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Electrophoretic, immunological and biological properties of a population of *Semisulcospira* transmitting *Paragonimus westermani* in Japan

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Introduction

Paragonimus westermani, the human lung fluke, is Asian pandemic. Reports indicate that certain genera in the 2 cerithiacean families Pleuroceridae and Thiaridae are implicated in the role of first intermediate host for this helminthic parasite. In Japan and Korea subgeneric taxa of the pleurocerid genus *Semisulcospira* are involved, particularly, *S. libertina* (Gould).

Species of *Semisulcospira* were described on the basis of adult conchology without other characters until 1960. Itagaki (1960) presented a general gross anatomy of *S. libertina*. Kajiyama & Habe (1961) made excellent use of embryo shells to aid in species discrimination. Burch & Davis (1967) discovered that a complex cytological situation existed within the genus *Semisulcospira* and that a number of different karyotypes enabled a discussion of fundamental subgeneric taxa. Davis (1969) discussed 10 taxa of *Semisulcospira* correlating embryo shell morphology, intra-brood chamber embryo shell growth patterns and adult shell characters; all data were grouped according to previously reported cytological findings.

With these recent findings it is becoming more possible to define species of *Semisulcospira* so that a unique genotype is understood which will encompass biochemical, physiological, cytological and developmental factors. An understanding of these factors is necessary to assess the genetic relationship of infected populations within a framework of species concepts derived from biosystematic studies. Such an assessment is basic to understanding mechanisms governing parasite transmission.

The genetic variability within the species *Semisulcospira libertina* is unknown. Abbott (1948) pointed out that the species is "... extremely variable, and until larger collections are made, the nomenclature of its many forms, races and subspecies will remain confused." Abbott (1948) placed 17 named taxa from Taiwan, Japan and Korea in synonymy under *S. libertina*. Kuroda (1963) ranked *S. libertina* along with 14 other named Japanese taxa under *S. bensoni*. Justification for synonymy was not given. Accordingly, the extent of genetic variability included under the name *S. libertina* (*S. bensoni*) is not known.

Topotypes of *S. libertina* were collected in large numbers from Shimoda and characterized in terms of cytology (Burch & Davis, 1967), embryo and adult shell morphology (Davis, 1967 b, 1969), electrophoretic and immunological data (Davis, 1967 b). Accordingly, something of the genetic makeup of genuine *S. libertina* is now known. In November, 1967, parasitologists of the 406th Medical Laboratory crushed 780 *Semisulcospira* snails collected from Nahari Town, Kochi Prefecture, Shikoku and found a 0.26 per cent infection with *Paragonimus westermani*. The purpose of this paper is to present malacological data derived from the Nahari population of snails and compare these data with those derived from topotype *S. liber-*

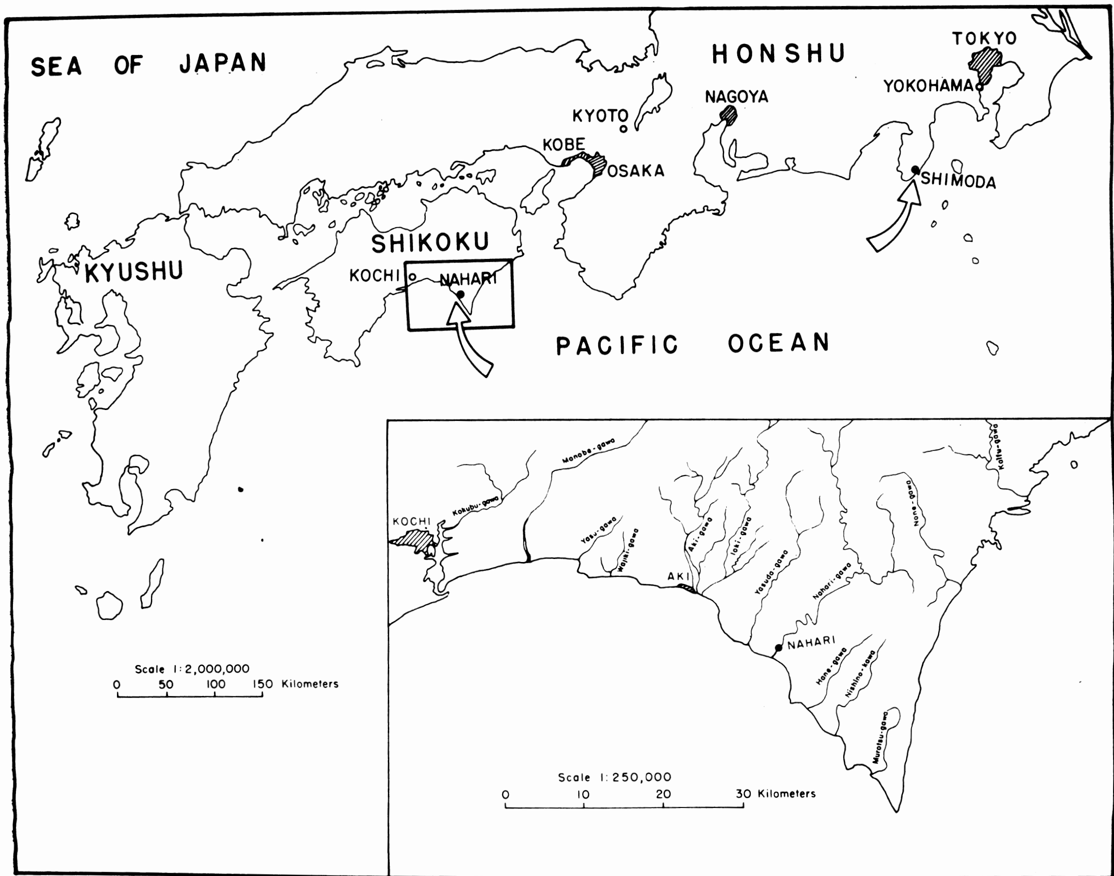

Fig. 1 The localities of the Shimoda population on Honshu and the Nahari population on Shikoku are indicated by the arrows. The inset shows an enlargement of the Nahari region.

tina. Factors included are 1) an analysis of adult shell features; 2) embryo shell morphology and intra-brood chamber development patterns; 3) cytological information on chromosome number; 4) electrophoretic analysis of proteins from foot muscle extract; 5) an immunological analysis. The genetic relationship of the Nahari population to topotype *S. libertina* and other species of the genus in Japan is discussed.

Materials and Methods

Source of Snails:

Snails were collected from Japan, Shikoku, Kochi Prefecture, Nahari Town (Fig. 1) on 8 May and 5 June, 1967. Over 1,000 living snails were collected on both occasions from a narrow stream running parallel to the Nahari River. Snails were brought to the laboratory for subsequent tests. The topotype locality of *S. libertina* at Shimoda is shown in Figure 1; conchological data from topotype snails were previously presented (Davis, 1967 b, 1969).

Cytology:

A number of snails were removed from their shells. The gonad together with the digestive gland was fixed and preserved in Newcomer's (1953) fluid. Gonadal tissue was prepared for studies of the chromosomes using the La Cour (1941) acetic—orcein squash technique. Observations were made at magnifications of 100X and

1000X using a Nikon compound microscope and 10X oculars. Drawings were made with the aid of a camera lucida.

Shell Morphology:

The largest 10 per cent of the population was selected for each 1,000 snails on the criterion of length of body whorl as discussed by Davis (1969). A series of snails was chosen at random from these large snails for studies of adult shell morphology and embryo shell characters. Snails for the former analysis were boiled in commercial Clorox (5.25 per cent sodium hypochlorite) which cleared the shell of all organic and inorganic deposits and enabled a study of detailed shell sculpture and color patterns (technique of Walter, 1962). Measurements and observations were made on the spire increment angle, number of basal cords, length of the body whorl, length and width.

To study embryos, adult shells were cracked open and the animal removed. The pallial brood pouch of those females containing embryos was dissected free and placed, one per vial, in Clorox. The Clorox destroyed all eggs and tissue leaving only the cleaned embryo shells. The number of embryo shells per female was recorded as were the following; length and width of embryo at each whorl stage, length of the body whorl of the shells at each whorl, sculptural characteristics.

Electrophoresis:

Snails from Shimoda and Nahari were studied using freshly prepared extracts of foot muscle and the polyacrylamide electrophoretic technique used by Davis (1967 a, b, 1968 and Davis & Lindsay, 1967). Components separated electrophoretically were stained for proteins. A total of 35 experiments was conducted with snails from Nahari. Each experiment consisted of simultaneously "running" 10 to 12 tubes containing aliquots of a single extract. Of these, 15 runs were selected for densitometric analysis on the basis of their being excellent runs, i.e. the human blood serum controls were normal, staining quality was excellent and the bands were sharp and straight. In all, 40 experiments were conducted with snails from Shimoda and of these, 12 were chosen, as above, for densitometric analysis.

The physical characteristics of the experiments were as follows. The standard 7.5 per cent acrylamide gel was used. Potassium ferricyanide was not used to inhibit polymerization of the lower gel and accordingly the N,N,N,N', tetramethylethylenediamine was reduced to 0.03 ml per 100 ml mixed lower gel. A tris-glycine buffer was used (pH 8.2–8.4). The current was maintained at 5 ma per tube. Gels were stained in amidoschwartz for 2 hours and destained in 8 per cent acetic acid (electrically). Gels were stored in tubes with 8 per cent acetic acid.

Densitometric tracings were made using a Photovolt Corp. Densicord Densitometer—542 using a quasi logarithmic setting of D-1. Separated components were analyzed both in terms of overall densitometric pattern and Rf values (ratio of distance from origin to a given band and origin to front). Rf values were calculated as in Davis & Lindsay (1967). Total experimental error was calculated to be equivalent to an Rf of 0.016 of which 0.014 was attributed to measurements in calculating the Rf value (Davis & Lindsay, 1967). Fractions in different gels which differed by a Rf or 0.016 or less were not considered significantly different. Table 9 was constructed on this basis.

