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Disc electrophoretic patterns of adult *Paragonimus iloktsuenensis* Chen, 1940, with special reference to *P. ohirai* Miyazaki, 1939

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Morphological differentiation between adult *Paragonimus iloktsuenensis* Chen, 1940 and *P. ohirai* Miyazaki, 1939 is difficult. Chen (1940) suggested that a differentiation could be made by two features: 1) the shape and arrangement of the cuticular spines and 2) the shape of the central mass in the testes (*P. iloktsuenensis* having slender central masses). However, Miyazaki (1944 a, b & 1947) stated that it was impossible to distinguish *P. iloktsuenensis* from *P. ohirai* because of similarities in their cuticular spines (i.e., shape and arrangement) and ovaries. Tanabe (1950) made a detailed investigation on the anatomy of adult lung flukes, *P. westermani*, *P. ohirai* and *P. iloktsuenensis* using solid models. He stated that it was very difficult to differentiate between *P. iloktsuenensis* and *P. ohirai* because their ovaries, cuticular spines, ratio of the testes to the ovary, and the central masses of the testes were very similar.

Isshiki (1953 & 1954) reported that the two species could be differentiated by characteristics of the intra-uterine eggs, i.e., shape of the egg, position of the maximum width of the egg, size of the egg and ratio of egg length to width, when many eggs were observed and measured.

Tomimura (1959) compared the ovary and testes of *P. iloktsuenensis* with those of *P. ohirai*. He stated that the ratio of the testes to the ovary as well as that of the length to width of the testes were markedly larger in the former than in the latter, suggesting that these features could be used to identify them even in whole mount specimens.

Yoshimura (1969 a) reported that each of the three species of adult lung flukes, *P. westermani*, *P. ohirai* and *P. miyazakii*, had a species characteristic disc electrophoretic pattern. The electrophoretic profile of *Paragonimus* sp. distributed on Sado Island (quite recently designated as *Paragonimus sadoensis* by Miyazaki *et al.* (1968)) was essentially identical to that of *P. ohirai* although slight differences in densities of several protein bands and Rf values were found between the two species (Yoshimura, 1969 b). The present studies were conducted to determine if disc electrophoresis could be utilized to establish definitive species characteristics for *P. iloktsuenensis*.

Materials and Methods

In May, 1968, a total of 129 *Potamon miyazakii* Miyake et Chiu, 1965, was collected at Alilao village in the Shimen district of Taipei County, Taiwan. Chiu (1962) first reported this area as endemic for paragonimiasis iloktsuenensis. These crabs were immediately brought to the authors' laboratory. The livers were removed and the remaining body parts cut into small pieces using a pair of scissors. The metacercariae from several crabs were morphologically determined to be *P. iloktsuenensis*. Twenty-nine adult laboratory white rats were used as the experimental definitive hosts.

They were starved for 3–5 days before infection. As described by Miyazaki (1944 a), individual rats were fed with the liver tissues and remaining parts of the bodies of 3～10 crabs (see Table 1).

All infected rats were autopsied 50 days later and the adult worms harvested. All of the worms were kept in 0.9% physiological saline solution for 4～5 hours in an incubator at 28°C. The intestinal metabolites, and eggs which were shed, were collected; the latter being used for other experiments. Worms removed from the incubator were washed twice with 0.9% physiological saline solution and once with distilled water before lyophilization.

Adult worm saline extracts were made by homogenizing 50 mg of the dry worm in 1 ml of 0.9% physiological saline solution first with a motor driven tissue grinder and then twice with a microhomogenizer (50,000 rpm/30 seconds) suspended in an ice water bath. The resulting homogenate was centrifuged twice at 1,500 rpm for 5 minutes at 2°C. The supernatant obtained was used in this study.

Disc electrophoresis was performed by the method described by Ornstein & Davis (1962) and Davis (1964) using a standard 7.5% acrylamide lower gel. The electrophoresis apparatus was designed according to a modification described by Davis & Lindsay (1967). The details of the electrophoretic technique employed in this study have been previously described by Yoshimura (1968).

The protein value of the whole worm saline extract was 35.06 mg/ml as determined by the Biuret reaction.

The best protein separations were observed when the extracts were diluted with the 5% acrylamide spacer gel (in contrast with usual 3.75% spacer gel) at ratios of 1:5～1:7. These dilutions were employed throughout this study. From the protein value previously determined, the amount of protein placed in each gel column was 584 μg for the 1:5 dilution and 438 μg for the 1:7 dilution.

