[Jap. J. Parasit., Vol. 26, No. 4, 222-229, 1977]

Studies on Chromosomes of the Lung Flukes in Japan

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(Received for publication; May 13, 1977)

There are five species of the lung fluke in Japan such as *Paragonimus westermani* (Kerbert, 1878), *P. miyazakii* Kamo *et al.*, 1961, *P. ohirai* Miyazaki, 1939, *P. sadoensis* Miyazaki *et al.*, 1968, and *P. iloktsuenensis* Chen, 1940. They are distinguishable from one another by the characters of suckers, ovaries, testes, cuticular spines, and eggs in the adult stage, as well as morphological features of their metacercariae.

Walton (1959) phylogenetically studied on the chromosome numbers of more than 50 species of helminthic parasites and showed that they were provided with definite or basic numbers in the genus or the family level. However, there have been very few studies on the karyotype of the lung fluke. Chen (1937) reported that chromosome number of *P. kellicotti* Ward was 2n=16 and n=8 without showing its karyotype. In recent years Sakaguchi and Tada (1975, 1976a, 1976b), clearly showed the karyotype of *P. ohirai* and *P. miyazakii* to be 2n=22 and n=11 by an air-drying method (Takagi and Oshimura, 1973), and supposed that the chromosome number on *P. westermani* was thirty three (triploid).

The present author performed karyotypic analyses on all of the five species of the lung flukes in Japan using their ovaries and testes by the air-drying method for an interest of their phylogeny and cytology (Terasaki *et al.*, 1976).

Materials and Methods

As shown in Table 1, various crabs known to be the intermediate hosts of the five species of the lung fluke were collected and searched for the metacercariae. The metacercariae were experimentally given to dogs and/or rats. The mammals were sacrificed 3–6 months after infection, and adult flukes obtained were subjected to cytological examination.

A simple cell cultivation method of Ando and Uchida (1973) was modified and used. One ovary and two testes per an adult fluke were taken out with micropin under a dissecting microscope, and separately put in 2 ml of culture fluid in a conical glass for three hours at 37C. The composition of the culture medium used was: Nissan 199 (Nissan Seiyaku) 0.99 g, sodium bicarbonate 0.10 g, distilled water 100 ml, and 1 mg/ml colchicine (Nakarai Kagaku Yakuhin) 10 ml. Then each of ovary and testes was put on a slide glass with a few drops of 0.6% sodium citrate, and was broken with micropin under a dissecting microscope. Germ cells were spread so as to be scattered on the slide glass and were kept in a room temperature for thirty minutes. Then these slide glasses were put into a moisture box which contained Carnoy solution (methyl alcohol 1: acetic acid 1). After thirty minutes Carnoy solution was put on the slide glasses with a pipette. After five minutes the solution was shed and dried by blowing (air-drying method). The slides were stained with 10% Giemsa's fluid for thirty minutes. Thus, three preparations were made from an adult fluke.

Good metaphase figures of mitosis and meiosis found in each preparation were photographed under magnification of ×2,500. Twenty photographs of metaphase figures of mitosis in each species of the fluke and five of

Table 1 Materials

Species of lung flukes	Species of crabs collected	Localities of collection	Animals infected with metacercariae	Duration of infection	Number of adult flukes observed
P. westermani	*Eriocheir japonicus*	Amakusa, Kumamoto	Dogs	About 6 months	48
P. miyazakii	*Potamon dehaani*	Iwakuni, Yamaguchi	Dogs	5–6 months	15
P. ohirai	*Sesarma dehaani* *Helice tridens*	Sendai, Kagoshima	Dogs and rats	About 3 months	42
P. sadoensis	*Potamon dehaani*	Sadogashima, Niigata	Dogs and rats	About 3 months	24
P. iloktsuenensis	*Sesarma dehaani* *Helice tridens*	Sendai, Kagoshima Amami-oshima, Kagoshima	Rats	About 3 months	51

those of meiosis in the same species were used for investigation. The chromosomes were arranged in order of their sizes as shown in Fig. 1. The length of long and short arms of each chromosome at metaphase in mitosis were measured by a slide caliper. From the results, relative arm lengths (the ratio of each chromosome length to sum of all chromosome lengths) and arm ratios were calculated. In the arm ratio, the results of plotting the frequency distribution on the normal probability papers showed to be nearly a linear line in all of five species. Therefore, the frequency distribution of the arm ratios was assumed to be a normal curve. The relative arm lengths of each chromosome in meiosis was calculated as same as above.