Immunology:

Antigens—The same extract used for electrophoresis was employed as a source of antigens. All experiments (except where noted otherwise) were conducted using lyophilized antigens. Extracts from fresh foot muscle tissue were pooled and the protein content determined using the Biuret reaction (fide Kabat and Mayer, 1961) prior to lyophilization (see Davis, 1968). The stock of lyophilized antigen was maintained at $-20°C$. In experiments the lyophilized extracts were reconstituted with distilled water and so adjusted as to have equal protein concentrations between 5.0 to 6.0 mg per ml.

Antiserum—Antisera were produced in 9 rabbits (6–7 lbs, albino, virgin female). Pre-

injection control serum was obtained. Extracts were injected intravenously (IV) or subscapular (SS) according to schedules given in Tables 1 and 2. Note that 2 rabbits (A and B) were injected using freshly prepared foot-muscle extract. SS injections involved emulsification of extract with an equal volume of Freund's complete adjuvant. Bleedings were from the ear as indicated in the Tables. Titers of the antisera were determined as discussed by Davis (1967 b). In this paper as in that of Davis (1967 b) excellent antisera produced 6 or more precipitin systems; good antisera produced 3 to 5; poor antisera, 2 or less.

Micro-Ouchterlony Diffusion—Techniques involving microdiffusion systems were previously presented by Davis (1967 b, 1968). Glass rings (22 mm inside diameter) were cemented on microscope slides with a paraffin wax mixture. Wells formed by the rings were washed with

Table 1 Schedule for injecting 5 rabbits (A to E) with antigens extracted from foot muscle tissue of Shimoda topotype *Semisulcospira libertina*, and titers of the antisera obtained

Day	Fresh extract		Lyophilized extract		
	A	B	C	D	E
1	4.5 mg	5.0 mg	5.0 mg	5.0 mg	5.0 mg
3	5.0	5.0	5.0	5.0	5.0
5	8.5	7.0	10.4	10.4	10.0
7	11.0	11.0	10.4	10.4	10.0
9	14.5	12.5	10.4	10.4	10.0
rest	21 days	21 days	15 days	15 days	21 days
1	4.5	5.0	5.2	5.2	5.0
3	5.0	5.5	5.5	5.2	5.0
5	8.5	10.0	10.4	10.4	10.0
7	11.0	10.0	10.4	10.4	10.0
9	14.5	12.0	10.4	10.4	10.0
12	bleed	bleed			
13+14			bleed	bleed*	bleed
rest		(6 months)		(30 days)	
1		s.s. 70.0 mg		s.s. 40.0 mg	
rest		8 days		14 days	
1		7.5 mg		5.0 mg	
3		9.0		5.0	
5		12.5		10.0	
7		15.5		10.0	
9		15.0		10.0	
12		bleed*		†bleed (13+14d)	
Titer:					
30 min.	1/128	1/128*	1/512	1/256* (1/256)†	1/256
60 min.	1/256	1/256*	1/512	1/512* (1/256)†	1/512
Antiserum quality	excellent	excellent	good	good	excellent

s.s.: subscapular d: day

Table 2 Schedule for injecting 4 rabbits (F to I) with lyophilized antigens extracted from foot muscle tissue of Nahari *Semisulcospira* and the titers of antisera obtained

Day	Rabbit F	G	H	I
1	2.0 mg	2.0 mg	2.0 mg	2.0 mg
2	2.0	2.0	2.0	2.0
3	2.0	2.0	2.0	2.0
4	2.0	2.0	2.0	2.0
5	2.0	2.0	2.0	2.0
6	2.0	2.0	2.0	2.0
7	2.0	2.0	2.0	2.0
rest	21 days	21 days	21 days	21 days
1	2.0 mg	2.0 mg	2.0 mg	2.0 mg
2	2.0	2.0	2.0	2.0
3	2.0	2.0	2.0	2.0
4	2.0	2.0	2.0	2.0
5	2.0	2.0	2.0	2.0
6	2.0	2.0	2.0	2.0
7	2.0	2.0	2.0	2.0
11+12	bleed	bleed	bleed	bleed
Titer 30 min	1/128	1/256	1/256	1/256
60 min	1/256	1/512	1/512	1/512
Quality	good	excellent	good	excellent

a 1 : 1,000 Siliclad Solution, rinsed in distilled water and dried. A 1 per cent solution of special Agar Noble (0.45% of saline; 1 : 10,000 merthiolate) was pipetted into the rings (2.5 ml/ring). The agar template used to cut three patterns was from a design of Dr. George M. Nace, University of Michigan, Ann Arbor, Midhigan, U. S. A.

In the 5 hole pattern (Fig. 9, Pl. 5) each well has a 3 mm diameter (volume of 46 μl). The 7 hole pattern (Fig. 9) has a center well of 4.5 mm diameter and a capacity for 96 μl; the satellite wells have a 3 mm diameter. Wells of the diamond pattern (Fig. 9) are 4 mm diameter and hold 82 μl.

Absorption Technique—Absorbed and unabsorbed antisera were used. Absorption was accomplished as follows. 1) 1.5 ml of antiserum was added to 5.0 to 6.0 mg lyophilized heterologous antigen along with 0.5 ml of distilled water. The solution was kept at room temperature 1 hour with intermittent shaking and then refrigerated for 12 to 15 hours after which the precipitate was removed by centrifugation (375 $\times$g for 5 min.). 2) Heterologous antigen (1 ml) was added to prepared sheep red blood cells (9×10^6 cells per ml) and the mixture was incubated 1 hour at 37°C with intermittent shaking after which time the cells were recovered by centrifugation. 3) The partially absorbed antiserum from step 1 was added to the antigen coated cells and the mixture was maintained at room temperature for 1 hour with intermittent shaking after which it was refrigerated for 12 to 15 hours. 4) The cells were removed by centrifugation and discarded; the completely absorbed antiserum was ready for experimental use.

In the micro-Ouchterlony diffusion systems, once antigens and antisera were added to the

wells, the plates were maintained at 23±2°C for 2 days after which the agar discs were removed from the rings, sliced in half, washed with 0.05 per cent cadmium sulfate for 5 minutes and subsequently washed in barbital buffer (pH 8.6) for 2 to 3 days (buffer changed thrice daily). The gels were then stained in amidoschwartz for 2 hours and destained in 8 per cent acetic acid; they were subsequently washed in water, mounted and dried on microscope slides.

Immunoelectrophoresis—Microscope slides (75 ×25 mm) were wiped with lens paper soaked in 0.5 per cent Siliclad, dried, and evenly covered with 4.0 ml of 2 per cent special Agar Noble (made with barbital buffer pH 8.2, 0.1 ionicity [Crowle, 1961] and 1 : 10,000 merthiolate). Wells cut into the agar were 4 mm diameter (25 μl capacity). The slot cut between the wells was 3 mm from the edge of each well.

A voltage gradient of 6–7 v/cm was maintained across each slide. Ice packs served to maintain the temperature at 14±2°C. The current was maintained for 75 min. The buffer was that given above. With termination of a run, the slots were cleared of agar and antiserum was added. Slides were kept in moist chambers at 23±2°C for 36 hours, after which they were soaked in 0.05 per cent cadmium sulfate solution for 5 minutes, washed in buffer for 2 days and then dried at room temperature. Completely dried slides were stained in amidoschwartz for 1 hour, destained in 8 per cent acetic acid, rinsed in water and dried.

In all, 25 immunoelectrophoretic experiments were conducted using 10 to 12 slides each experiment.

Results

1. Shell Morphology:

Adult Shells—Statistics of shell features measured or counted involving Nahari and Shimoda populations are given in Table 3. Adult shells are shown in Fig. 5 (Pl. 1) (Nahari) and Fig. 6 (Pl. 2) (Shimoda, a—d; Amami-Oshima, e—f). The snails from Amami-Oshima are also considered to be *S. libertina* (see discussion of Davis, 1969).

The shell parameters studied were not significantly different. Ribbed shells were not present in these populations (only 3 per cent of the shells from Shimoda had a trace of ribbing, Fig. 6a). Spiral cords were prominent only on shells from Amami-Oshima. In all the populations the basal cords are the most pronounced with an average of 8.7 to 9.3 in number.

There were differences between topotype *S.*

Table 3 Comparison of adult shells of Shimoda and Nahari *Semisulcospira* in terms of basic shell features and dimensions (mm)

Populations	Shimoda (N=25)			Nahari - I (N=16)			Nahari - II (N=16)		
Statistic	X	S	Se	X	S	Se	X	S	Se
Spire angle	22.2	3.13	0.63	22.3	2.75	0.69	22.2	3.44	0.86
Basal cords	9.2	1.55	0.31	8.7	1.30	0.33	9.3	1.41	0.35
Length B.W.	19.2	1.67	0.33	20.0	1.21	0.32	20.5	0.97	0.24
No. Whorls (eroded)		4 to 5			3 to 4			3 to 4	
Shell length	28.6	—	—	29.4	—	—	30.8	—	—
Shell width	13.0	1.29	0.26	13.3	0.69	0.17	13.5	0.63	0.16

N no. of snails; S standard deviation; B.W. body whorl
X mean; Se standard error of the mean

Table 4 Comparison of adult shells of Shimoda and Nahari *Semisulcospira* in terms of color patterns

Banding patterns	Shimoda (N=1000)		Nahari - I (N=100)		Nahari - II (N=100)	
	% of population		% of population		% of population	
	Shell predominantly: Yellow	Purple	Shell predominantly: Yellow	Purple	Shell predominantly: Yellow	Purple
1 Uniform color	60.0	0.0	23.0	10.0	26.0	10.0
2 1 band, adapical or basal	9.0	0.0	0.0	0.0	6.0	0.0
3 2 bands, mid and adapical or mid and basal	0.5	0.0	9.0	0.0	0.0	0.0
4 2 bands, adapical and basal	15.5	0.0	5.0	6.0	11.0	7.0
5 3 bands	15.0	0.0	24.0	23.0	15.0	25.0
Total %	100.0	0.0	61.0	39.0	58.0	42.0

