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Nucleo-cytoplasmic Hybrids between *Rana brevipoda* and *Rana plancyi chosenica*

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(With 4 Plates)

INTRODUCTION

When two species are so remotely related to each other as their interspecific hybrids die at the late blastula or early gastrula stage, the reciprocal nucleo-cytoplasmic hybrids between these two species can not live beyond the same stage (BRIGGS and KING, 1952; MOORE, 1958, '60; HENNEN, 1963). Reciprocal nucleo-cytoplasmic hybrids obtained by GURDON (1962) from *Xenopus laevis* and *X. tropicalis* stopped their development at the neurula stage or a little later. From these two species interspecific hybrids had not been produced even by artificial fertilization. A similar kind of nucleo-cytoplasmic hybrids had been obtained by HENNEN (1964, '65, '67) from the combination of *Rana pipiens* and *R. palustris*.

Differing from the nucleo-cytoplasmic hybrids noted above, those obtained by KAWAMURA and NISHIOKA (1963a, b) from *Rana nigromaculata* and *R. brevipoda* as well as from *Rana japonica* and *R. ornativentris* developed into matured frogs which were fertile to a certain extent. By reciprocal crosses the former two species can easily produce interspecific hybrids which attain their sexual maturity, although the males are nearly completely sterile. From the latter two species, interspecific hybrids which are all sterile males can be produced by mating female *japonica* with male *ornativentris*. Recently, GALLIEN (1970) obtained reciprocal nucleo-cytoplasmic hybrids from *Pleurodeles waltlii* and *P. poireti*. A few of them became adults which were fertile.

In contrast with the interspecific nucleo-cytoplasmic hybrids, intraspecific ones can develop into normal frogs which are quite similar to the nuclear species in every character, as observed by MCKINNELL (1960), GURDON (1961) and ORTOLANI, FISCHBERG and SLATKINE (1966).

Among the above stated nucleo-cytoplasmic hybrids, those which developed into adults are permitted to pursue the effects of the egg cytoplasm upon their specific or subspecific characters. Such effects were found only in the interspecific nucleo-cytoplasmic hybrids obtained by KAWAMURA and NISHIOKA. Besides, the same authors found a difference in reproductive ability between nucleo-cytoplasmic and ordinary hybrids of two different species. It seemed to

be interesting to the present authoress to examine the activity of nuclei combined with foreign cytoplasm in manifestation of specific characters and reproductive capacity by producing another kind of nucleo-cytoplasmic hybrids which can attain their sexual maturity.

MATERIAL AND METHODS

In this research, eight females and two males of *Rana brevipoda* ITO and two females and two males of *Rana plancyi chosenica* OKADA were used as material. The former species was collected from the suburbs of Okayama in the breeding season of 1963, while the latter was from Seoul, Korea, by the courtesy of the late Mr. K. K. KIM, a famous gardener, in April of the same year. Eggs were obtained from the oviducts of the females after pituitary transplantation. The nuclei of unfertilized eggs were removed by PORTER's method (1939), and blastula nuclei were transferred to the enucleated eggs by BRIGGS and KING's method (1952, '53).

Reciprocal hybrids were produced between *Rana brevipoda* and *Rana plancyi chosenica* as one of the control series. The ploidy of the individuals of the experimental and the control series was identified by the tail-tip method on the tadpoles which were about 30 days after the nuclear transplantation or fertilization. Chromosome preparations were made by the squash technique after treat-

TABLE 1

Production of nucleo-cytoplasmic hybrids between two species, *R. brevipoda* and

Individual no. of parents		No. of eggs	No. of cleaved eggs		No. of blastulae		No. of gastrulae	
Enucleated egg	Blastula nucleus		Norm.	Abnorm.	Norm.	Abnorm.	Norm.	Abnorm.
(B)63.1	PP. 1	30	16 (53.3%)	13	14 (46.7%)	2	8 (26.7%)	3
(B)63.2	PP. 1	30	15 (50.0%)	13	14 (46.7%)	1	10 (33.3%)	2
(B)63.3	PP. 1	30	21 (70.0%)	8	18 (60.0%)	3	11 (36.7%)	5
(B)63.4	PP. 1	30	19 (63.3%)	9	16 (53.3%)	3	11 (36.7%)	3
(B)63.5	PP. 2	40	25 (62.5%)	13	24 (60.0%)	1	13 (32.5%)	4
(B)63.6	PP. 2	30	15 (50.0%)	12	11 (36.7%)	4	6 (20.0%)	3
(B)63.7	PP. 2	35	20 (57.1%)	15	18 (51.4%)	2	11 (31.4%)	2
(B)63.8	PP. 2	50	41 (82.0%)	9	36 (72.0%)	5	23 (46.0%)	4
Total		275	172 (62.5%)	92	151 (54.9%)	21	93 (33.8%)	26

ment with distilled water. In such a case as more than ten good mitotic figures were not obtained in a tadpole, the regenerated tail-tip of the same tadpole was cut off again and the chromosomes were examined by the same squash technique. The gonads of frogs were fixed in NAVASHIN's fluid, cut into paraffin sections and stained with HEIDENHAIN's iron hematoxylin for histological observation.