Each run consisted of 8～9 gel columns charged with the worm saline extracts and 1 or 2 columns with normal human sera, the latter being used as a control to check on the gel condition.

Ten good gel columns with protein migrations of 34.5～35.5 mm were selected for analyses. The methods for analyzing the data have been previously reported by Yoshimura (1968). Results reported previously by Yoshimura (1969 a, b) for *P. westermani*, *P. ohirai*, *P. miyazakii* and *P. sadoensis* were utilized for comparative studies.

Results

The results of experimentally infecting rats with *P. iloktsuenensis* are summarized in Table 1. Two negative rats were found in the group fed with 4 crabs. All of the remaining rats yielded adult worms. The mean infection rate was 93.1% (27/29) and the mean worm burden per rat was 6.6 (Table 1).

A total of 18 electrophoretic runs was made. A minimum of 23 distinct protein bands was separated, of which 19 had a very high rate of reproducibility, i.e., identified from 100% of the gels. The mean Rf values, occurrence frequencies for the separated bands, and the standard deviations of the Rf values for the prominent bands are listed in Table 2.

Numerical identification of the bands separated from the present species was made by comparing Rf values, electrophoretic patterns and staining qualities with similar bands from *P. ohirai*, *P. westermani* and *P. miyazakii*, which were previously established by Yoshimura (1969 a).

Few variations were observed in the electrophoretic patterns (A-C of Fig. 1). A faint band 21a was found in only 30% of the gels and appeared to be separated from band 22 which was very blurred. When band 21a was detected, then band 22 appeared as a narrow, sharp disc (A of Fig. 1). A faint band 8 was observed between bands 7 and 9 in 50% of gels (A & C of Fig. 1). A faint band 11 was seen between bands 10 and 12 in only 30% of the gels (C of Fig. 1). Band 9a was fuzzy in appearance and seemed to be separated from band 10 which had a very high density with a wide disc. Both bands 13 and 15 were usually

Table 1 Experimental infection of rats with *Potamon miyazakii*

Groups			No. of rats		Total No. of worms recovered (range)	No. of worms recovered per rat
No. of crabs used to infect each rat	Ranges of crab size in mm (mean)	No. of crabs used	Infected	Positive (%)		
3	12-25 (20.0)	3 (♂2, ♀1)	1	1 (100)	3	3.0
4	7-31 (21.9)	100 (♂75, ♀25)	25	23 (92.0)	155 (0-24)	6.7
8	11-17 (14.6)	16 (♂14, ♀2)	2	2 (100)	17 (8-9)	8.5
10	11-14 (12.6)	10 (♂10)	1	1 (100)	4	4.0
Total		129 (♂101, ♀28)	29	27 (93.1)	179	6.6

Table 2 Mean Rf values for the protein bands separated from adult *Paragonimus iloktsuenensis*

Protein bands	Mean Rf values	S D	Occurrence frequency of band (%)
1	0.011		100
*2 (A)	0.040	0.002	100
4	0.068		100
5	0.082		100
6	0.113		100
7	0.157		100
8	0.181		50
*9 (C)	0.213	0.008	100
9a	0.239		20
*10 (C-1)	0.271	0.017	100
11	0.310		30
*12 (E)	0.380	0.017	100
13	0.435		100
15	0.492		100
16	0.532		100
*18 (H-1)	0.570	0.011	100
*19 (H-2)	0.625	0.017	100
20	0.651		100
21a	0.716		30
22	0.755	0.026†	100
*25 (K)	0.842	0.018	100
26	0.914		100
27	1.000		100

* Prominent band.
SD Standard deviation.
† Greatest deviation.
Letters in parentheses correspond to prominent peaks in the densitometric tracings.

faint. In only 10% of the gels, a very faint disc was observed between bands 26 and 27.

A minimum of 7 prominent peaks was always obtained in the densitometric tracings (A-C of Fig. 1). These peaks correspond to A, C, C-1, E, H-1, H-2 and K shown in Fig. 1. Prominent peaks are identified C-1, H-1 and H-2 to indicate that they have significantly different Rf values compared with densitometrically prominent peaks characterizing other species of *Paragonimus* labeled C and H. This does not mean that other taxa are devoid of bands at Rf values analogous to C-1, H-1 and H-2. When they occur in other species, the bands do not show appreciable densities enough to characterize the species in contrast with the bands labeled C-1, H-1 and H-2 from *P. iloktsuenensis*.

Comparisons of the protein bands obtained from the four species of adult lung flukes, *P. iloktsuenensis*, *P. ohirai*, *P. westermani* and *P. miyazakii*, are summarized in Table 3.