N. No. of shells studied.

Table 5 Comparison of Shimoda and Nahari *Semisulcospira* females in terms of numbers of embyos brooded

Population	Shimoda N=11			Nahari - I N=8			Nahari - II N=15		
Statistic	X	S	Se	X	S	Se	X	S	Se
No. embryos per female	351.5	241.7	72.9	94.8	85.0	30.1	87.0	51.0	13.2
Total range		168–979			6–208			2–153	

Table 6 Comparison of embryos from the pallial brood chambers of Shimoda and Nahari *Semisulcospira* in terms of dimensions (mm) and ratios per whorl size

Population	Shimoda				Nahari - I						Nahri - II				
Whorl size	<2.0	2.0	2.5	3.0	<2.0	2.0	2.5	3.0	3.5	4.0	<2.0	2.0	2.5	3.0	3.5
% embryos at each whorl size	47.6	35.6	16.1	0.7	29.8	18.5	21.6	19.8	9.6	0.7	28.0	9.2	24.4	22.5	15.9
1. X length		0.95	1.15	1.35		0.93	1.22	1.50	1.75	2.05		1.00	1.28	1.59	1.91
2. X width		0.74	0.84	0.93		0.71	0.86	1.02	1.14	1.19		0.77	0.94	1.09	1.22
3. X LBW		0.78	0.90	0.99		0.80	0.98	1.15	1.31	1.33		0.87	1.06	1.25	1.40
4. L/W		1.28	1.37	1.45		1.31	1.42	1.47	1.54	1.72		1.30	1.36	1.46	1.57

X mean W width
L length LBW length of body whorl

libertina and the Nahari snails in color pattern, a class of traits least reliable for discrimination of species and the most variable between populations (Davis, 1969). As shown in Table 4 none of the Shimoda shells had a predominant or uniform purple color while 39 to 42 per cent of the Nahari snails were purplish. The purple color of some Nahari snails was due to 1) a fusion of wide purple bands (Fig. 5e) or 2) the fact that purple bands were so wide that very little inter-band yellow shell could be discerned (Fig. 5d). The banding patterns listed in Table 4 were variations of a basic pattern of 3 bands; basal, mid-whorl and subsutural.

Embryo Shells—As shown in Table 5, the Nahari females have significantly fewer embryos per pallial brood chamber than females from Shimoda. As shown, there is an extremely great variability in the numbers brooded by the females. The populations are compared in Table 6 in terms of 1) the largest whorl stage attained in the brood chamber, 2) the shell dimensions of embryos at each whorl stage and 3) the percentage of embryos at each whorl stage. It is evident that embryos attain a greater whorl stage in the brood chambers of Nahari females than in those of Shimoda females. This is somewhat variable as embryos of 4.0 whorls were found in the May collection (Nahari–I) while those of only 3.5 whorls were found in the June collection (Nahari–II). Several collections from Shimoda over a 3 year period have yielded embryos of only 3.0 whorls.

Four populations are compared in Table 7 on the basis of measurements of shell features of 3 whorl embryo shells. The lengths of shells from Nahari females collected 1 month apart are significantly different (t test, p .01), a fact possibly related to the fact that the collection having the larger embryos had embryos of only 3.5 whorls maximum in the brood chamber. It is pointed out that the order of magnitude of difference is small, that of 0.09 mm between of the means. The lengths of embryo of both Nahari collections are significantly greater than those of embryos from Shimoda or Amami-

Table 7 Comparison of 4 populations of *Semisulcospira libertina* in terms of statistics of shell dimensions (mm) for embryos of 3.0 whorls

Population	No. of snails	Shell features measure	Statistic		
			X	S	Se
Nahari - II	25	L (length)	1.59	0.121	0.024
		W (width)	1.09	0.096	0.019
		LBW (length of body whorl)	1.25	0.115	0.023
Nahari - I	25	L	1.50	0.112	0.022
		W	1.02	0.070	0.014
		LBW	1.15	0.098	0.020
Amami-Oshima	25	L	1.39	0.018	0.039
		W	0.99	0.026	0.013
		LBW	1.08	0.035	0.018
Shimoda	16	L	1.35	0.094	0.023
		W	0.93	0.050	0.013
		LBW	0.99	0.059	0.015

X Mean
S Standard deviation
Se Standard error of the mean

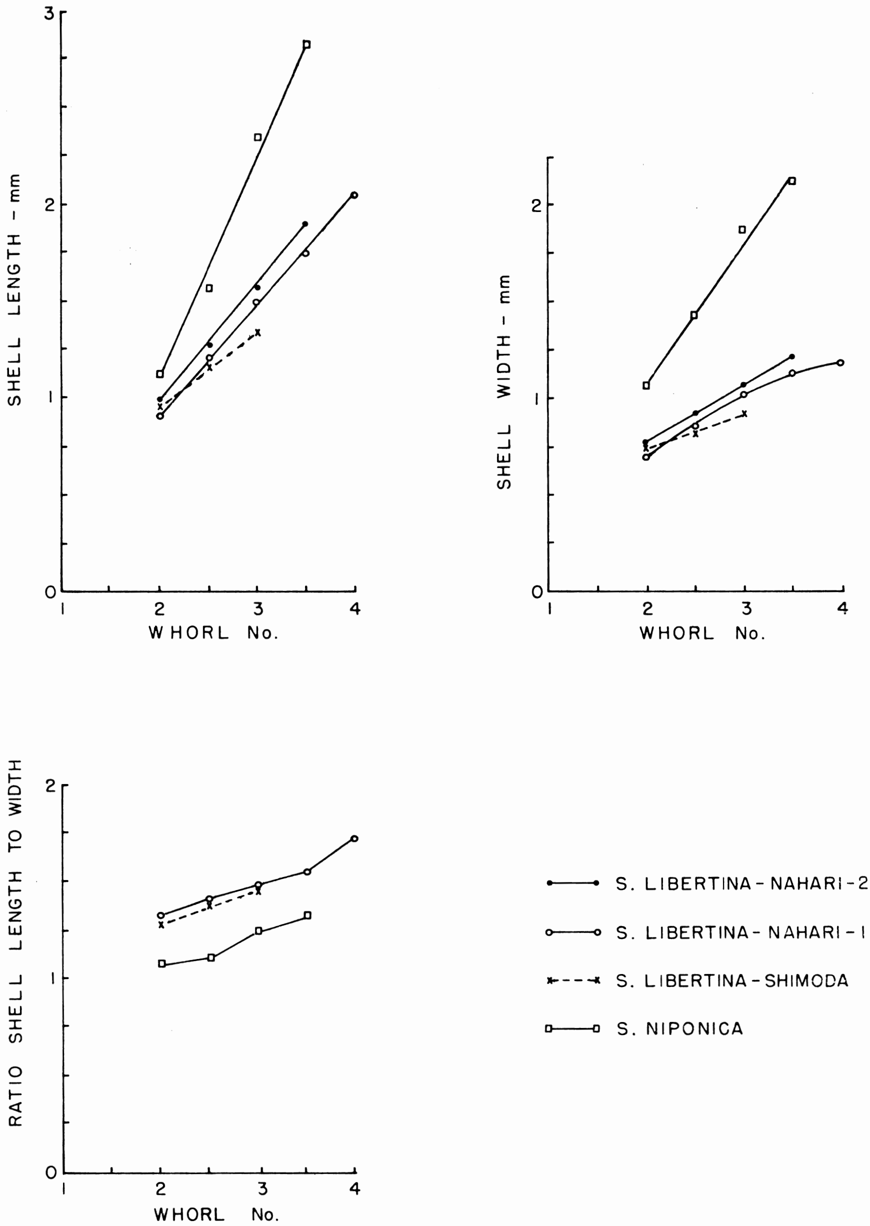

Fig. 2 Changes in dimensions and ratio of embryo shells from the female brood chamber of three populations of *Semisulcospira libertina* and *S. niponica*. Data from the latter species are added to show contrast when embryos of *S. libertina* are compared with those of another distinct species. There is a slight difference in slope of length and width of shell per whorl increment between the Nahari and Shimoda snails. The data for Nahari-II snails are omitted from the plots of the ratio of shell length to width as they were nearly identical with those of Nahari-I snails.

Oshima snails.

Data from Table 6 are plotted in Figure 2. Also compared are similar data for *Semisulcospira niponica* from Lake Biwa, Shiga Prefecture, Japan. The embryos of the Nahari population (collections 1 and 2) have greater slopes for increment of length or width per shell whorl stage than that derived from data on Shimoda snails. These differences, while significant, are relatively minor when compared with the data for embryos of *S. niponica.*

Embryos from the Nahari and Shimoda populations are shown in Fig. 7 (Pl. 3). Embryos from snails of both localities are yellow-brown to brown. Those from Nahari snails have a faint cancellate sculpture due to faint spiral lines crossing growth lines; on the base of the shell are several spiral grooves. Spiral cords are very faint; only 1 cord develops and this was absent in 28 per cent of the 3.0 to 3.5 whorl embryos of the June collection and it was absent in 36 per cent of the 3 whorl and 16 per cent of the 3.5 whorl embryos of the May collection (see Table 8). Embryos from Shimoda snails have 2 very prominent cords (70.4 per cent of those with 3.0 whorls) and a strongly developed cancellate sculpture. None of the Nahari embryos had an indication of ribbing while 1 among 1000 Shimoda embryos had ribs on the body whorl (Fig. 7j).

2. Cytology:

Definitive chromosome data were derived from several cells of each of 10 males. In no case did the females studied yield satisfactory data. The haploid number is n=18 as shown by the diakinesis stage in Figure 3. The same number was counted from several males with cells in metaphase I. No exact count of 2n=36 was possible from the preparations made. The inferred diploid number is 36.

