[Jpn. J. Parasitol., Vol. 37, No. 4, 195–202, August, 1988]

Population Genetic Studies on Isozymes in *Paragonimus westermani* from the Philippines

TAKESHI AGATSUMA[1], SHIGEHISA HABE[2], KENJIRO KAWASHIMA[3], and BAYANI L. BLAS[4]

(Received for publication; October 31, 1987)

Abstract

Fourteen enzymes (seventeen loci) of *Paragonimus westermani* from the two localities, Leyte and Sorsogon, of the Philippines were investigated by means of starch gel electrophoresis. Eight enzymes (AK, EST, GDH, GPT, HK, ME, 6PGD and PGM) were all single-banded and monomorphic in the two populations. Four enzymes, DIA, G6PD, MDH and TO, showed two staining zones, indicating two genetic loci involved, without any variation in both of the populations. Polymorphism was seen at *GOT* and *GPI* loci in Leyte and Sorsogon, respectively. Nei's (1975) genetic distance between the two populations was 0.00588.

When the two Philippine populations were compared with a Japanese diploid population (Ohita), a great difference was seen in allelic composition at 7 loci, *GOT, G6PD-I, GPI, GPT, MDH-I, 6PGD* and *PGM*. Nei's genetic distance was, in average, 0.5234. Further, the Philippine populations showed 0.5755 (in average) of the genetic distance for a Taiwan diploid population (Taipin). These values suggested that the Philippine lung fluke may be a separate species.

When the Philippine and Japanese triploid populations were compared, any common allele was not found at the three loci, GOT, HK and PGM, in the Philippine lung flukes. Therefore, the Philippine lung fluke was found not to be related to the ancestor of the Japanese triploid of *P. westermani* in terms of the gene introgression, which is considered to have been involved in the origin of the triploid type of the Japanese *P. westermani*.

Key words: *Paragonimus westermani*, isozyme, electrophoresis, genetic differentiation, Philippines

Introduction

The main agent of paragonimiasis in Japan, *Paragonimus westermani*, has been known as triploid, while all other Japanese species of *Paragonimus* are diploid (Sakaguchi and Tada, 1976a, b; Terasaki, 1977). Afterwards, Miyazaki (1977) discovered a new type of *P. westermani* which preserves numerous sperm in seminal receptacle, and stated that this new type may be bisexual and can be easily differentiated from the conventional triploid *P. westermani*. The new type was found to be diploid later (Terasaki, 1980). Since then, the existence of the diploid type of *P. westermani* has been confirmed in various areas of Japan (Nishida *et al.*, 1981; Habe and Miyazaki, 1982; Shibahara, 1982; Sugiyama *et al.*, 1983; Yoshimura *et al.*, 1983; Sugiyama *et al.*, 1984; Kanazawa *et al.*, 1986; Shibahara, 1986; Shiwaku *et al.*, 1986; Yokogawa *et al.*, 1986).

On the other hand, *P. westermani* distributing in the Philippines was reported to mature even in the albino rat (Yokogawa *et al.*,

[1] *Department of Parasitology, Kochi Medical School, Oko-cho, Nankoku City, Kochi 781 -51, Japan.*
[2] *Department of Parasitology, School of Medicine, Fukuoka University, Fukuoka 814-01, Japan.*
[3] *School of Health Sciences, Kyushu University, Fukuoka 812, Japan.*
[4] *Schistosomiasis Research and Training Center, Palo, Leyte, the Philippines.*

This study was supported by the research grant Nos. 60041052 and 61043047 under the International Scientific Research Program from the Ministry of Education, Science and Culture, Japan.

吾妻　健（高知医科大学寄生虫学教室）
波部重久（福岡大学医学部寄生虫学教室）
川島健治郎（九州大学医療技術短期大学部）

1977), and has been suspected to be a different species from the Japanese *P. westermani.* The Philippine lung fluke was also found to be diploid (Terasaki, 1983). As to the taxonomical status of the Philippine lung fluke, Miyazaki (1978a) and Ito *et al.* (1978) studied the adult and larval morphology in detail, and stated independently that the lung fluke was a separate species, naming it *P. filipinus* and *P. philippinensis,* respectively. Afterwards, Miyazaki amended his species after re-examining a lot of specimens to be a subspecies of the Japanese *P. westermani* (Miyazaki and Habe, 1979). At present, there is still controversy on the classification of the Philippine lung fluke.