In addition to the above mentioned method, on the testes of 10 individuals of *P.*

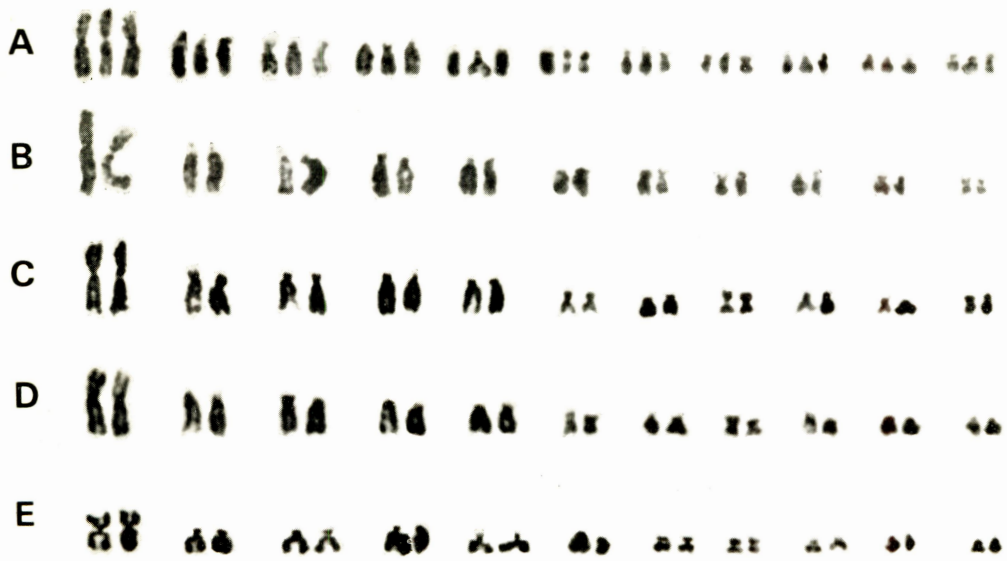

Fig. 1 Karyotypes of germ cells of the lung flukes in Japan;
A: *Paragonimus westermani*, B: *P. miyazakii*, C: *P. ohirai*, D: *P. sadoensis*, and E: *P. iloktsuenensis*.

westermani, the specimens were prepared with squash method (Makino, 1963), and observed by microscopy.

Further, sections of testes in each two individuals of *P. ohirai* and *P. westermani* were prepared, stained by haematoxylineosin, and microscopically observed.

Results

On the metaphase figures of mitosis of four species except *P. westermani*, 11 pairs of chromosomes were recognized with the air-drying method (Fig. 1). On the other hand, on almost all of the metaphase figures of mitosis in *P. westermani*, thirty-three chromosomes were recognized, made up as follows: three large chromosomes having

Table 2 Number of chromosomes observed in metaphase figures of mitosis in *Paragonimus westermani*

Number of chromosomes/cell	Number of metaphase figures (%)
34	3(3.37)
33	72(80.90)
32	11(12.36)
31	2(2.25)
30	1(1.12)
	Total 89(100.0)