3. Electrophoresis:

The results of the electrophoretic study of proteins from freshly prepared foot muscle extract are given in Table 9 (Rf values) and Fig. 8 (Pl. 4) (densitomitric tracings of stained gels and 2 prints of gels). The taxon specific densitometric profile for topotype *Semisulcospira libertina* is that given previously (Davis, 1967 b). Such a pattern results when an electrophoretic separation of proteins yields a unique combination of prominent, stable, differentially spaced fractions from all subunits of a given taxon. The major fractions which are the basis for the specific pattern are marked by asterisk beside the band number in Table 9. These

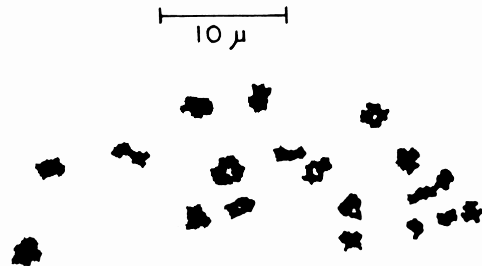

Fig. 3 Diakinesis stage of male meiotic division reveals the haploid chromesome number to be 18. The drawing was provided by Dr. J. B. Burch, University of Michigan who confirmed the haploid number.

Table 8 A comparison of the Shimoda and Nahari populations of *Semisulcospira* in terms of the development of cords on the embryo shell in successive whorl stages

Population	Shimoda			Nahari - I					Nahari - II			
Whorl stage	2.0	2.5	3.0	2.0	2.5	3.0	3.5	4.0	2.0	2.5	3.0	3.5
% Shell with:												
0 Cord	24.3	11.0	3.7	96	72	36	16	0	96	64	28	28
1 Cord	47.0	29.0	25.9	4	28	64	84	100	4	36	72	72
2 Cords	28.7	60.0	70.4	0	0	0	0	0	0	0	0	0

Table 9 Congruency or lack of congruency of protein fractions separated electrophoretically from fresh foot muscle extracts of Shimoda and Nahari *Semisulcospira*

BAND No.	Shimoda	% Runs where present	Nahari	% Runs where present	Significant different (SD)
1	.015*	100	.019*	100	
2	.067	100	.059	100	
3	.092	83	.088	100	
4	.110	92	.111	53	
5**	.144	100	.138	100	
6	.175	33	.170	63	
7**	.198	100	.192	100	
8**	.215	100	.213	87	
9	.250	42	.240	73	
10	.285	100	.276	63	
11	.312	33	.305	67	
12**	.345	100	.345	100	
13	.391	100	.388	93	
14**	.448	100	.433	100	
14a	—	—	.476	27	SD
15	.516	75	.499	87	
16	.578	92	.556	87	SD
17	.629	100	.622	87	
18**	.680	100	.687	100	
19	.725	67	—	—	SD
19a	—	—	.751	73	SD
20**	.794	100	.786	100	
21	.835	100	.831	80	
22	.890	92	.882	87	
23	.936	17	.937	47	
24	1.000	1000	1.000	100	

* Rf value
** dense, distinct bands as shown in Fig. 8, Plate 4

Table 10 An analysis of variation in Rf value for selected fractions separated electrophoretically in polyacrylamide gel

	Shimoda N=12			Nahari N=15			
Band No.	$\overline{X}$	S	Se	X	S	Se	Significant difference
5	.144	.0090	.0026	.138	.0116	.0029	No
12	.345	.0081	.0023	.345	.0145	.0037	No
14	.448	.0152	.0043	.433	.0167	.0042	No
18	.680	.0091	.0026	.687	.0173	.0044	No
20	.794	.0150	.0043	.786	.0125	.0055	No

N Number of tubes analyzed (different experiments)
X mean
S Standard deviation
Se Standard error of the mean

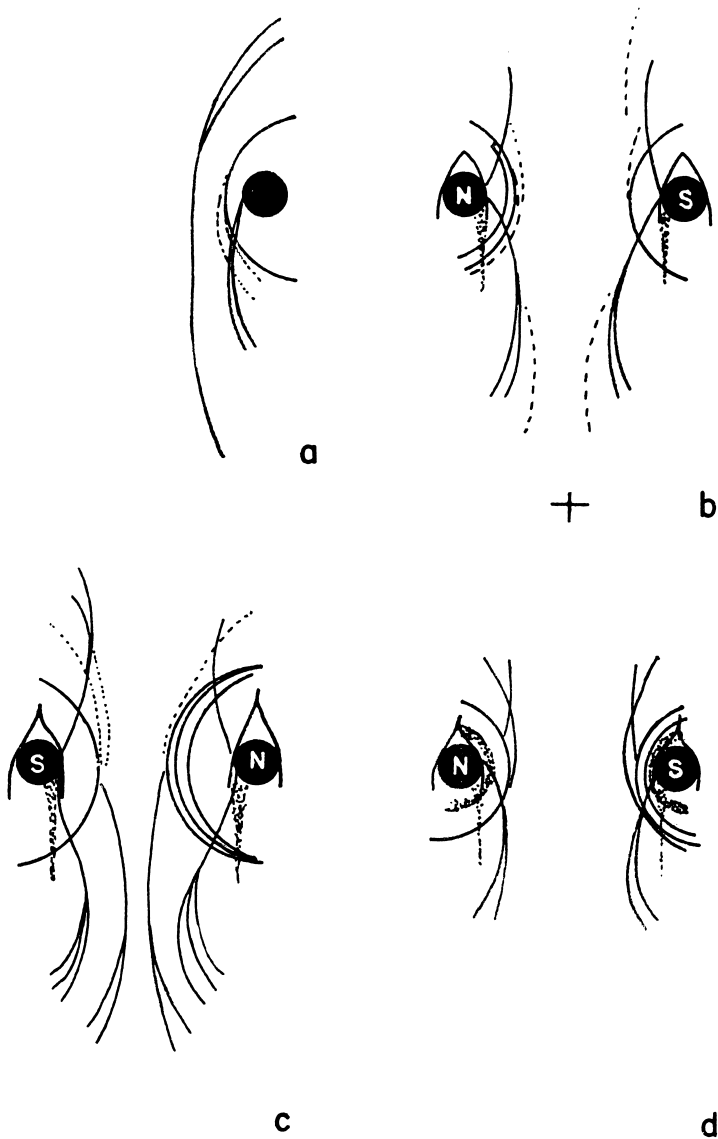

Fig. 4 Schematic drawings to clearly show all antigen-antibody precipitin systems using 4 different antisera. In a, all systems compared using Nahari and Shimoda antigens were corresponding. In b to d are shown differences in precipitin systems between the two taxa. The heterologous reaction yielded complex arcs immediately opposite the antigen wells. Results in b and c indicate qualitative differences. The dotted lines represent faint systems. N, is Nahari antigen; S, Shimoda antigen.

involve 7 of the 26 fractions separated. The twin bands 7 and 8 after the prominently dense band 5 are due to 2 very thin yet sharp dense fractions. Between prominent peaks 14 and 20 is an area containing 3 or 4 faint fractions. Band 18 is usually dense; it is found in all runs.

As shown in Table 9, proteins from extracts of fresh foot muscle of Nahari snails yielded 25 fractions of which only number 16 was significantly different in migration of the protein fractions found in both populations. Three other differences were due to faint components found in extracts of 1 population but not the other. There was complete congruency of fractions found in 100 per cent of the runs. One major quantitative difference was noted in that fraction 18 of Nahari extract was more dense and prominent than fraction 20; the situation is reversed for Shimoda snail extract (Fig. 8).

In electrophoretic studies a small degree of variability occurs. A study of Figures 8a and 8c reveals that in some experiments fractions 12 and 14 were not particularly dense (less than 15 per cent of experiments) when compared with results in Figures 8b and 8d. Resolution of fractions between 11 and 14 has at times been problematic, a problem relating to excellence in gel polimerization and chemical purity. What frequently resulted was band 13 being greatly curved and in some cases diffuse. At times bands 12, 13 and 14 appeared diffuse and distorted as was the case demonstrated in Figure 8c.

In subsequent studies of extracts from Shimoda snails I have demonstrated fractions 14a and 19a which occur at low frequency (about 10 to 20 per cent). I have been unable to demonstrate fraction 19 in extracts from Nahari snails. It is important to note that all fractions are not necessarily demonstrated in each experiment. From 50 to 70 per cent of the fractions were clearly resolved in 90 to 100 per cent of the experiments.

The Rf values presented in Table 9 are averages. The position of a fraction relative to the front band is often slightly variable between experiments. An example of such variability for 5 fractions is shown in Table 10.

4. Immunology:

Results with the micro-Ouchterlony diffusion experiments using each of the antisera, indicated that all precipitin systems compared between taxa were homologous (Fig. 9a, b; Pl. 5). There were no discrete bands or spurs unique to one or the other taxon. This phenomenon was not changed by diluting the antigens two or four-fold (Fig. 9c). Results with partially absorbed sera (Fig. 9d, f) or wholly absorbed sera (Fig. 9e) indicated that the antigen—antibody systems of both taxa were homologous.

Precipitin patterns resulting from immunoelectrophoretic experiments were identical for homologous and heterologous antigen mixtures with 70 per cent of the antisera tested. With the other 30 per cent slight differences between the taxa were found as shown in Fig. 4b, c, d, (corresponding to Fig. 10 h, c, d, Pl. 6). As shown in the stylized drawings of Figure 4a to d, the complex of 3 precipitin arcs exactly opposite the center well resulted in the heterologous reaction when either anti-Nahari or anti-Shimoda sera were used. Several qualitative differences involved faint systems (dotted lines). However, in each case, absorption tests (Fig. 10 j to l) indicated that there were no unique antigenic differences which would serve to differentiate the populations studied.

All control sera were negative.

Discussion

1. A note on the families of snail transmitting *Paragonimus westermani*.

It has been evident for some time that snails previously considered to be "*Melania*" actually belong to a number of genera. Further, the name *Thiara* Röding in the Bolten Catalog of 1798 has precidence over *Melania* Lamarck (1799). In this consideration the family Thiaridae replaced the "Melaniidae" as reviewed by Wenz (1938–1944). The family Thiaridae was reduced in scope by Morrison (1951, 1954) who considered that "The family of Melanians in the broad sense, is a biological absurdity." He arranged the genera of Thiaridae defined

by Wenz into three families; Thiaridae, Pleuroceridae and Melanopsidae. This arrangement has been accepted by Taylor and Sohl (1962).