Meanwhile, after his morphological and ecological search, Miyazaki (1978b) proposed that the two types of *P. westermani* should be different species, and named the triploid *P. pulmonalis.* Hirai *et al.* (1985) performed the chromosome analysis of the diploid and triploid *P. westermani* and found the triploid alloploidy, supporting Miyazaki's statement. Agatsuma and Habe (1985) also tried to examine 15 enzymes of both types by starch gel electrophoresis, and found that out of 15 enzymes examined, the following five enzymes, DIA, GOT, HK, LGG and PGM, showed different electrophoretic patterns between the two types, that is, the triploid type always possessed additional bands in the five enzyme loci, showing remarkably high level of heterogeneity. They, therefore, stated that the existence of this high heterogeneity in the triploid can not be explained just by accumulation of single mutations, but by genome introgression as a result of hybridization. From these genetic studies, the triploid type of *P. westermani* was considered a hybrid species which had, recently, been brought about by hybridization between the Japanese diploid *P. westermani* and an unknown subspecies of *P. westermani* or its closely-related species, and has drastically spread throughout Japan (Agatsuma and Habe, unpublished data).

Main purpose of this study is to analyze genetic relationship between the Philippine and Japanese *P. westermani* for detecting the unknown taxon of *Paragonimus* which has been suggested by Agatsuma and Habe (1985) and Hirai *et al.* (1985). In addition, the taxonomical status of the Philippine lung fluke will be discussed from a standpoint of population genetics.

Materials and Methods

Collections were made at the same localities of two provinces, Leyte (Leyte Island) and Sorsogon (Luzon Island) in the Philippines, as Ito *et al.* (1978) and Miyazaki and Habe (1979) surveyed previously. The metacercariae collected were inoculated into dogs and adult worms were recovered about 6 months after inoculation. The adults were homogenized individually and centrifuged at 3,000 rpm, and the supernatants were applied for starch gel electrophoresis. The 14 enzymes (17 loci) examined in this study were as follows: adenylate kinase (*AK*), diaphorase (*DIA-I*), esterase (*EST*), glutamate dehydrogenase (*GDH*), glutamic-oxaloacetic transaminase (*GOT*), glucose-6-phosphate dehydrogenase (*G6PD-I, -II*), glucosephosphate isomerase (*GPI*), glutamic-pyruvate transaminase (*GPT*), hexokinase (*HK*), malate dehydrogenase (*MDH-I, -II*), malic enzyme (*ME*), 6-phosphogluconate dehydrogenase (*6PGD*), phosphoglucomutase (*PGM*), tetrazolium oxidase (*TO-I, -II*).

Electrophoretic conditions and enzyme staining methods followed the way described previously (Agatsuma and Habe, 1985; Agatsuma *et al.*, 1987).

The normalized identity of gene (*I*) and the standard genetic distance or average net codon difference (*D*) (Nei, 1975) were used for the discussion of relative phylogenetic relationships among local populations of *Paragonimus westermani.*

Results

1) *Description of electrophoretic patterns of 14 enzymes in* P. westermani *from two localities of*

the Philippines.

The lung fluke numbers examined for each enzyme are shown in Table 1. The following eight enzymes showed a single band with no variations either within or between populations: AK, EST, GDH, HK, GPT, ME, 6PGD and PGM. There were two staining zones on the gel in the three enzymes, DIA, G6PD, and TO, indicating involvement of two loci. No variations, however, were detected at either locus of the three enzymes (Out of two loci of DIA, only one locus, *DIA-I*, was examined). In the MDH pattern, multiple bands were observed, showing at least two loci (*MDH-I, -II*) involved. *MDH-I* locus encodes the bands migrating into the cathod, while *MDH-II* was responsible for the anodal one. But, no variants were found at either loci. Only the two enzymes, GPI and GOT, showed variations in the populations. At *GPI* locus, the Sorsogon population showed polymorphism, while the Leyte was monomorphic, possessing the allele, *Gpi-c*, which was predominant in Sorsogon. Low polymorphism at the *GOT* locus was found in Sorsogon and a predominant allele in Sorsogon was the same as the one fixed in the Leyte population.