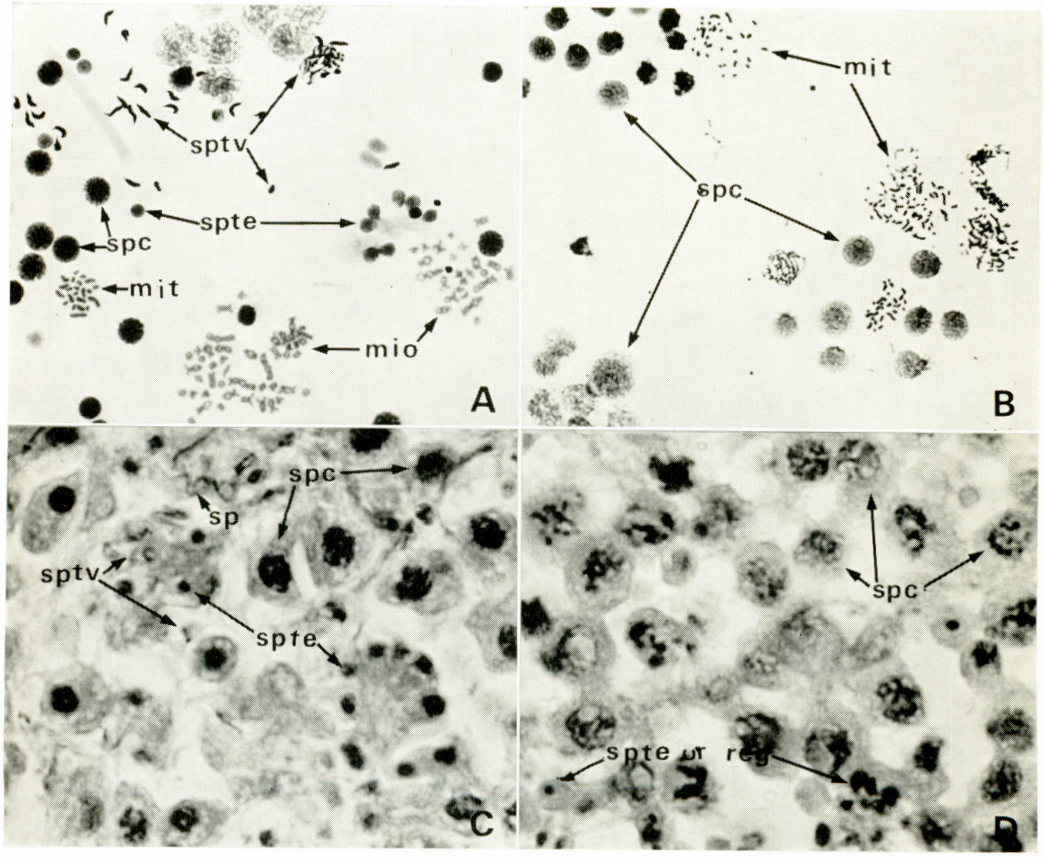

Fig. 2 Each specimen of testis on *Paragonimus ohirai* with air-drying method (A), *P. westermani* with air-drying method (B), *P. ohirai* with section method (C), and *P. westermani* with section method (D).
mio; metaphase figure in meiosis, mit; metaphase figure in mitosis, reg; cell on the regressive change (?), sp; nearly perfect sperm, spc; spermatocyte, spte; spermatid of early stage, sptv; spermatid of various stages.