The family Melanopsidae is European. Representatives of the Thiaridae and Pleuroceridae are found in Asia. The family Pleuroceridae is characterized by snails in which the eggs pass to the exterior via a pallial oviduct or where there is ovoviviparity and development is in a pallial brood chamber. The adult shells are devoid of colored patterns or have spiral color bands (purple, red-purple, brown, etc.). The edge of the mantle is smooth, males are present in the population (commonly 10 to 50 per cent).

The family Thiaridae is primarily defined as those freshwater or brackish water snails brooding embryos in a post cephalic chamber (ovoviviparity or release of veliger larvae) which one finds by dissecting down into the head-neck region of the snail. In the Thiaridae one group (subfamily) has shells with verticle flamulate color patterns, the edge of the mantle is fimbriated (fleshy protrusions) and males are absent or very scarce (much less than 5 per cent of the population). Genera such as *Thiara*, *Melanoides* and *Stenomelania* of Asia are within this grouping. Another group has shells with spiral color bands, a smooth mantle edge and males[1] are prevelant (the genus *Brotia* of S.E. Asia).

It is pointed out that too little is known about the anatomy and biology of numerous species still known only as "*Melania*" to ascertain which of those traits discussed above will be most useful in the diagnosis of the two families in the future. For example, fimbriation of the mantle may be a character common to both families yet predominating in the Thiaridae. Abbott (1948) stated that the mantle edge of *Paludomus* had 20 to 25 "fleshy papillae." Morrison (1954) places *Paludomus* in the Pleuroceridae. I have studied a species from South Vietnam which has a smooth mantle border yet all the other traits were thiarid.

The family Pleuroceridae is found throughout Asia. Freshwater Thiaridae overlap the pleurocerids from the Ryukyu Islands through Formosa to S. E. Asia and India. The Thiaridae predominate in S. E. Asia. Taxa of the pleurocerid genus *Semisulcospira* transmit *Paragonimus westermani* in Japan, Korea, Taiwan and mainland China. Taxa of the thiarid genera *Thiara*, *Brotia* and "*Melania*"[2] are implicated in Taiwan, mainland China and S. E. Asia (see review of Yokogawa et al., 1960).

2. The *Semisulcospira libertina* complex.

Burch and Davis (1967), Davis (1967 b, 1969) studied the species of *Semisulcospira* of Japan and discussed 10 distinct taxa from Japan. These are shown in Figures 11 and 12. The descriptions of the new taxa *S. habei* and *S. habei yamaguchi* are in press (Davis, 1969). The taxa were placed in 1 of 2 species complexes (Table 11) on the basis of chrome-

Table 11 Chromosome numbers of *Semisulcospira from* Japan*

Species Complex	Chromosome Number(n)	Locality
S. libertina complex		
S. libertina	18	Shimoda
*S. kurodai***	18	Hioki, Jyoto-cho
S. reiniana	20	Uji River
S. niponica complex		
S. habei habei	7	Uji River
S. habei yamaguchi	7	Lake Biwa
S. niponica	12	Lake Biwa
S. decipiens	12	Lake Biwa
S. reticulata	12	Lake Biwa
S. nakasekoae	13	Uji River
S. multigranosa	14	Lake Biwa

* Adapted from Burch and Davis (1967)

** Burch and Davis (unpublished) cited by Patterson, 1967

[1] I have studied *Brotia paludiformis* with Dr. Brandt at the SEATO Medical Research Laboratory and noted the male anatomy and male-type gonad in snails representing more than 10 per cent of the sample. Histological evaluation of the gonads of 2 individuals revealed they were packed with sperm. I collected this species from Thailand, Phitsanuloke, Nakhon Thai District, Kaeng So Pha Waterfall, Kaek River.

[2] As yet these so-called "*Melania*" are not assigned to a valid genus.

some number, number of basal cords on the adult shell and number of embryos retained in the pallial brood chamber. The *S. libertina* complex is defined as those taxa of *Semisulcospira* where cytologically n=18, the adult shell has 7 or more basal cords and there are numerous young in the brood chamber (usually 100 or more; see Discussion in Davis, 1969). Snails of the second group, the *S. niponica* complex have n=7 to 14; there are 2 to 6 basal cords and less than 50 embryos per female brood chamber (usually 35 or less).

Nahari *Semisulcospira* belong to the *S. libertina* complex as cytologically n=18; there are, on the average, more than 7 basal cords (Table 3) and numerous embryos (averaging 94.8 and 87.0, Table 5), greatly in excess of the 50 or less characterizing the *S. niponica* complex.

3. The specific identity of the Nahari *Semisulcospira.*

Semisulcospira of the Nahari I and II populations belong to the widespread and variable species *S. libertina* (Gould). This is confirmed by results of cytological analysis, data from studies of adult shell features (Table 3) and embryo size and number. Electrophoretic and immunological data indicate that the Nahari snails do not differ significantly from Topotype *S. libertina* from Shimoda. The four fraction differences of the electrophoretic analysis are very minor and are indicative of slight differences between populations of a species which are of less magnitude than those discussed by Davis and Lindsay (1967). The taxon-specific densitometric patterns of Shimoda and Nahari snails were identical. The antigen-antibody systems studied qualitatively were homologous as shown by the absorption tests thus confirming the close genetic alliance of the taxa. However, a degree of imbalance and asymmetry in the immunoelectrophoretic results using several of the antisera revealed that all antigen-antibody systems, whether qualitative or quantitative, were not equal between the Shimoda and Nahari populations. In every case where the same antigen mixture was run in both wells of the slide the precipitin patterns which resulted were symmetrical and balanced.

A provision must be made about the utilization of the immunological methods discussed here for species discrimination. I have noted (Davis, unpublished) that the degree of antigen heterogeneity between non pleurocerid genera and *Semisulcospira* is great. I have found (Davis, 1968), using the same micro-Ouchterlony techniques, that 3 taxa defined as subspecies of *Oncomelania hupensis* (Hydrobiidae) did not have 1 or more antigen-antibody systems present in the homologous reactions with foot-muscle extract of *O. h. formosana*. However, it has yet to be shown that distinct species of *Semisulcospira* have discrete antigens in extracts of their foot muscle.

4. Subspecific variability.

In comparing Shimoda and Nahari *S. libertina* a number of differences were found which are indicative of infra specific variability and which expand our concept of the species *S. libertina* in terms of genetic diversity.

The fact that Nahari snails brood, on the average, relatively few embryos compared with the Shimoda snails (Table 5, 87 or 95 vs. 352) indicates that one must be circumspect in using the criterion of numbers of embryos per female to justify a taxonomic position. It is not known if such a difference in average number as reported here is due to ecological or genetic factors. It is pointed out, however, that the average number of 87 embryos per female with a range of 2 to 153 is very high relative to the numbers brooded by species of the *S. niponica* complex.

Nahari *S. libertina* broods embryos of larger whorl size than does Shimoda *S. libertina*. Two factors should be mentioned related to this. 1) The larger sized embryos may be correlated with the fact that there were fewer embryos per female Nahari snail. 2) The fact that the largest size of embryo in the brood chamber was 4.0 whols in one month's collection and only 3.5 whorls in another shows that the trait of "largest size of embryo in terms of whorl size" must be used with caution in describing and characterizing species. This problem was

initially raised by Davis (1969) who stated that *S. libertina* from Shimoda had embryos of no more than 3.0 whorls throughout the year, yet it was wondered if variability in this trait did occur, and the extent which it might reach. As only 0.7 per cent of the embryos of the Nahari—I population reached 4.0 whorls and as 10 to 16 per cent of the Nahari I and II populations had 3.5 whorls (Table 6) it is concluded that the size of 3.5 whorls is more characteristic of the maximum size attained by the Nahari snails. *S. libertina*, depending on the population, broods embryos up to 3.0 or 3.5 whorls with exceptional cases of embryos attaining 4.0 whorls before birth.

Size and sculptural differences of the embryos of the Nahari and Shimoda population again indicate types of variability to be expected within the scope of the species concept for *S. libertina*. Yet, the embryos are still the yellow-brown to brown, small, rather plain embryo characterizing the species in contrast with the very large and/or highly ornate embryos of other species (Davis, 1969).

As previously reported (Davis, 1969), color patterns in the adult shell are highly variable between populations of *Semisulcospira libertina*. Banding patterns are the least reliable of all traits to be used in characterizing a species.

5. A note on other named *Semisulcospira* of Japan reported to transmit *Paragonimus westermani*, and synonyms of *Semisulcospira libertina*.

Semisulcospira bensoni was named by Philippi (1851, Zeitsch. Mal., p. 82) as a species from "Liew—Kiew" (Riew—Kiew=Ryukyu Islands). No specific type locality on the island chain was indicated. The species was not illustrated by Philippi (1851) or Brot (1874). It was only mentioned by name by von Martens (1861), Boettger (1886); it was not mentioned by Kobelt (1879). Kuroda (1929) lists *Melania bensoni* as an unclarified species.

Itagaki (1960), Kajiyama and Habe (1961), Kuroda (1963) and Habe (1965) considered *Semisulcospira libertina* to be a synonym of *S. bensoni*. Considering *S. libertina* a synonym of *S. bensoni* is not advocated at this time for the following reasons. No biological data are available for typical *S. bensoni*. No one population of *Semsiulcospira* from the Ryukyu Islands has been confirmed as *S. bensoni* by comparison with the type specimens. No justification has been given for such synonymy. On the other hand *Semisulcospira libertina* is well known in terms of type specimens, type locality and biological data. The name is well established in the literature of medically important snails.

Semisulcospira japonica (Reeve, 1859, Conch. Icon.) is currently considered to be a species transmitting *P. westermani* in Japan (Yokogawa et al., 1960). *S. japonica* is a synonym of *S. libertina* (Gold, 1859) and was considered thus by some early workers (von Martens, 1877; Kobelt, 1879). No locality data for the species other than "Japan" were given with the type description. As a result no identifiable population serves as the type population from which one can obtain material for biological studies. Various populations throughout Japan have snails conforming to the type description and illustration by Reeve (1859, Fig. 125) and that of Brot (1874, Pl. 6, Fig. 13). These populations have the biological characteristics of *S. libertina* as defined in Davis, 1969 and given above. Shells with 2 or 3 bright spiral color bands, traits at times used to identify *S. japonica*, are likewise found in populations of *S. libertina*.

