.

Table 1. *Summary of the second intermediate host, experimental definitive host and polyploidy of* Paragonimus westermani *sampled from each locality*

	Mie (1)	Mie (2)	Ohita	Tsushima	Amakusa	Yaku	Gang Wond
Second intermediate host*	*G.d.*	*G.d.*	*G.d.*	*E.j.*	*E.j.*	*G.d.*	*C.s.*
Experimental definitive host	Dog	Dog	Dog	Dog	Dog	Cat	Dog
Ploidy	2n	2n	2n	3n	3n	3n	3n
Reference†	(1)	(1)	(2)	(3)	(4)	(5)	(6)

* *G.d.*, *Geothelphusa dehaani*; *E.j.*, *Eriocheir japonicus*; *C.s.*, *Cambaroides similis*.

† (1) Sugiyama, Okuda, Sonoda, Tomimura & Nishida (1983); (2) Habe & Miyazaki (1982); (3) Terasaki (1980*b*); (4) Terasaki (1977); (5) Habe & Terasaki (1982); (6) Hirai, Sakaguchi & Habe (unpublished data).

MATERIALS AND METHODS

Populations studied

The metacercariae of the triploid type were obtained from the crab host, *Eriocheir japonicus*, collected in 1981 at Tsushima Is. and Amakusa Is. and from *Geothelphusa dehaani* collected at Yaku Is., Japan in 1981, and from the crayfish, *Cambaroides similis*, at Gang Wond, Korea in 1983. Diploid type metacercariae were obtained from *G. dehaani*, sampled at Ohita, Mie (1) and Mie (2), Japan, in 1981, 1982 and 1983, respectively. Metacercariae from all these localities except Yaku were separately administered *per os* to dogs and those of Yaku were fed orally to a cat. About 4 months after inoculation of the metacercariae, the animals were sacrificed and the adult flukes were recovered from worm cysts in the lungs. Table 1 is a list of the second intermediate and experimental definitive hosts and their references with regards to polyploidy.

Electrophoretic methods

All samples of adults were washed with 0·8 % physiological saline, and subsequently stored at −80 °C until used. The extracts were prepared by homogenizing each individual in 100 μl of 0·1 M phosphate buffer solution (pH 7·5) with a Teflon homogenizer in an ice water bath. The homogenized worms were centrifuged at 3000 r.p.m. for 3 min at room temperature. The supernatant fractions obtained were used in this study. Gels for electrophoresis were prepared from hydrolysed potato starch (Connaught Laboratories, Ontario). The amount of starch used was usually 12·0 g/100 ml of gel buffer. Samples were absorbed onto 5 × 4 mm strips of filter paper before insertion into gels. The buffer systems and electrophoretic conditions are given in Tables 2 and 3. The staining procedures for these enzymes were taken from Shaw & Prasad (1970), and Selander, Smith, Yang, Johnson & Gentry (1971). It was confirmed that the bands, which appeared in each reaction mixture, showed specific enzyme activity, because no bands were detected without substrate in the mixtures. The solution for the enzyme under study was poured on the surface of the cut gel and incubated at 37 °C until the bands of enzyme activity developed fully.

Genetic interpretations

In the absence of a formal genetic cross, tentative genotypes were assumed on the basis of the number of bands, their mobility and the relative intensity of staining,

Table 2. *Electrode and gel buffers used in the present study*

System	Electrode buffer	Gel buffer
SI	0·155 M Tris, 0·043 M citrate (pH 7·0)	Dilute 66·7 ml of electrode buffer to 1 litre
S13	0·378 M Tris, 0·165 M citrate (pH 6·0)	Dilute 33·3 ml of electrode buffer to 1 litre
S18	0·1 M Tris, 0·1 M maleic acid 0·01 M $Na_2EDTA.2H_2O$, 0·01 M $MgCl_2$, 0·13 M NaOH (pH 7·4)	Dilute 100 ml of electrode buffer to 1 litre
FWL	0·1 M $LiOH.H_2O$, 0·38 M boric acid (pH 7·5)	0·003 M Citrate, 0·014 M Tris 0·01 M $LiOH.H_2O$, 0·038 M borate (pH 8·0)
POULIC	0·3 M Boric acid, 0·06 M NaOH (pH 8·2)	0·076 M Tris, 0·005 M citrate (pH 8·7)