2) *Genetic variability in the Philippine population.*

Proportion of polymorphic loci (*P*) was 0.0588 for both populations, showing a extremely low value. Average heterozygosity ($\bar{H}$) was also very small in value, being 0.0031 (Leyte) and 0.0253 (Sorsogon). ($\bar{H} = 1 - \overline{\Sigma q_i^2}$, where q is the frequency of *i*-th allele, and the average is taken over the examined loci.)

3) *Comparison of genetic structure between two Philippine populations, Leyte and Sorsogon.*

No differences were seen in allele composition at 15 enzyme loci. Only two loci, *GOT* and *GPI*, showed different allele frequencies between them. The Nei's genetic distance (*D*) was 0.00588 (Table 3).

4) *Comparison of genetic structure between the Philippine and Japanese diploid (Ohita) populations.*

The Ohita population was re-examined for

Table 1. The individual numbers examined, and genotype and allele frequencies at 17 enzyme loci in *P. westermani* from the two localities, Leyte and Sorsogon, in the Philippines

Enzyme loci	Populations	
	Leyte	Sorsogon
1. *AK*		
a/a*	26	30
2. *DIA-I*		
a/a	55	35
3. *EST*		
a/a	50	30
4. *GDH*		
a/a	50	30
5. *GOT*		
b/b	52	35
b/f	3	0
b†	0.973	1.000
f	0.027	0.000
6. *G6PD-I*		
b/b	50	30
7. *G6PD-II*		
a/a	50	30
8. *GPI*		
c/c	54	14
c/d	0	20
d/d	0	1
c	1.000	0.686
d	0.000	0.314
9. *GPT*		
b/b	50	30
10. *HK*		
a/a	50	30
11. *MDH-I*		
a/a	55	35
12. *MDH-II*		
a/a	55	35
13. *ME*		
a/a	50	30
14. *6PGD*		
a/a	31	30
15. *PGM*		
g/g	50	30
16. *TO-I*		
a/a	45	30
17. *TO-II*		
a/a	55	35

* : presumptive genotype
† : allele

Table 2. Comparison of allele frequencies at 17 loci in *P. westermani* among 4 populations from the Philippines, Taiwan and Japan

	Enzyme loci	Populations			
		the Philippines		Japan*	Taiwan†
		Leyte	Sorsogon	Ohita	Taipin
1.	*AK*				
	a	1.000	1.000	1.000	1.000
2.	*DIA-I*				
	a	1.000	1.000	1.000	1.000
3.	*EST*				
	a	1.000	1.000	1.000	1.000
4.	*GDH*				
	a	1.000	1.000	1.000	1.000
5.	*GOT*				
	a			1.000	0.756
	b	0.973	1.000		0.222
	f	0.027			
	others				0.022
6.	*G6PD-I*				
	b	1.000	1.000		
	c			1.000	1.000
7.	*G6PD-II*				
	a	1.000	1.000	1.000	1.000
8.	*GPI*				
	a			1.000	0.671
	c	1.000	0.686		
	d		0.314		
	others				0.329
9.	*GPT*				
	a			1.000	1.000
	b	1.000	1.000		
10.	*HK*				
	a	1.000	1.000	1.000	0.740
	b				0.260
11.	*MDH-I*				
	a	1.000	1.000		
	b			1.000	1.000
12.	*MDH-II*				
	a	1.000	1.000	1.000	1.000
13.	*ME*				
	a	1.000	1.000	1.000	1.000
14.	*6PGD*				
	a	1.000	1.000		
	b			1.000	1.000
15.	*PGM*				
	a			1.000	0.195
	g	1.000	1.000		
	others				0.805
16.	*TO*				
	a	1.000	1.000	1.000	1.000
17.	*TO-II*				
	a	1.000	1.000	1.000	
	b				1.000

* : Cited from Agatsuma and Habe (1985)

† : Cited from Agatsuma *et al.* (1987)

comparison in this study. The result of this population survey was the same as previous one (Agatsuma and Habe, 1985).