Discussion

The electrophoretic characteristics of *P. iloktsuenensis* are distinctly different from those of *P. ohirai*, *P. westermani*, *P. miyazakii* and *P. sadoensis*, which had been previously established by Yoshimura (1969 a, b). Special attention was given to a comparison between *P. iloktsuenensis* and *P. ohirai* since their morphological features have been reported to be very similar. The major differences between both species are observed in the mid-gel region between bands 10～22. Bands 16～21 are

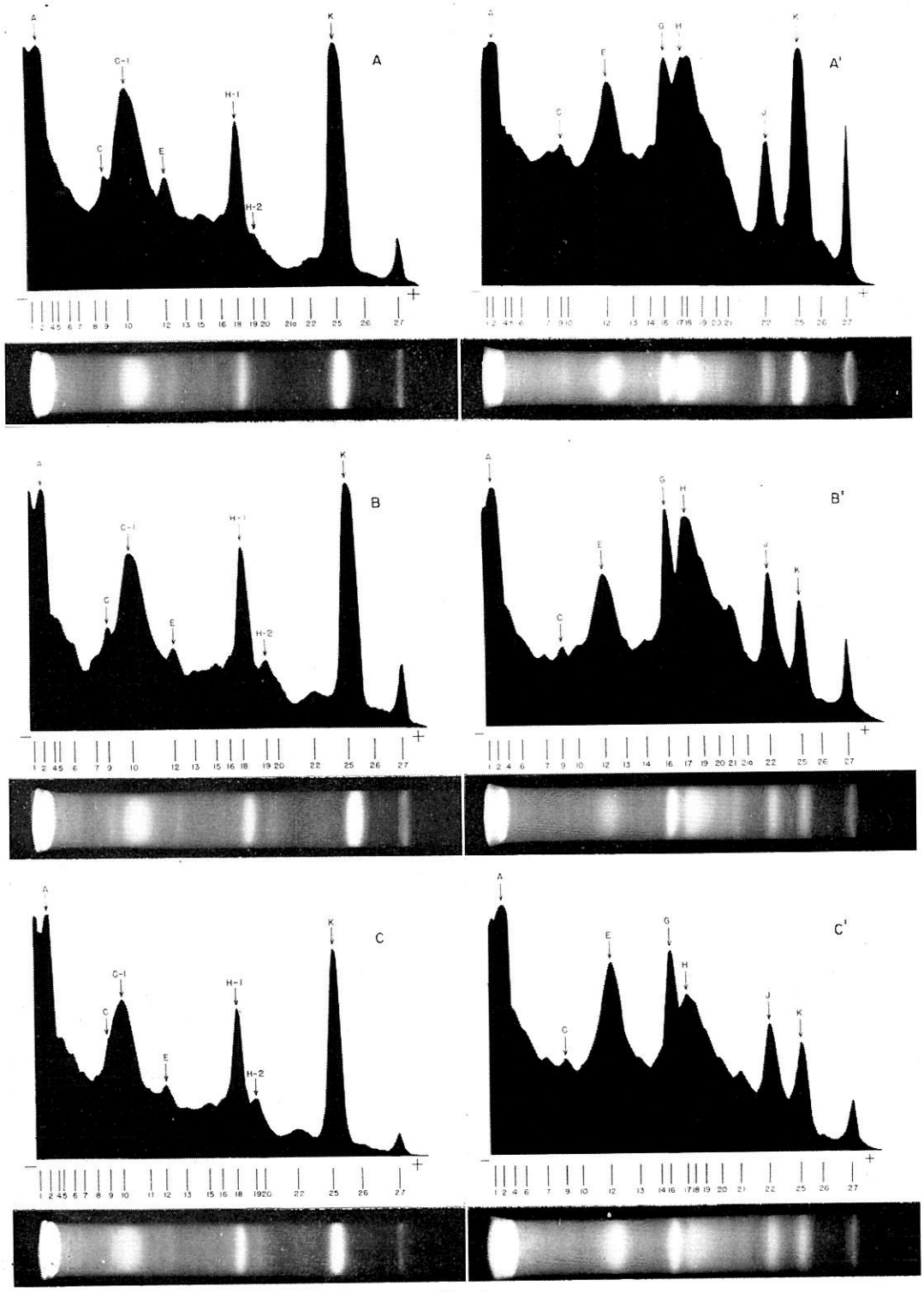

Fig. 1

Table 3 Comparisons of the protein bands identified from four species of adult lung flukes, *Paragonimus iloktsuenensis*, *P. ohirai*, *P. westermani* and *P. miyazakii*