Table 3. *Electrophoretic conditions for each enzyme examined*

System	Enzymes*	Conditions
SI	HK MDH	50 mA current constant, 4·5 h
S13	AK ME PGM	60 mA current constant, 5·5 h
S18	GPI	70 mA current constant, 15·0 h
FWL	GDH EST TO TDH	150 V voltage constant, 5·5 h
POULIC	ALP DIA GOT LAP LGG	250 V voltage constant, 5·5 h

* For abbreviations, see Table 4.

according to the conventional procedures which have been adopted for parasitic organisms (Vrijenhoek, 1978; Fletcher & LoVerde, 1981). It has been demonstrated that, when a given genotype is A/A/B, each of the 3 genes is active to the same degree and so the band corresponding to an allele, A, becomes stained relatively stronger than the other one, B, due to gene dosage (Neaves & Gerald, 1968; 1969). In this paper, all of the phenotypes obtained will be explained genetically in this way. We have also found isozyme variants of GOT, LGG, DIA, PGM and GPI in the natural populations of the other *Paragonimus* species, *P. ohirai*, *P. iloktsuenensis* and *P. sadoensis*, and the breeding data showed that these variants are controlled by simple Mendelian inheritance (Agatsuma & Habe, unpublished data).

RESULTS

Electrophoretic forms of enzymes and variation within each type

Tables 4 and 5 list the enzymes and the number of individuals examined in each population. In total, 15 enzymes were surveyed for all of the individuals sampled from each locality of diploid and triploid types.

In the Ohita population of the diploid type, all the individuals showed the same banding pattern in all enzymes examined; no variation in any enzyme was found within the population. The result indicated that all individuals were homozygous at all the enzyme loci examined. On the other hand, 2 populations from Mie were polymorphic at the PGM and GPI loci. Considerable polymorphism of GPI was found in Mie (2). At

Table 4. *List of enzymes examined*

Enzymes	Abbreviation	
(1) Adenylate kinase	AK	EC 2.7.4.3
(2) Alkaline phosphatase	ALP	EC 3.1.3.1
(3) Diaphorase	DIA	EC 1.6.2.2
(4) Esterase	EST	EC 3.1.1.1
(5) Glucosephosphate isomerase	GPI	EC 5.3.1.9
(6) Glutamate dehydrogenase	GDH	EC 1.4.1.3
(7) Glutamic-oxaloacetic transaminase	GOT	EC 2.6.1.1
(8) Hexokinase	HK	EC 2.7.1.1
(9) Leucine aminopeptidase	LAP	EC 3.4.1.1
(10) Leucylglycylglycine aminopeptidase	LGG	EC 3.4.1.3
(11) Malate dehydrogenase	MDH	EC 1.1.1.37
(12) Malic enzyme	ME	EC 1.1.1.40
(13) Phosphoglucomutase	PGM	EC 2.7.5.1
(14) Tetrazolium oxidase	TO	EC 1.15.1.1
(15) Threonine dehydrogenase	TDH	EC 1.1.1.103

Table 5. *Number of two types, diploid and triploid, of* Paragonimus westermani *obtained from 7 localities*

	Mie (1)	Mie (2)	Ohita	Tsushima	Amakusa	Yakushima	Gang Wond
(1) AK	35	20	42	57	28	15	19
(2) ALP	35	20	42	35	23	15	19
(3) DIA	35	20	42	57	23	15	19
(4) EST	35	20	22	43	28	15	19
(5) GPI	57	20	31	109	13	15	19
(6) GDH	35	20	42	57	28	15	19
(7) GOT	35	20	42	38	28	15	19
(8) HK	35	20	42	57	28	15	19
(9) LAP	35	20	42	45	28	15	19
(10) LGG	35	20	42	35	23	15	19
(11) MDH	35	20	42	57	28	15	19
(12) ME	35	20	42	54	28	15	19
(13) PGM	55	20	42	32	28	15	19
(14) TO	35	20	84	114	84	75	38
(15) TDH	35	20	46	45	23	15	19

the PGM locus in Mie (1), also, there was polymorphism and significant deviation from the Hardy–Weinberg equilibrium was observed. It could be that this population consisted of 2 subpopulations. Details of the GPI and PGM polymorphism in diploid populations are shown in Table 6.