The loci having different alleles between them were *G6PD-I, GPI, GPT, MDH-I, 6PGD,* and *PGM* (Fig. 1). One allele, *Got-b,* at the *GOT* locus was shared by both populations. The other 10 loci were found to be occupied by the same fixed alleles between them. Average genetic distance (*D*) was 0.5234 (Tables 2 and 3).

5) *Comparison of genetic structure between the Philippine and Japanese triploid (Tsushima)*

Table 3. Matrices of genetic distances (D) (above the diagonal) and normalized identity of genes (I) (below the diagonal) between each pair of 4 populations from the Philippines, Taiwan and Japan

D / I	the Philippines		Japan	Taiwan
	Leyte (LT)	Sorsogon (SG)	Ohita (OT)	Taipin (TP)
LT		0.00588	0.52908	0.58151
SG	0.99414		0.51780	0.56955
OT	0.58915	0.59583		0.11079*
TP	0.55906	0.56578	0.89513	

* : One locus, *LAP,* was excluded from this study (Agatsuma *et al.*, 1987).

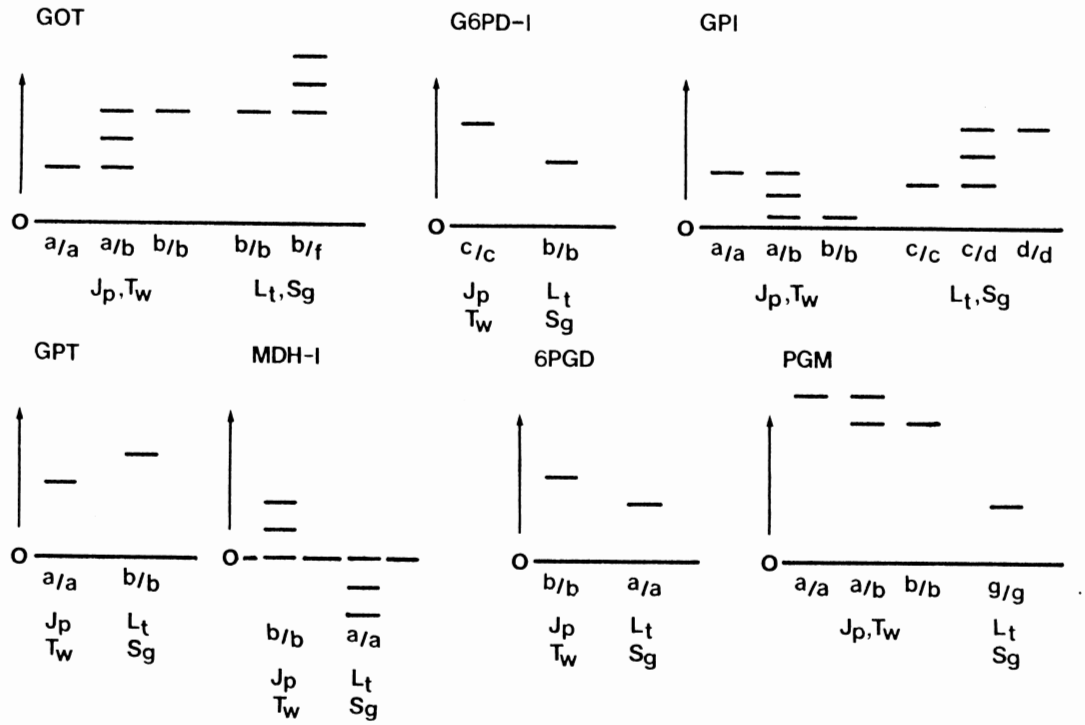

Fig. 1. Electrophoretic patterns of seven enzymes, which showed differences, in *Paragonimus westermani* from the Philippines (Lt: Leyte, Sg: Sorsogon), Taiwan (Tw: Taipin), and Japan (Jp: Ohita). Presumptive genotypes for the respective patterns were also given (a/a, a/b, and etc.,). O: origin. Arrows show electrophoretic direction

populations.