Table 3 Results of chromosome measurements in the lung flukes in Japan

Pair number	*P. westermani*	*P. miyazakii*	*P. ohirai*	*P. sadoensis*	*P. iloktsuenensis*	Nomenclature by Levan *et al.* (1964)
1	20.41±1.79 (1.33±0.10)	20.12±1.43 (1.39±0.09)	19.23±1.19 (1.27±0.10)	18.86±1.11 (1.32±0.13)	19.35±1.27 (1.44±0.16)	m
2	11.97±0.63 (4.52±0.86)	11.98±0.54 (5.46±0.91)	12.38±0.45 (3.87±1.16)	12.19±0.45 (4.51±1.15)	12.94±0.48 (5.41±1.21)	st
3	11.15±0.48 (3.69±1.03)	11.09±0.39 (4.98±1.25)	11.46±0.44 (3.59±1.01)	11.46±0.48 (3.98±0.97)	11.92±0.52 (5.13±1.22)	st
4	10.16±0.42 (4.35±0.78)	10.45±0.38 (5.42±0.86)	10.75±0.53 (4.20±1.08)	10.62±0.40 (3.99±1.16)	11.12±0.64 (4.53±1.07)	st
5	9.01±0.44 (4.28±1.12)	9.86±0.34 (5.02±1.26)	9.85±0.49 (4.03±0.83)	9.94±0.53 (4.03±1.09)	10.19±0.72 (3.90±0.76)	st
6	7.39±0.43 (1.87±0.67)	6.96±0.52 (1.84±0.70)	7.09±0.36 (1.80±0.47)	7.11±0.47 (1.75±0.41)	6.72±0.39 (1.82±0.27)	smm
7	6.75±0.63 (3.02±0.84)	6.46±0.38 (2.61±0.90)	6.63±0.31 (2.59±0.84)	6.64±0.31 (2.65±0.70)	6.32±0.35 (2.89±0.41)	sm or st
8	6.38±0.49 (1.91±0.54)	6.29±0.32 (1.96±0.71)	6.26±0.34 (1.67±0.45)	6.30±0.28 (1.75±0.54)	6.03±0.27 (1.68±0.46)	smm
9	5.98±0.51 (3.35±1.15)	6.01±0.36 (2.71±1.07)	5.98±0.38 (2.47±0.82)	6.03±0.34 (2.87±1.12)	5.61±0.20 (2.67±0.60)	sm or st
10	5.57±0.52 (3.55±1.14)	5.70±0.47 (2.63±0.95)	5.48±0.32 (2.74±0.93)	5.66±0.27 (3.00±1.23)	5.06±0.31 (2.40±0.50)	sm or st
11	5.11±0.55 (1.89±0.71)	5.09±0.46 (1.60±0.34)	4.89±0.42 (1.73±0.32)	5.20±0.40 (1.44±0.35)	4.73±0.33 (1.56±0.25)	smm

The above shows averages and standard deviation of relative arm length and (arm ratio).

the centromeres at their median region were distinct, and the other chromosomes were separable into ten groups, each of which was evenly composed of three chromosomes (Fig. 1 and Table 2). Table 3 shows the average and standard deviations of the relative arm lengths and arm ratios in each chromosome at metaphase in mitosis on germ cells of the five species.

On the specimens made from the gonads of the four species by the air-drying method, eleven chromosomes were easily recognized on the meiosis figures, and spermatids of various stages of spermatogenous process were found in testes (Fig. 2, A). However, no figure of meiosis was found on all of preparations made from 48 individuals of *P. westermani* with the same method, nor transformed spermatid in the spermiogenesis could be recognized in testes (Fig. 2, B), although a few spermatoid bodies were visible in the specimens prepared by the squash method. In the sectioned specimens of *P. ohirai*, spermatocytes and spermatids of various stages from early stage of spermatid to nearly accomplished sperm were recognized as in those specimens by the air-drying method (Fig. 2, C). On the other hand, in those of *P. westermani*, the majority of the testes were occupied by spermatocytes, and spermatoid bodies seemed to be in the course of spermiogenesis and cells with concentrated nuclei were fewly recognized, but a few transformed spermatids with slender nuclei were visible (Fig. 2, D).

Discussion

In the four species other than *P. westermani*, the chromosome number has been shown to be 2n=22, on almost all of the metaphase figures of mitosis (Fig. 1 and Table 3). These numbers are the same as those of Sakaguchi and Tada (1975, 1976a) in *P. ohirai* and *P. miyazakii*. Among these four species, the metaphasic chromosomes are divided into three groups (large, medium, and small) by their sizes. According to the nomenclature recommended by Levan *et al.*

(1964), the karyotype had one pair of large-sized 'm', four pairs of medium-sized 'st', three pairs of small-sized 'smm', and three pairs of small-sized 'sm' or 'st'. The average of the relative arm lengths and arm ratios showed few differences among the four species. Further, on the average of relative arm lengths in meiosis, large difference was not found between the species as same as mitosis.