In the triploid type, no variation in any enzyme was found either within or between populations, as shown in Table 7; that is, the 4 populations which were derived from remote areas were monomorphic at all the enzyme loci examined.

Comparison and genetic interpretation of the electrophoretic patterns of the two types

When enzyme forms were compared, 5 of 16 showed different patterns between the diploid and triploid types. The 5 enzymes showing different patterns were DIA, GOT, HK, LGG and PGM (Fig. 1).

Table 6. *Polymorphism at* GPI *and* PGM *loci found in two different populations of diploid type of* Paragonimus westermani *from Mie locality in Japan*

	Genotypes				Alleles		Heterozygosity‡
	a/a	a/b	b/b	Total	a	b	(H)
			(1) GPI				
Mie (1)	54*	3	0	57	0·974	0·026	0·0506
	(54·07)†	(2·89)	(0·04)				
Mie (2)§	3	13	4	20	0·475	0·525	0·4988
	(4·51)	(9·98)	(5·51)				
			(2) PGM				
Mie (1)‖	28	13	14	55	0·627	0·373	0·4677
	(21·60)	(25·73)	(7·65)				

* Number observed.
† Number expected from Hardy–Weinberg's law.
‡ $H = 1 - \Sigma q_i^2$, where q_i is the frequency of the ith allele at a locus.
§ $0{\cdot}2 > P > 0{\cdot}1$ (D.F. = 1).
‖ $P < 0{\cdot}001$ (D.F. = 1).

Table 7. *Presumptive genotype frequencies in* 3 *populations of diploid type and* 4 *populations of triploid types of* Paragonimus westermani *at* 6 *enzyme loci, where their allele compositions were found to be different between* 2 *types from electrophoretic patterns*

Loci and genotypes*	DIA		GOT		GPI			
	a/a	a/a/b	a/a	a/a/b	a/a	a/a/a	a/b	b/b
Diploid								
Mie (1)	35	—	35	—	54	—	3	—
Mie (2)	20	—	20	—	3	—	13	4
Ohita	42	—	42	—	31	—	—	—
Triploid								
Tsushima	—	57	—	38	—	109	—	—
Amakusa	—	23	—	28	—	13	—	—
Yakushima	—	15	—	15	—	15	—	—
Gang Wond	—	19	—	19	—	19	—	—

Loci and genotypes*	HK		LGG-3		PGM			
	a/a	a/a/b	a/a	a/a/b (a/b/c)†	a/a	a/b	a/b/b	b/b
Diploid								
Mie (1)	35	—	35	—	28	13	—	14
Mie (2)	20	—	20	—	20	—	—	—
Ohita	42	—	42	—	42	—	—	—
Triploid								
Tsushima	—	57	—	35	—	—	32	—
Amakusa	—	28	—	23	—	—	28	—
Yakushima	—	15	—	15	—	—	15	—
Gang Wond	—	19	—	19	—	—	19	—

* For abbreviations, see Table 4.
† LGG showed rather obscure band pattern in triploid type of *Paragonimus westermani*, although the pattern was clearly different between the two types. So, we considered its genotype tentatively to be a/a/b or a/b/c at the 3rd locus (*LGG-3*).