The Tsushima population was used for comparison. There was no difference between the present and previous results in the electrophoretic patterns of the triploid (Agatsuma and Habe, 1985).

Three loci, *GOT, HK* and *PGM,* were compared in the present study. No common aliele at each of the three loci was found between the two populations.

6) *Comparison of genetic structure between the Philippine and Taiwan populations.*

The Taiwan *P. westermani* was surveyed for comparison, and no difference was found in electrophoretic data between this study and previous one (Agatsuma *et al.*, 1987).

The loci showing different alleles between the two populations were *G6PD-I, GPI, GPT, MDH-I, 6PGD, PGM* and *TO-II* (Fig. 1). The other 10 loci revealed no difference in allele frequencies between the populations. Average genetic distance (*D*) was 0.5755 (Tables 2 and 3).

Discussion

In this study, *Paragonimus westermani* from the Philippines was compared with the Japanese and Taiwan *P. westermani* using enzyme electrophoresis, and the genetic distance or the degree of genetic differentiation (Nei, 1975) was estimated between them. Comparing the two local Philippine populations, the Nei's genetic distance, 0.00588, was obtained. When the Philippine populations were compared with the diploid population from Japan and Taiwan, the average genetic distances were 0.5234 and 0.5755, respectively. According to the data which have been reported in different groups of plant and animal species, the value between intra-specific local populations falls into the range of 0.013–0.058, the value between subspecies populations may be in the range of 0.163–0.306, and the value between separate species tends to be in 0.559–1.066 (Avise, 1975). Comparing these values with ones obtained from this study, the genetic distance between the two local populations was within the range of the conventional values for inter-local populations. However, the value between the Philippine and Japanese or Taiwan populations fell within the range of the conventional inter-species level. Incidentally, the value (0.1108) between Japanese and Taiwan populations fall within the inter-subspecies range (Agatsuma *et al.*, 1987). Figure 2 showed a dendrogram constructed from the data in Table 3. The dendrogram also indicates that the Philippine populations are considerably differentiated genetically from the cluster consisting the Japanese and Formosan populations. The present results, thus, correspond the previous study which showed that the Philippine *P. westermani* can easily mature even in the albino rat (Yokogawa *et al.*, 1977), and also support the statement by Ito *et al.* (1978) that the Philippine lung flukes should be

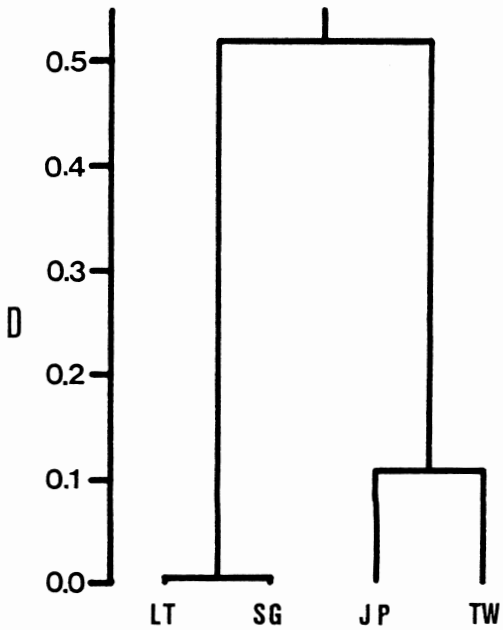

Fig. 2. Dendrogram constructed from Nei's genetic distances (*D*) (Table 3) between 4 different populations of *P. westermani* derived from the Philippines, Taiwan and Japan. For abbreviation see Fig. 1.

classified into a separate species.