Protein bands	*P. iloktsuenensis* from rats Mean Rf	*P. ohirai*† from rats Mean Rf	*P. ohirai*† from cats Mean Rf	*P. westermani*† from cats Mean Rf	*P. miyazakii*† from cats Mean Rf
1	0.011	0.014	0.016	0.012	0.013
1a	—	0.033	0.023	—	—
2	*0.040 (A)	*0.035 (A)	*0.044 (A)	*0.045 (A)	*0.035 (A)
3	—	—	—	—	0.050
4	0.068	0.069	0.076	0.074	0.068
5	0.082	0.083	0.087	—	0.090
6	0.113	0.105	0.110	0.113	—
7	0.157	0.176	0.175	0.162	*0.159 (B)
8	0.181	—	—	0.190	0.193
9	*0.213 (C)	*0.218 (C)	*0.223 (C)	*0.236 (C)	—
9a	0.239	—	—	—	—
10	*0.271 (C-1)	0.272	0.263	—	0.262
11	0.310	0.314	0.307	0.293	*0.291 (D)
12	*0.380 (E)	*0.347 (E)	*0.343 (E)	*0.358 (E)	0.363
12a	—	—	0.361	—	—
12b	—	—	—	—	0.414
13	0.435	0.419	0.424	—	*0.444 (F)
14	—	0.466	0.459	0.457	—
15	0.492	—	—	0.489	0.491
16	0.532	*0.506 (G)	*0.508 (G)	0.530	+
17	—	*0.556 (H)	*0.561 (H)	—	0.555
18	*0.570 (H-1)	0.574	0.578	0.581	—
19	*0.625 (H-2)	0.610	0.619	0.608	0.615
20	0.651	0.649	0.657	*0.654 (I)	*0.660 (I)
21	—	0.694	0.690	0.674	—
21a	0.716	0.725	—	0.720	—
22	0.755	*0.782 (J)	*0.775 (J)	*0.779 (J)	0.752
23	—	—	—	0.814	0.793
24	—	—	—	0.834	—
25	*0.842 (K)	*0.871 (K)	*0.867 (K)	*0.877 (K)	*0.877 (K)
26	0.914	0.932	0.935	0.946	0.936
26a	+	+	+	+	+
27	1.000	1.000	1.000	1.000	1.000

† Previous data described by Yoshimura (1969a).

* Prominent band.

Letters (A—K) correspond to prominent peaks in the densitometric tracings.

Fig. 1 Comparison of densitometric tracings of disc electrophoretic patterns from *Paragonimus iloktsuenensis* (A—C) and *P. ohirai* (A′—C′) adult worms obtained from rats.
The bands are numbered below the densitometric tracings and the most characteristic bands for the species are lettered.

high density components in *P. ohirai* while, in *P. iloktsuenensis*, this area consists of relatively low density components, except for band 18 (Fig. 1). Conversely, both species have similar patterns at each end of the gel column. Two prominent bands (A and K) identified from these regions appear to be genus specific proteins as previously pointed out by Yoshimura (1969 a). In the densitometric tracings, the two species yield at least seven prominent peaks, of which four (A, C, E and K) are common to both species (Fig. 1). The remaining three peaks (C-1, H-1 and H-2) are characteristics of *P. iloktsuenensis*. Band 18, corresponding to peak H-1 in *P. iloktsuenensis*, is also a high density component of both *P. ohirai* and *P. sadoensis*. These peaks were never seen in *P. westermani* and *P. miyazakii* (see Yoshimura, 1969 a).

Band 22 from *P. iloktsuenensis* is markedly low in density when compared to band 22 (peak J) from *P. ohirai* and a significant difference in Rf value was found ($p<5.0\%$). However, no significant difference was found in the Rf values of band 22 when comparisons were made between *P. iloktsuenensis* from rats and *P. ohirai*, *P. westermani* or *P. miyazakii* from cats. Thus, this band can probably be regarded as identical in all four species (Table 3). The patterns of both bands 22 and 25 are somewhat similar to those from *P. miyazakii* and *P. sadoensis* (see Yoshimura, 1969 a, b).

Rf values of both bands 25 (peak K) and 26 were significantly different from those of *P. ohirai* at the respective levels of $p<0.1\%$ and $p<1.0\%$. However, both bands appear to be closely related based on their staining qualities and over all electrophoretic patterns. The Rf value of band 25 from *P. iloktsuenensis* is very close to the value (0.844) from *P. sadoensis* (Yoshimura, 1969 b). These observations are supported by Sibley (1960) who stated that "...electrophoretically identical peaks in different species of the same genus result from proteins nearly identical in basic structure." However, the final identification of individual bands must be made by analyzing the separated fractions with various biochemical or immunochemical techniques in the future since the same Rf values for bands in different species may not necessarily mean homology and vice versa when differences in Rf values are not too large.