The following abbreviations are used in the present paper.

B — A set of *Rana brevipoda* chromosomes
P — A set of *Rana plancyi chosenica* chromosomes
(B)— *Rana brevipoda* cytoplasm
(P)— *Rana plancyi chosenica* cytoplasm

RESULTS

I. Production of nucleo-cytoplasmic hybrids

For the purpose of producing nucleo-cytoplasmic hybrids between *Rana brevipoda* and *Rana plancyi chosenica*, diploid nuclei taken out of blastulae of the latter species were transferred to enucleated eggs of the former species. The results of experiments are shown in Table 1.

Blastula nuclei were transferred to 275 enucleated eggs obtained from eight females (Nos. 1~8). Out of these eggs, 172 cleaved normally, while 92 did abnormally and the other 11 did not at all. Twenty-one of the normally cleaved eggs became partial blastulae, 32 stopped their development at the blastula

R. plancyi chosenica by the nuclear transplantation method

No. of neurulae		No. of tail-bud embryos		No. of hatched tadpoles		No. of analysed tadpoles	Ploidy	
Norm.	Abnorm.	Norm.	Abnorm.	Norm.	Abnorm.		2n	3n
5 (16.7%)	3	5 (16.7%)	0	3 (10.0%)	2	2 (6.7%)	2 (6.7%)	0
5 (16.7%)	5	4 (13.3%)	1	2 (6.7%)	2	1 (3.3%)	1 (3.3%)	0
4 (13.3%)	7	2 (6.7%)	2	2 (6.7%)	0	1 (3.3%)	1 (3.3%)	0
9 (30.0%)	2	8 (26.7%)	1	5 (16.7%)	3	4 (13.3%)	4 (13.3%)	0
10 (25.0%)	3	8 (20.0%)	2	7 (17.5%)	1	5 (12.5%)	5 (12.5%)	0
3 (10.0%)	3	2 (6.7%)	1	2 (6.7%)	0	0	0	0
6 (17.1%)	5	5 (14.3%)	1	4 (11.4%)	1	3 (8.6%)	3 (8.6%)	0
12 (24.0%)	11	12 (24.0%)	0	10 (20.0%)	2	6 (12.0%)	6 (12.0%)	0
54 (19.6%)	39	46 (16.7%)	8	35 (12.7%)	11	22 (8.0%)	22 (8.0%)	0

TABLE 2

Production of various kinds of tadpoles in the control series

Parents		No. of eggs	No. of cleaved eggs		No. of gastrulae		No. of neurulae		No. of tail-bud embryos		No. of hatched tadpoles	
Female	Male		Norm.	Abnorm.	Norm.	Abnorm.	Norm.	Abnorm.	Norm.	Abnorm.	Norm.	Abnorm.
(B)BB. 1	(B)BB. 1	50	50 (100 %)	0	44 (88.0%)	6	43 (86.0%)	1	41 (82.0%)	2	40 (80.0%)	1
(B)BB. 2	(B)BB. 1	35	32 (100 %)	0	30 (85.7%)	2	30 (85.7%)	0	30 (85.7%)	0	30 (85.7%)	0
(B)BB. 3	(B)BB. 1	50	47 (94.0%)	0	32 (64.0%)	15	32 (64.0%)	0	31 (62.0%)	1	31 (62.0%)	0
(B)BB. 4	(B)BB. 1	40	36 (90.0%)	0	25 (62.5%)	11	25 (62.5%)	0	25 (62.5%)	0	24 (60.0%)	1
(B)BB. 5	(B)BB. 2	40	40 (100 %)	0	36 (90.0%)	4	35 (87.5%)	1	32 (80.0%)	3	30 (75.0%)	2
(B)BB. 6	(B)BB. 2	50	47 (94.0%)	0	46 (92.0%)	1	45 (90.0%)	1	45 (90.0%)	0	44 (88.0%)	1
(B)BB. 7	(B)BB. 2	50	49 (98.0%)	0	49 (98.0%)	0	48 (96.0%)	1	44 (88.0%)	4	43 (86.0%)	1
(B)BB. 8	(B)BB. 2	60	55 (91.7%)	0	55 (91.7%)	0	52 (86.7%)	3	50 (83.3%)	2	49 (81.7%)	1
Total		375	356 (94.9%)	0	317 (84.5%)	39	310 (82.7%)	7	298 (79.5%)	12	291 (77.6%)	7
(B)BB. 1	(P)PP. 1	56	55 (98.2%)	0	55 (98.2%)	0	54 (96.4%)	1	54 (96.4%)	0	54 (96.4%)	0
(B)BB. 2	(P)PP. 1	75	73 (97.3%)	0	73 (97.3%)	0	73 (97.3%)	0	73 (97.3%)	0	73 (97.3%)	0
(B)BB. 3	(P)PP. 1	47	45 (95.7%)	0	44 (93.6%)	1	42 (89.4%)	2	41 (87.2%)	1	41 (87.2%)	0
(B)BB. 4	(P)PP. 1	73	72 (98.6%)	0	72 (98.6%)	0	72 (98.6%)	0	72 (98.6%)	0	72 (98.6%)	0