The main purpose of this study was to find an unknown subspecies of *P. westermani* or its related species of *Paragonimus* which is considered as an ancestral lung fluke of the Japanese triploid *P. westermani*, that is, to detect the five set of alleles present at the 5 loci of the triploid in a certain taxon of *Paragonimus* from some habitats (Agastuma and Habe, 1985). We tried to find out 3 kinds of alleles in question at the respective three loci, GOT, HK and PGM present in the Japanese triploid. However, we failed to find any allele out of the 3 alleles in the Philippine populations. It was, thus, concluded that the Philippine *P. westermani* is very remote genetically from the Japanese diploid and triploid *P. westermani* and bears no relation to the unknown ancestral species (or subspecies) in terms of the genetic structure.

Acknowledgements

We wish to express our thanks to Dr. A. T. Santos Jr., the Director of Schistosomiasis Control and Research Service, Ministry of Health, Manila, and the staffs of Ministry of Health in Leyte and Sorsogon, the Philippines, for their excellent cooperation in this study. We are also indebted to Dr. E. G. Garcia and Dr. B. D. Cabrera, Professor and Former Professor of the Institute of Public Health, University of the Philippines, for their generous support to perform this investigation. We are greatly indebted to Dr. I. Miyazaki, Emeritus Prof. of Kyushu University and Dr. M. Yokogawa, Emeritus Prof. of Chiba University for their encouragement. We wish to thank Prof. N. Suzuki and Associate Prof. Y. Hashiguchi, Kochi Medical School, Department of Parasitology, for their invaluable comments and reviews of this manuscript.

References

1) Agatsuma, T. and Habe, S. (1985): Electrophoretic studies on enzymes of diploid and triploid *Paragonimus westermani.* Parasitology, 91, 489–497.
2) Agatsuma, T., Habe, S. and Yokogawa, M. (1987): Population genetic studies on isozymes of *Paragonimus westermani* from Taiwan and Japan. – With special reference to the origin of the triploid *P. westermani* –. Jpn. J. Parasitol., 36, 302–310. (in Japanese)
3) Avise, J. C. (1975): Systematic value of electrophoretic data. Systematic Zool. 33, 465–481.
4) Habe, S. and Miyazaki, I. (1982): On the occurrence of the bisexual type of *Paragominus westermani* (Kerbert, 1878) found in Innaimachi, Oita Prefecture, Japan. Jpn. J. Parasitol., 31, 275–280. (In Japanese)
5) Hirai, H., Sakaguchi, Y., Habe, S. and Imai, H. T. (1985): C-banding analysis of six species of lung flukes, *Paragonimus* spp. (Trematoda: Platyhelminthes), from Japan and Korea. Z. Parasitenkd., 71, 617–629.
6) Ito, J., Yokogawa, M., Araki, K. and Kobayashi, M. (1978): Studies on the morphology of larval and adult lung fluke in the Philippines, with a proposition of new name, *Paragonimus Philippinensis* n. sp. Jpn. J. Parasitol., 27, 97–112.
7) Kanazawa, T., Hata, H., Kanzaki, T., Niimura, M., Kojima, S. and Yokogawa, M. (1986): *Paragonimus westermani,* diploid type, from Otaki-machi in Chiba Prefecture, Japan. Jpn. J. Parasitol., 35, 527–533.
8) Miyazaki, I. (1977): A newly introduced question on *Paragonimus westermani* (Kerbert, 1878). Jpn. Med. J., 2788, 43–46.
9) Miyazaki, I. (1978a): *Paragonimus filipinus* sp, n. found in Leyte, the Republic of the Philippines. Med. Bull. Fukuoka Univ., 5, 5–10.
10) Miyazaki, I. (1978b): On two types of the lung fluke which has been called *Paragonimus westermani,* with proposition of the new Japanese name. Jpn. Med. J., 2819, 43–48 (in Japanese).
11) Miyazaki, I. and Habe, S. (1979): *Paragonimus westermani filipinus* MIYAZAKI, 1978, stat. n. occurring at Jaro, Leyte, the Philippines. Med. Bull. Fukuoka Univ., 6, 447–462. (in Japanese)
12) Nei, M. (1975): Molecular population genetics and evolution. North-Holland, Oxford, 288pp.
13) Nishida, H., Sakai, M., Uemoto, K., and Shibahara, T. (1981): On the lung fluke, *Paragonimus westermani* -diploid type-, found from the fresh water crab, *Geothelphusa dehaani,* in Shiga Prefecture, Japan. Jpn. J. Parasitol., 30, 331–336. (in Japanese)
14) Sakaguchi, Y. and Tada, I. (1976a): A comparative karyotype study of lung flukes, *Paragonimus ohirai* and *P. miyazakii.* Jpn. J. Parasitol. 25, 5–7.
15) Sakaguchi, Y. and Tada, I. (1976b): Chromosomes of a lung fluke, *Paragonimus westermani.* Chrom. Inf. Serv. 20, 23–24.
16) Shibahara, T. (1982): Studies on the lung fluke, *Paragonimus westermani* -diploid type-, in northern part of Hyogo Prefecture, Japan. I. Geographical distribution in Toyooka City and Izushi-gun, and morphological characteristics of