In *P. westermani*, Sakaguchi and Tada (1976b) reported that chromosome numbers were 33 in mitosis. In the present studies, the quite same results were obtained. The averages of the relative arm lengths and arm ratios of each three chromosomes of eleven groups are shown in Table 3, indicating similar values to these of the other species. These findings show that the chromosome of *P. westermani* is triploidy. No figure of meiosis was observed nor complete sperm could be recognized on any preparation of 48 individuals with the air-drying method. Further, spermatoid bodies were rarely recognized and no typical sperm could be recognized on preparations by the squash method. On the sections of testes in *P. westermani*, a few transformed spermatoid bodies with slender nuclei were visible. Examination of these specimens reveals that it can not be concluded that the cells with the more deeply staining and smaller nuclei than those of spermatocytes are homologous with real spermatids, since the spermatids are considerably resemble to the cells having regressive change such as pyknosis and karyorrhexis. However, no phagocyte-like body was recognized in any sections. It seemed that the greater part of cells in testes of *P. westermani* remained at the stage of the spermatocyte, although it could not be confirmed for few specimens whether the least part of them proceeded to the spermatids through spermatocytes or turned in the regressive change without meiosis.

Judging from absence of enough sperms for fertilizing many eggs and presence of triploidy, it is doubtful that the sperms and the eggs completely carry out fertilizations, and it seems that parthenogenesis may be carried out by *P. westermani*, as same as some nematodes (Zaffagnini, 1973) and cestodes (Jones and Mackiewicz, 1969). It is also interesting that Sakaguchi and Nakagawa (1975) reported the occurrence of the triploidy in *Fasciola* sp. in Japan.

Differences of the averages of the relative arm lengths and the arm ratios of each chromosome between each species were surveyed by t-test and the results are shown in Tables 4 and 5. The pair numbers in which significant differences are seen between two variances, are shown in the tables. Sakaguchi and Tada (1976a) reported that differences were seen in the pair Nos. 2, 3, and 7 between *P. miyazakii* and *P. ohirai*. The present results are the same with their report in the pair Nos. 2. and 3 between *P. miyazakii* and *P. ohirai*. However, as Sakaguchi and Tada (1976a) pointed out, the statistically significant differences might be accounted for the mensural artifacts due to incorrect homologue matching of certain morphologically similar members.

While, on almost specimens of testes in all of the five species subjected with air-drying method, masses of many chromosomes as same as polyploidy besides metaphase figures showing 22 or 11 chromosomes were recognized in both mitosis and meiosis (Fig. 2, A and B). It may be considered that the mass of chromosomes was derived from several cells together. However, nothing such as a mass was recognized on any specimens of ovaries with the same method. From these facts, it seems that this may depend on a difference between intercellular junctions of ovarian and testicular germ cells. This matter should be investigated in the future.

Summary

To investigate the relationship of five species of the lung flukes (the genus *Paragonimus*) in Japan, the author analyzed their karyotypes by the air-drying method using ovaries and testes. Further, the specimens made from testes of *P. ohirai* and *P. wes-*

Table 4 Results of t-test on averaged relative lengths of chromosomes of five species of the lung flukes in Japan

	P. westermani	*P. miyazakii*	*P. ohirai*	*P. sadoensis*	*P. iloktsuenensis*
P. westermani		5	4,5	(1) 4,5	2,3(4) (5)6 (9) (10)
P. miyazakii	5			1	2,3(4) (9) (10)
P. ohirai	4, 5				2,3, 6,7 (9) 10
P. sadoensis	(1) 4, 5	1			2, 7,8(9) (10)11
P. iloktsuenensis	2,3(4) (5)6(9) (10)	2,3(4) (9) (10)	2,3, 6,7(9)10	2, 7,8(9) (10)11	

Figures indicate pair numbers of chromosomes.
Level of significance : $p<0.01$
( ) : A significant difference is seen between the two variances by F-test.