Natural intermediate hosts for *P. iloktsuenensis* in Taiwan are different from those in both Japan and main land (Canton) China; the intermediate hosts for the former are a fresh water snail, *Tricula chiui* Habe et Miyazaki, 1962 (Miyazaki & Chiu, 1962) (quite recently designated as *Oncomelania hupensis chiui* by Davis (1967 & 1968)) and a fresh water crab, *Potamon miyazakii* (Chiu, 1962) whereas those for the latter two areas are two brackish water snails, *Assiminea lutea* A. Adams (Chen, 1940) and *A. parasitologica* Kuroda, 1958 (Tomimura *et al.* 1960) and three species of brackish water crabs, *Sesarma dehaani* H. Milne-Edwards (Chen, 1940; Miyazaki, 1944 a), *S. sinensis* H. Milne-Edwards (Chen, 1940) and *Helice tridens tridens* de Haan (Mannoji, 1952). Chiu and Tsai (1964) studied the parasitism of *P. iloktsuenensis* metacercariae in *Potamon miyazakii* crabs, stating that there was no significant difference in metacercarial infestations between these crabs and brackish water crabs. Chiu (1965) studied the larval development of the species in *T. chiui*. He reported that regardless of the type of snail (fresh water or brackish water), there was no significant difference in the larval development and morphology. Sibley (1960) stated that "protein structure is genetically determined." Thus, it can be assumed that the Japanese strain would have identical electrophoretic characteristics with the strain of *P. iloktsuenensis* from Taiwan. The patterns for the Japanese strain must be established to determine whether the difference in two intermediate hosts can significantly affect the worm protein components.

From the present data, it can be concluded that the four species of lung flukes, *P. westermani*, *P. ohirai*, *P. miyazakii* and *P. iloktsuenensis*, can be easily differentiated by their disc electrophoretic patterns and that disc electrophoretic procedures can be employed as

an aid for the identification of these lung flukes.

Summary

The present studies were conducted to determine if disc electrophoretic patterns of adult *Paragonimus iloktsuenensis* could be used for identification of the species.

Electrophoresis was made on whole worm saline extracts prepared from lyophilized worms.

A total of 23 distinct protein bands was separated, of which 19 were highly reproducible. In the densitometric tracings, a minimum of 7 prominent peaks was always identified. This species is characterized by the presence of prominent protein bands found in the midgel region.

The patterns of *P. iloktsuenensis* are distinctly different from those of *P. westermani*, *P. ohirai*, *P. miyazakii* and *P. sadoensis* which were previously established by Yoshimura (1969). Thus, it is suggested that electrophoretic patterns can be employed as a mean of differentiating *P. iloktsuenensis*.

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小型大平肺吸虫（*Paragonimus iloktsuenensis* Chen, 1940）成虫のディスク電気泳動像，特に大平肺吸虫（*P. ohirai* Miyazaki, 1939）との比較について

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小型大平肺吸虫（*Paragonimus iloktsuenensis* Chen, 1940）成虫のディスク電気泳動像が本種の分類学上の種的標徴となり得るかどうかを検討するため本実験を行なつた．特に成虫の形態学的特徴が相互に酷似する大平肺吸虫との比較に重点を置いた．

中華民国台湾省台北県石門郷阿里荖村において採集したミヤザキサワガニ（*Potamon miyazakii*）の肝臓ならびに細切した体部をラットに試食感染させて成虫を得た．電気泳動実験には凍結乾燥虫体の0.9％生理食塩水抽出液を用いた．

本種からは合計23本の明瞭な蛋白泳動帯が分離され，その中19本は再現性の極めて高いものであつた．densitometric tracings においては，少なくとも7本の著明なピークが認められた．本種はこれらの著明蛋白分画の存在によつて特徴づけられる．Yoshimura（1969）の報告した *P. westermani*, *P. ohirai*, *P. miyazakii* および *P. sadoensis* の泳動像と比較したところ，本種の泳動像はこれら4種のいずれとも明らかに異つており，特にゲルの中央部において明瞭な差異が認められる．従つて大平肺吸虫ならびに小型大平肺吸虫の成虫はディスク電気泳動像の差異によつても容易に区別する事ができる．