metacercariae. Jpn. J. Parasitol., 31, 545–559. (in Japanese)
17) Shibahara, T. (1986): Studies on the lung fluke, *Paragonimus westermani*-diploid type, in the northern part of Hyogo Prefecture, Japan. V. Geographical distribution in Mikata-gun and Kinosaki-gun. Jpn. J. Parasitol., 35, 17–23.
18) Shiwaku, K., Chigusa, Y., Kadosaka, T., Kaneko, K. and Kondo, S. (1986): Geographical distribution of *Paragonimus westermani* -diploid type- from *Geothelphusa dehaani* in the eastern part of Gifu Prefecture. Jpn. J. Parasitol., 35, 43–51. (in Japanese)
19) Sugiyama, H., Sonoda, J., Tomimura, T., and Nishida, H. (1983): Studies on the geographical distribution of the lung fluke, *Paragonimus westermani* (Kerbert, 1878), in the southern prefecture of the Kinki district, Japan: Observations on the incidence of encysted larvae of *P. westermani* in Geothelphusa dehaani in Higashi-Yoshino area, Nara Prefecture. Jpn. J. Vet. Sci., 45, 227–236.
20) Sugiyama, H., Sonoda, J., Okuda, M., Tomimura, T. and Nishida, H. (1984): Studies on the geographical distribution of the lung fluke, *Paragonimus westermani* (Kerbert, 1878), in the southern prefecture of the Kinki district, Japan: Observations on the incidence of metacercariae of *P. westermani* in *Geothelphusa dehaani* in Uda area, Nara Prefecture. Jpn. J. Parasitol., 33, 7–14. (in Japanese)
21) Terasaki, K. (1977): Studies on chromosomes of the lung flukes in Japan. Jpn. J. Parasitol., 26, 222–229.
22) Terasaki, K. (1980): Comparative studies of *Paragonimus westermani* (s. Str.) and *P. pulmonalis*. Jpn. J. Parasitol., 29, 239–243.
23) Terasaki, K. (1983): Karyotype of a lung fluke, *Paragonimus westermani filipinus* Miyazaki, 1978. Jpn. J. Vet. Sci., 45, 9–14.
24) Yokogawa, M., Kojima, S., Kobayashi, M., Hata, H., Kano, A. and Cabrera, B. D. (1977): Studies on the migration route of the larvae of *Paragonimus westermani* (Philippine strain) in rats. Jpn. J. Parasitol., 26, 37. (in Japanese)
25) Yokogawa, M., Sato, Y., Hasegawa, H., Takai, A., Otsuru, M., Asato, R., Kanazawa, T. and Hata, H. (1986): Studies on *Paragonimus* in Okinawa, Japan. Jpn. J. Parasitol., 35, 331–338. (in Japanese)
26) Yoshimura, H., Kondo, K., Ohnishi, Y., Akao, N., Okamoto, T. and Takamura, Y. (1983): Studies on lung fluke, *Paragonimus westermani,* found in naturally infected racoon dogs and freshwater crabs collected in mountainous area of Komatsu City, Ishikawa Prefecture. Hokuriku J. Public Health, 10, 7–13. (in Japanese)