It is necessary to point out that the term variety has little meaning or use in discriminating between molluscan taxa. Names should apply to the species or subspecies level where the taxonomic categories are defined in terms of real data indicative of basic genetic information. Several authors, notably von Martens (1877) and Kuroda (1929) stated that populations of *S. libertina* had within them snails exhibiting variable color and sculpture patterns. Where such variability occurs in an interbreeding population it is not appropriate to apply trinomial nomenclature to identify the varient classes. In this regard it is not acceptable to consider *japonica* to be a variety of *S. libertina*.

Von Martens (1877) described a number of

varieties on the basis of varying adult shell sculptural traits which he found in populations he considered to be *S. libertina*. These were: *plicosa, irrigua, decussata*. He included *Melania tenuisulcata* Dunker (1859 Malac. Blatt., p. 229) and *Melania ambidextra* von Martens (1860) in his list of "varieties." These are all considered synonyms of *S. libertina* for the reasons given above.

Davis (1967 b) justified placing *S. trachea* (Westerlund) as a synonym of *S. libertina*.

Summary

A population of *Semisulcospira* from the Nahari River, Kochi Prefecture, Shikoku Island, Japan was found infected with *Paragonimus westermani*. Cytological studies showed the taxon in question to be a member of the *S. libertina* complex as $n=18$. Embryos in the female were numerous, brown in color and without spines; thus, they resembled embryos of topotype *S. libertina* from Shimoda, Izu Peninsula, Honshu, Japan. Electrophoretic analysis of proteins from foot-muscle extract and immunological studies revealed that the Nahari snails were not different from those of the Shimoda population in terms of these basic parameters. The only differences found were those increasing our understanding of variablity (inter-population) within the species; these were 1) embryos of greater size and number of whorls in the female's brood pouch, 2) different developmental patterns of embryos in terms of the slope of the curve for length of shell per whorl stage and 3) sculptural characteristics of the embryos. As a result of this study it is concluded that the genetics of the snails of both populations are extremely similar, that the Nahari population is *S. libertina*, that we would predict that the topotype population is capable of transmitting *Paragonimus westermani*.

Acknowledgements

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Plate 1.

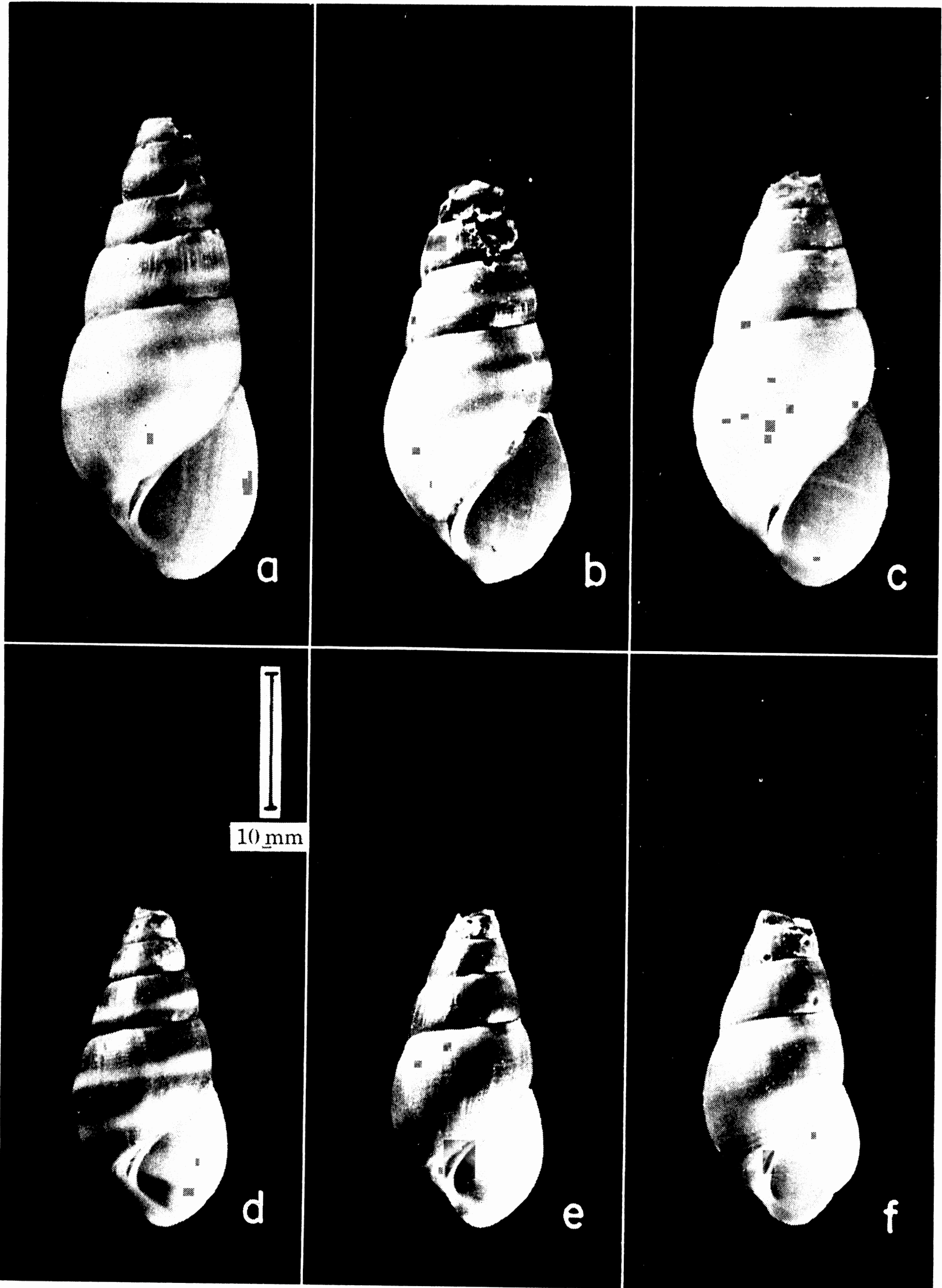

Fig. 5

Plate 2.