Table 5 Results of t-tests on averaged arm ratios of five species of the lung flukes in Japan

	P. westermani	*P. miyazakii*	*P. ohirai*	*P. sadoensis*	*P. iloktsuenensis*
P. westermani		2,3,4			3, 5 (10)
P. miyazakii	2,3,4		1,2,3,4(5)	2, 4	(5)
P. ohirai		1,2,3,4(5)			(1)2,3
P. sadoensis		2, 4			3
P. iloktsuenensis	3, 5(10)	(5)	(1)2,3	3	

Figures indicate pair numbers of chromosomes.
Level of significance : $p<0.01$
( ) : A significant difference is seen between the two variances.

termani with the squash method and the section method were histologically investigated. Results are as follows: (1) In the four species, *P. miyazakii*, *P. ohirai*, *P. sadoensis*, and *P. iloktsuenensis*, their spermatogonial and oogonial metaphases showed 22 chromosomes (2n=22, in mitosis), including one pair of large-sized 'm', four pairs of medium-sized 'st', three pairs of small-sized 'smm', and three pairs of small-sized 'sm' or 'st' (nomenclature recommended by Levan *et al.*, 1964). The relative arm lengths and arm ratios of those chromosomes were closely related among the four species. Each of their meiotic metaphases showed 11 chromosomes (n=11). (2) In *P. westermani*, the mitotic metaphases showed 33 chromosomes, including each of 11 groups constituted with three chromosomes, and each group showed the same karyotype as the other four species. However, no meiosis was recognized and spermiogenesis was not easily confirmed. Then, *P. westermani* may be triploid, and its reproductive process may be parthenogenetic.

Acknowledgements

I would like to thank Prof. I. Miyazaki and Prof. T. Kifune of our Department, and Mr. K. Ando of Department of Anatomy of our University for critical reading of the manuscript and grateful to suggestions in the experiments. Finally, I am very grateful to Mr. K. Iwata and Miss K. Yoshino of our Department for the assistance in the experiments.

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日本産肺吸虫の染色体に関する研究

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日本産肺吸虫5種（ウェステルマン肺吸虫，宮崎肺吸虫，大平肺吸虫，佐渡肺吸虫および小形大平肺吸虫）の系統分類学的関係や細胞学的興味から，各種肺吸虫の卵巣と精巣を材料に，エアードライ法で作成した標本を観察し，核型分析を行なつた．さらに大平肺吸虫とウェステルマン肺吸虫の精巣から，おしつぶし法や切片法で標本を作り組織学的な検討を行なつた．その結果は次のとおりである．

（1） 宮崎肺吸虫，大平肺吸虫，佐渡肺吸虫および小形大平肺吸虫の生殖腺で作つた標本で，体細胞分裂の核板では22個の染色体が認められた（2n＝22）．Levan *et al.* (1964) に従えば，その核板は1対の大型の‘m’，4対の中型の‘st’，3対の小型の‘smm’および3対の小型の‘sm’または‘st’染色体から構成されていた．それらの relative arm length や arm ratio は4種の間で非常によく似ており区別し難かつた．減数分裂の中期像では11個の染色体が認められた（n＝11）．

（2） ウェステルマン肺吸虫では，体細胞分裂の中期像で33本の染色体が認められ，それらはおのおの3個ずつの相同と思われる染色体からなり，各組は他の4種の各対と同様の核型を示した．しかし，ウェステルマン肺吸虫ではいずれの標本からも減数分裂像は認められず，精巣には精細胞様の細胞がきわめて少数認められた．それらのことはウェステルマン肺吸虫が三倍体であり，その増殖過程は単為生殖であることを暗示している．