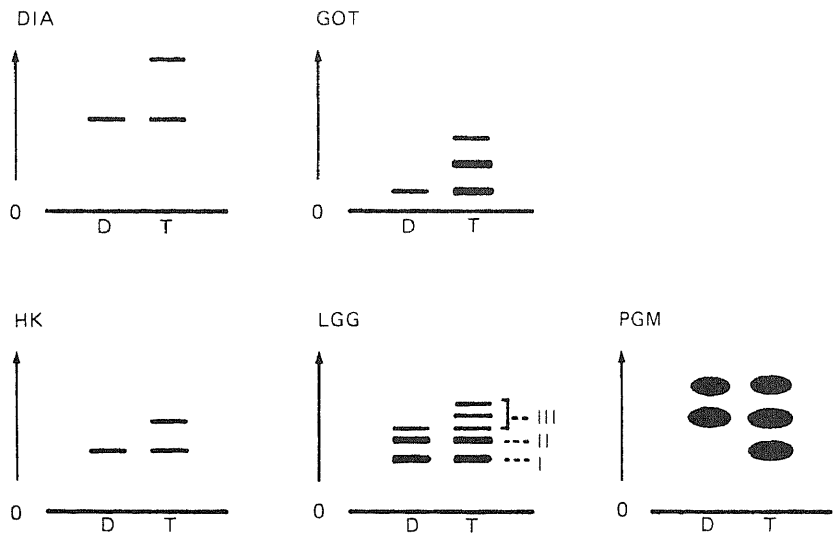

Fig. 1. Diagram of the electrophoretic patterns of 5 enzymes, diaphorase (DIA: EC 1.6.2.2), glutamic-oxaloacetic transaminase (GOT: EC 2.6.1.1), hexokinase (HK: EC 2.7.1.1), leucylglycylglycine aminopeptidase (LGG: EC 3.4.1.3) and phosphoglucomutase (PGM: EC 2.7.5.1), which revealed clear differences between the two types, diploid and triploid, of *Paragonimus westermani*. D, Diploid type; T, triploid type; O, origin.

Diaphorase (DIA)

Both types possessed a major band with the same mobility, but an additional broad band appeared in the triploid type. This pattern was puzzling, because earlier data had suggested that this enzyme may be a dimer in *P. ohirai* (Agatsuma & Habe, unpublished observations). It was tentatively considered that the genotype of the triploid could be a/a/b, and that of the diploid, a/a.

Glutamic oxaloacetic transaminase (GOT)

A 3-banded pattern was detected in the triploid type with relatively strong staining of the slowest band, while only a single band appeared in the diploid. GOT seems to be a dimer and highly polymorphic in *P. ohirai* populations (Agatsuma & Habe, unpublished observations) and it appears that GOT of *P. westermani* is also a dimer from the triplet pattern. Therefore, a genotype, a/a, has been assigned to the diploid, and a/a/b, to the triploid.

Hexokinase (HK)

A single band was detected in the diploid type, while 2 bands appeared in the triploid type. Of the 2 bands in the triploid, 1 band was identical with that of the diploid in mobility, and the other had faster mobility with a relatively lower intensity of staining. The diploid is considered to be a homozygote, a/a, and the triploid, a/a/b.

Leucylglycylglycine aminopeptidase (LGG)

This enzyme showed a complicated banding pattern, especially in the triploid, but the patterns were clearly different between the two types. We have some information about this enzyme in *P. ohirai*: that is, *P. ohirai* LGG was controlled by 3 loci, and each isozyme from these loci was a monomer and showed its own substrate specificity to

several other substrates. Moreover, it was found that the 3rd locus of *P. ohirai*, which corresponds to the 3rd locus in *P. westermani*, was highly polymorphic, and the variants were inherited in the simple Mendelian fashion (Agatsuma & Habe, unpublished data). In the present material, *P. westermani*, a similar electrophoretic pattern to that of *P. ohirai* was detected, and different substrate specificity was also observed in the respective 3 enzyme zones which indicate independent enzyme loci. From this information, it was considered that the LGG of *P. westermani* is controlled by 3 loci, and in the 3rd locus, the triploid type is heterozygous, a/a/b or a/b/c, while the diploid will be homozygous, a/a.

Phosphoglucomutase (PGM)

The triploid type had 3 bands, while the diploid showed 2 bands; their mobility was identical with 2 of the 3 bands in the triploid. Genetic interpretation can be performed on the basis of the pattern and frequency of variants found in the diploid populations (Table 6). In the triploid type, the slowest band which corresponds to an allele, b, was found to be broader and stronger in staining than the fastest band. This indicates that the genotype of the triploid will be a/b/b, and that of the diploid, a/a.

Other enzymes

The remaining 10 enzymes (MDH, TDH, EST, LAP, GDH, TO, ALP, ME, AK and GPI) were monomorphic in both types, since only a single band was detected in all of these enzymes in all the individuals examined, except for MDH and AK. In the case of MDH, a multiple banding pattern was seen in all individuals of both types. However, the number of loci involved was unknown, because no variation was detected either within or between types. In this enzyme, for the present, 1 structural gene was considered to be involved. Three bands were detected in AK, but it is apparent that the pattern was composed of sub-bands derived from a major one with the slowest mobility: this enzyme tends to produce a series of sub-bands running in an anodal direction, due to polymerization of molecules. Thus, it may at present be thought that a single locus is involved in this enzyme.