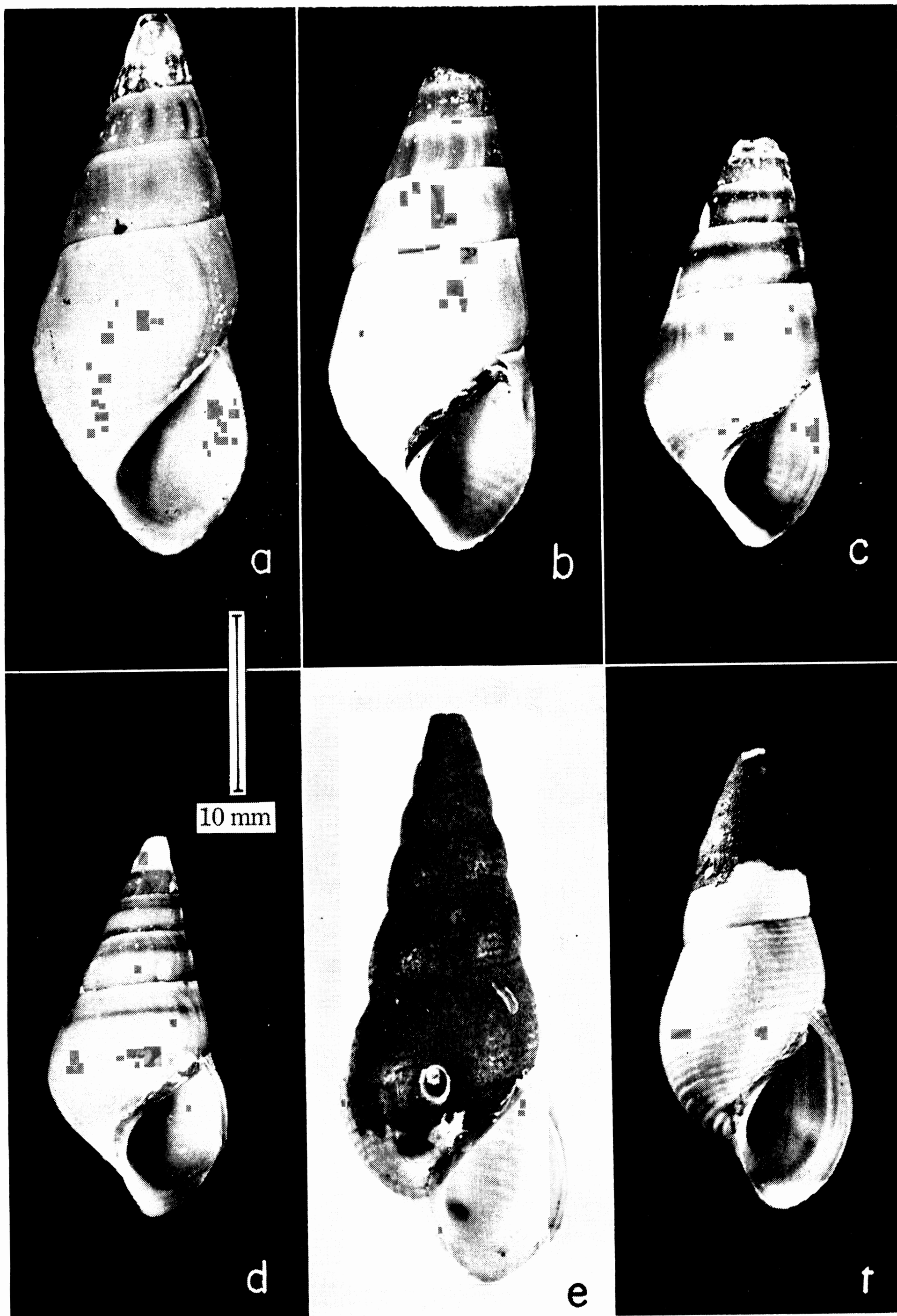

Fig. 6

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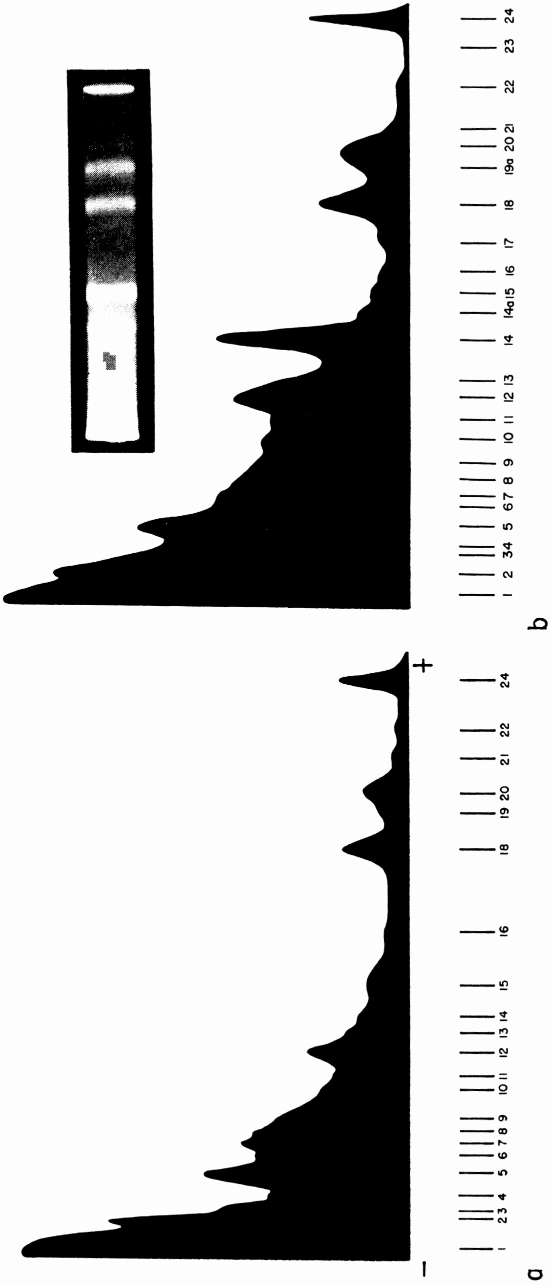


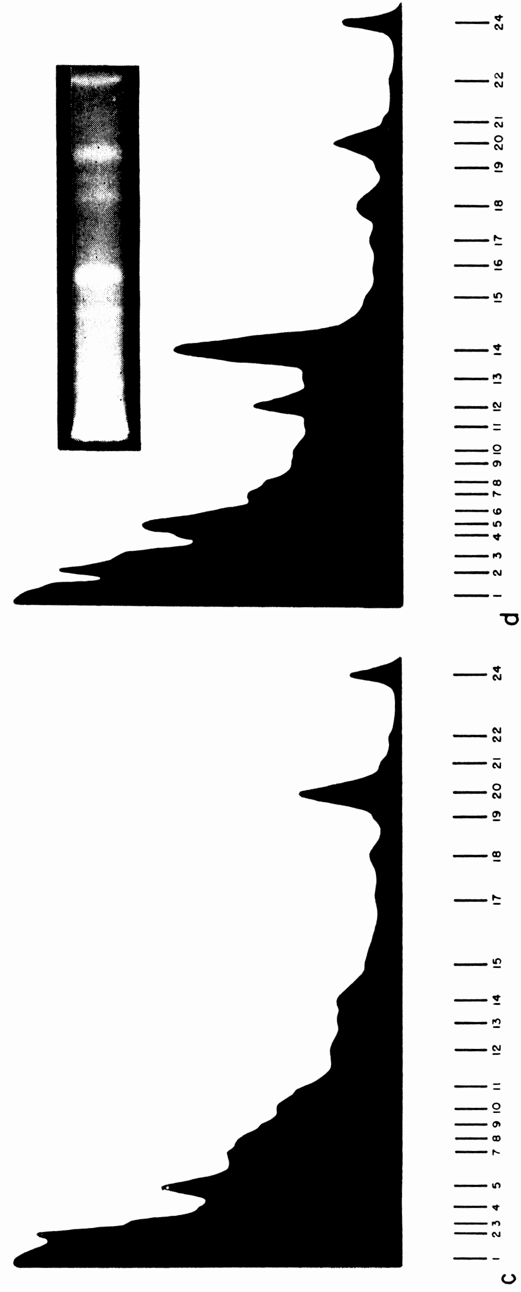


Fig. 8

Plate 3.

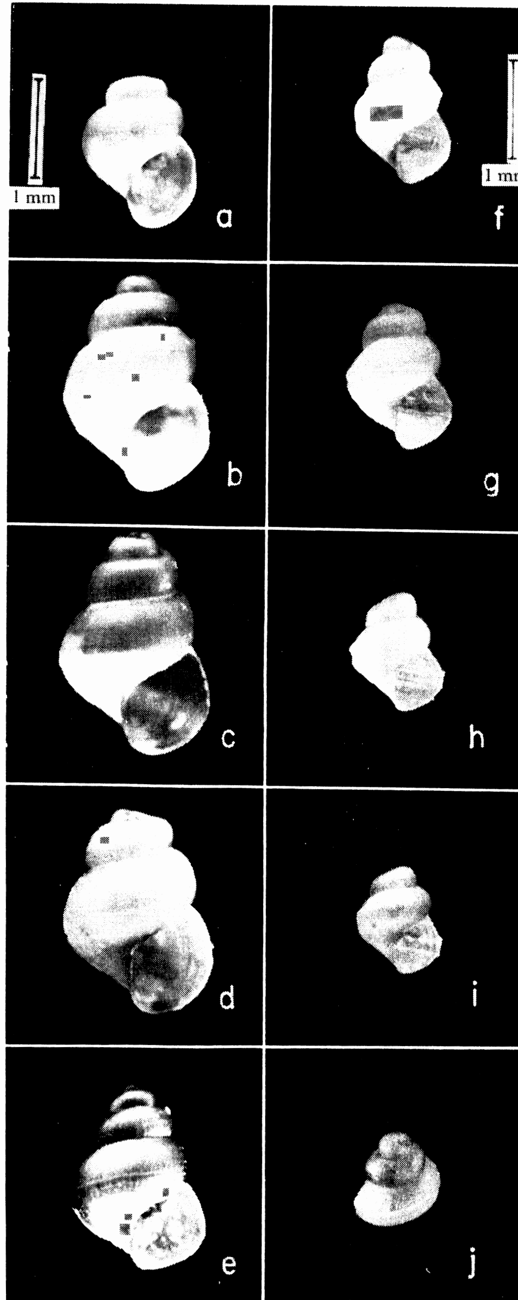
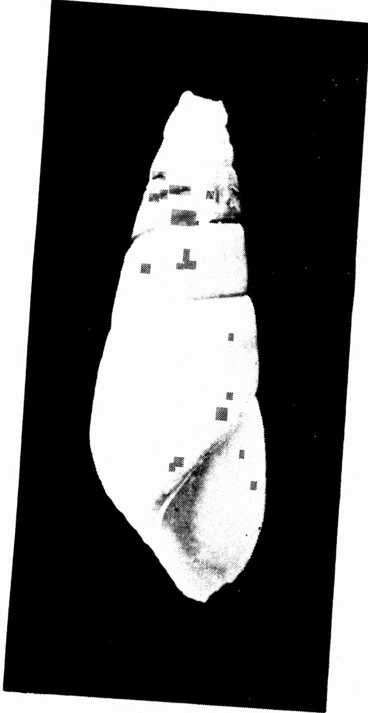