In the present study, a total of 18 loci, controlling 15 enzymes, was surveyed in both types of *P. westermani*. Zymograms of 5 enzymes showing different patterns between the two types are summarized in Fig. 1. The results showed that each of 5 loci possessed at least a different allele in the triploid type from that of the diploid one.

DISCUSSION

In this paper, genotypes of the variants were tentatively assigned to each enzyme form according to conventional criteria which have been successfully utilized in many organisms, particularly those where genetic cross experiments are thought to be difficult or impossible (e.g. Markert, 1875). However, in the present case it was also possible to compare the *P. westermani* situation with that relating to the genetics of enzymes in another *Paragonimus* species, *P. ohirai* where different patterns of GOT, PGM and LGG proved to be genetic variants controlled by a simple Mendelian mode of inheritance (Agatsuma & Habe, unpublished data). Uncertainty remains in the case of LGG, however, because the pattern of the triploid was rather obscure, although it seems that there is distinction of isozyme pattern between the two types.

Polymorphism was found at the GPI and PGM loci in Mie populations of the diploid, but consistent monomorphism was observed in all the other loci examined. On the other

hand, the Ohita population was monomorphic at all loci. Average heterozygosity (18 loci) was 0·035, 0·033 and 0·000, in Mie (1), Mie (2) and Ohita, respectively, and their ratios of polymorphic loci were 2/18 = 11·7 %, 1/18 = 5·9 % and 0/18 = 0·0 %, respectively. These values are rather low, compared with those of the other parasitic species of *Paragonimus* (Agatsuma & Habe, unpublished data), *Schistosoma mansoni* (Fletcher, LoVerde & Woodruff, 1981) and *Contracaecum* spp. (Vrijenhoek, 1978), in which relatively large numbers of loci (15–25 %) were polymorphic and average heterozygosity was also relatively high. Variation is lost in a population when the population size is drastically decreased by certain causes, such as the migration of a few individuals into a remote area or temporal fluctuation by environmental pressure, and/or when the population might be subject to a stabilized selection (Lewontin, 1974; Nevo, 1978). In cases of enzyme variation the role of natural selection may be small (Mukai & Voelker, 1977). Random genetic drift may largely affect the genetic structure of parasite populations (Fletcher *et al.* 1981). As the triploid types can self reproduce (Terasaki, 1977; Miyazaki, 1978), random drift may be more effective in these forms. In fact, we found that the triploid type had a considerable uniformity of genetic structure throughout all 4 populations, although they are located rather distantly from each other. The consistent monomorphism and low level of heterogeneity observed in the populations of the two types may be due to a founder effect resulting from the migration of a few individuals to make new populations. In the case of the triploid type especially, distance could not be a major factor for complete isolation because its main crab host, *E. japonicus*, has a strong migrating capability (the host can migrate to remote islands or land by ocean currents or by adhering to ships or flotsam) which make the genetic structure uniform even among the distant populations. This implies a recent origin for the 4 populations of the triploid type.

Out of 18 loci from both types, 5 (about 28 %) possessed different alleles in the triploid; that is, the triploid form was probably heterozygous at these 5 loci, though at least 1 allele of the triploid was always the same as that of the diploid form. Mutation and recombination are thought to be major causes of variation. But, since they will generally occur at random, the variation might be brought about not only among individuals at a given locus, but also among loci in a given individual. Thus, the present results may not easily be considered to have arisen due to accumulation of single mutations or recombination. It is suggested, rather, that the genetic variability in the triploid might be introduced by a process like hybridization with a subspecies, or a closely related species, leading to formation of triploid and/or parthenogenesis. So, the variability may have been preserved since the time of the origin of the triploid. Certainly, the triploid form studied here was found to be very different from the diploid form from the viewpoint of biochemical genetics, and we suggest that the triploid form may be an allotriploid. This suggestion was confirmed by the recent chromosome studies of Hirai *et al.* (personal communication). The triploid, therefore, seems to constitute a different biological entity. This finding lends support to the statement of Miyazaki (1978) that the two types are separate species.

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