Fig. 7

Plate 7.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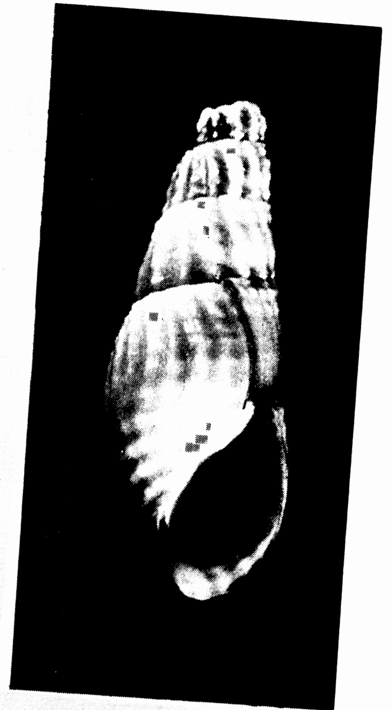
S. NIPONICA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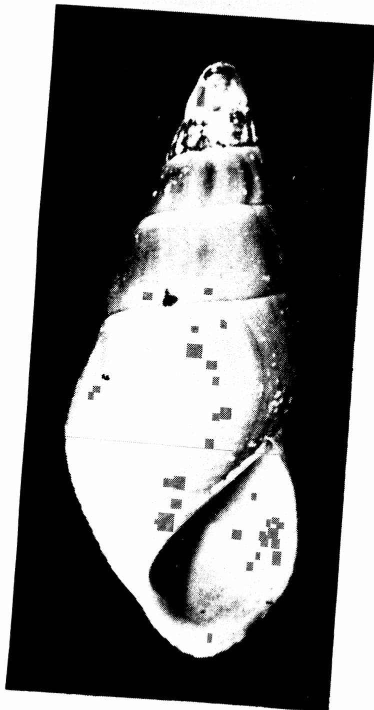
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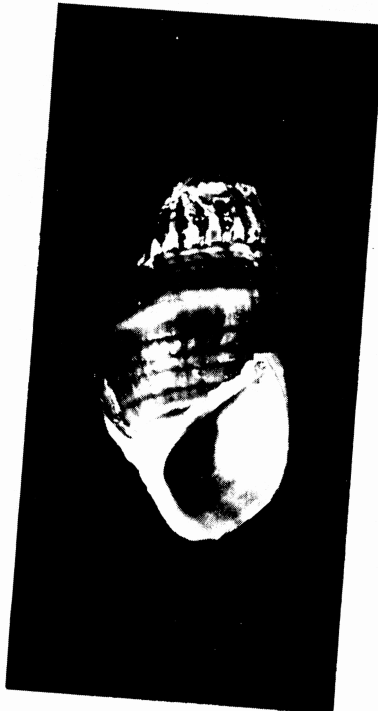
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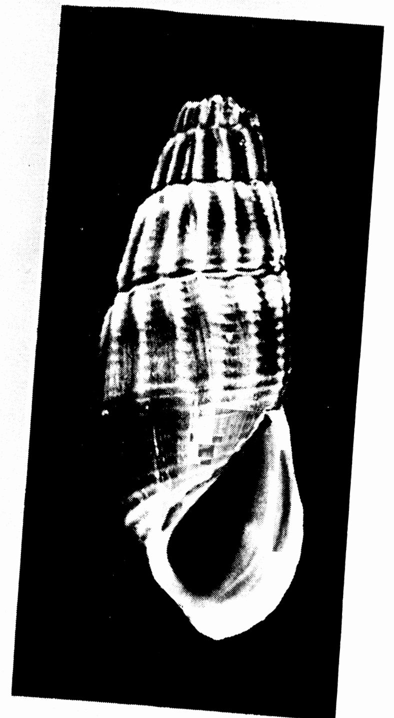
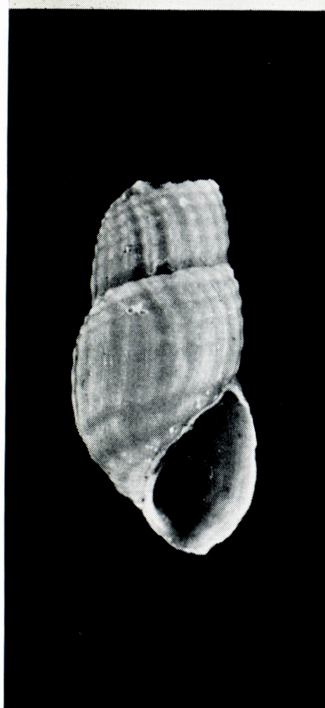
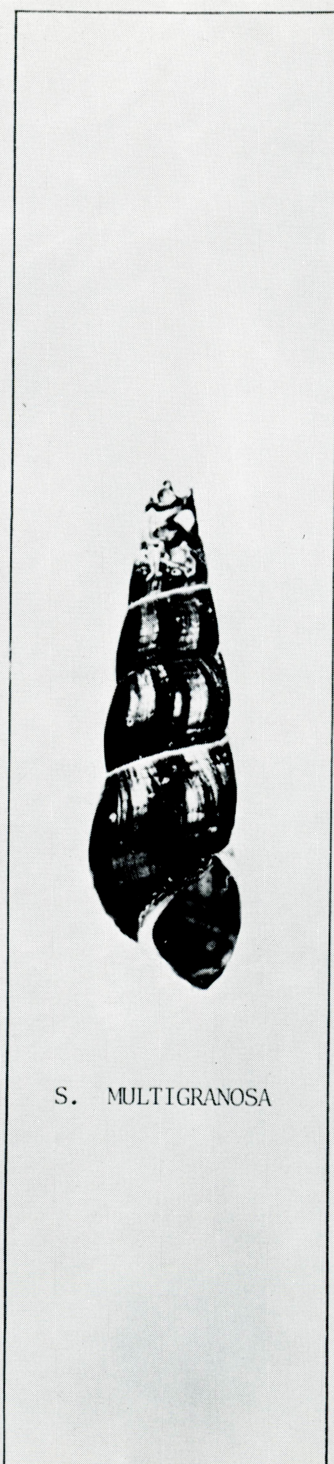

S. REINIANA

Fig. 11

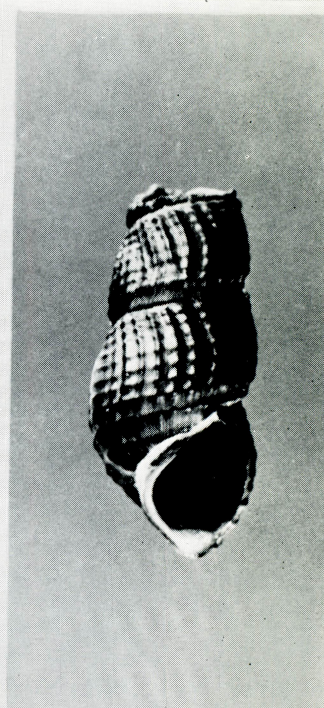
Plate 8.



S. HABEI YAMAGUCHI



S. MULTIGRANOSA



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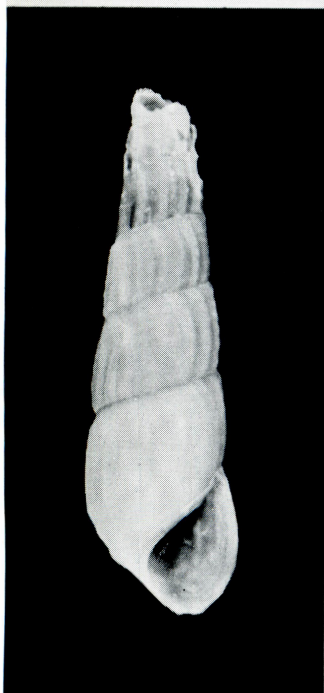
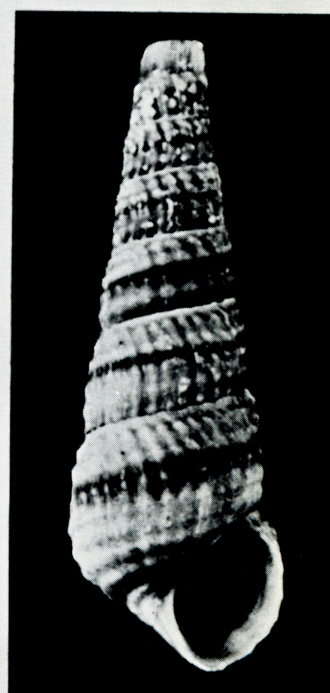


S. DECEPIENS



S. RETICULATA

Fig. 12

Fig. 5 Shells from the largest 10 per cent of the population of *Semisulcospira* from Nahari. The scale line equals 10 mm. Note banding pattern 5 (Table 4) of shell d and pattern 3 of shell b.

Fig. 6 Shells from the largest 10 per cent of the population of *Semisulcospira libertina* from Shimoda (a–d) and Amami-Oshima (e–f). The scale line equals 10 mm. Shells e and f show encrustment which occludes natural shell color. Shell d has banding pattern 2 (Table 4).

Fig. 7 Embryos from the brood chamber of adult females from Nahari (a–e) and Shimoda (f–j). The scale line equals 1 mm. Note the two spiral cords on the body whorl of the Shimoda embryo shells (g–i) and the ribs (j).

Fig. 8 Densitometric analysis of electrophoretic results when proteins from foot muscle extracts of the Nahari population (a–b) and Shimoda population (c–d) were studied. Prints of examples of the respective gels are shown. The protein fractions are numbered below each tracing.

Fig. 9 Micro-Ouchterlony diffusion plates showing straight diffusion where antigens of Nahari snail extract (N) and Shimoda snail extract (S) where 4.5 to 5.5 mg/ml. Antiserum (A) is in the central well (a–b). The antigens are diluted in c where 1–2 are 1 : 1, 3–4 are 1 : 2, 5–6 are 1 : 4. All systems are homologous. Results with partially absorbed antiserum are shown in d, f. In f, A is unabsorbed antiserum while A_1 is absorbed antiserum.

Fig. 10 Immunoelectrophoretic results where wells with the Shimoda antigens are marked S and the Nahari antigens are marked N. The antisera listed in Tables 1 and 2 were used as follows: Rabbit A, slide b; E–c; G–d; H–e; J–f; B–g; C–h; D–i. Slight differences in reaction were noted for slide c, d and h (see Fig. 4, c, d, b). Results in j to l were with absorbed antiserum (unabsorbed controls are in d to f). A protein migration control is shown (a).

Fig. 11 Adult shells of 6 of the 10 taxa of *Semisulcospira* considered to be valid species or subspecies in Japan.

Fig. 12 Adult shells of 4 of the 10 taxa of *Semisulcospira* considered to be valid species or subspecies in Japan. Note the smooth and nodulate morphs of *S. multigranosa*.

日本における ***Paragonimus westermani*** の中間宿主 ***Semisulcospira*** の電気泳動学的，免疫学的ならびに生物学的検討

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高知県奈半利川産の *Semisulcospira* が *Paragonimus westermani* に感染していることが判つた．このものは細胞学的には半数体数 n＝18 の *S. libertina* complex に属していることを知つた．雌貝中の胎貝 (embryo) はその数が多く，褐色をしており，殻表面に突起を持つていない．このような点で，この embryo は伊豆半島下田産の *S. libertina* (topotype) のそれに類似している．fooot-muscle 抽出液の蛋白質を電気泳動学的ならびに免疫学的に分析したところ，奈半利川産の貝と下田産のそれとの間にはその基本的な特徴において差異がないことが判つた．しかしながら両者の間には種内変異（inter-population）が認められた．なわち 1) 奈半利川産のものでは，雌の brood pouch 中の embryo が大きく，しかも螺層(whorl)の数も多いこと，2) embryo の発育パターン，すなわち whorl stage に対する殻長の比に差異が認められること，および 3) embryo の sculptural characteristics に差異が認められること，であつた．

以上の成績から，結論として，奈半利川産および下田産の貝は遺伝学的に相互に酷似していること，奈半利川産のものが *S. libertina* であり，したがつて，下田産 topotype のものも *Paragonimus westermani* の中間宿主となることができるものと思われる。