(B)BB. 5	(P)PP. 2	77	75 (97.4%)	0	75 (97.4%)	0	75 (97.4%)	0	73 (94.8%)	2	72 (93.5%)	1
(B)BB. 6	(P)PP. 2	64	62 (96.9%)	0	62 (96.9%)	0	62 (96.9%)	0	62 (96.9%)	0	62 (96.9%)	0
(B)BB. 7	(P)PP. 2	52	50 (96.2%)	0	50 (96.2%)	0	48 (92.3%)	2	48 (92.3%)	0	48 (92.3%)	0
(B)BB. 8	(P)PP. 2	59	55 (93.2%)	0	55 (93.2%)	0	55 (93.2%)	0	54 (91.5%)	1	54 (91.5%)	0
Total		503	487 (96.8%)	0	486 (96.6%)	1	481 (95.6%)	5	477 (94.8%)	4	476 (94.6%)	1
(P)PP. 1	(B)BB. 1	207	187 (90.3%)	0	169 (81.6%)	4	144 (69.6%)	25	139 (67.1%)	5	137 (66.2%)	2
(P)PP. 1	(B)BB. 2	158	158 (100 %)	0	144 (91.1%)	6	134 (84.8%)	10	131 (82.9%)	3	131 (82.9%)	0
(P)PP. 2	(B)BB. 1	219	219 (100 %)	0	177 (80.8%)	26	147 (67.1%)	30	126 (57.5%)	21	125 (57.1%)	1
(P)PP. 2	(B)BB. 2	116	114 (98.3%)	0	112 (96.6%)	2	111 (95.7%)	1	107 (92.2%)	4	100 (86.2%)	7
Total		700	678 (96.9%)	0	602 (86.0%)	38	536 (76.6%)	66	503 (71.9%)	33	493 (70.4%)	10
(P)PP. 1	(P)PP. 1	92	91 (98.9%)	0	91 (98.9%)	0	91 (98.9%)	0	91 (98.9%)	0	91 (98.9%)	0
(P)PP. 2	(P)PP. 2	101	98 (97.0%)	0	96 (95.0%)	2	95 (94.1%)	1	95 (94.1%)	0	94 (93.1%)	1
Total		193	189 (97.9%)	0	187 (96.9%)	2	186 (96.4%)	1	186 (96.4%)	0	185 (95.9%)	1

stage and 26 became abnormal at the gastrula, while the remaining 93 finished their gastrulation normally. At the neurula stage 39 embryos died of various abnormalities in the formation of the neural tube or becoming edematous at the site of the abdomen. Eight and eleven embryos died of edema or other abnormalities at the tail-bud and hatching stage, respectively. Eventually 35 hatched normally and became swimming tadpoles.

Three and four tadpoles died by the feeding stage from breaking out blisters and becoming edematous, respectively. Six more tadpoles died of ill-development or edema afterwards within 20 days after the nuclear transplantation. Thirty days after this operation there were only 22 healthy tadpoles, 8% in number of total eggs. The ploidy of these tadpoles was examined by the squash technique applied on the tail-tip. More than ten good mitotic figures, 13.4 on an average, were observed to identify the ploidy of each tadpole. As the results, it was found that all the 22 tadpoles were diploids, having 26 chromosomes, that is, 10 large and 16 small.

In the control series four kinds of matings were made in and between *Rana brevipoda* and *Rana plancyi chosenica*. The results of these matings are shown in Table 2. In the matings between eight female and two male *Rana brevipoda*, 291 (77.6%) out of 375 eggs became hatched tadpoles, while 185 (95.9%) out of 193 eggs did in the matings between two female and two male *Rana plancyi chosenica*. Reciprocal crosses between the two species were made by the use of the same females and males as those of the above matings. In the crosses between the eight female *Rana brevipoda* and the two male *Rana plancyi chosenica*, 476 (94.6%) out of 503 eggs passed normally through the hatching stage, while in the reciprocal crosses between the two females and the two males, 493(70.4%) out of 700 eggs attained the same stage. Accordingly, it was quite clear that there was no hybrid inviability between female *Rana brevipoda* and male *Rana plancyi chosenica* up to the hatching stage at least, while there was a slight degree of hydrid inviability between female *Rana plancyi chosenica* and male *Rana brevipoda*: much more embryos died at the gastrula, neurula and tail-bud stages in this kind of cross than in the other.

The identification of the ploidy of the tadpoles in the control series was performed 30 to 40 days after the fertilization by the use of ten tadpoles for each mating. By observing the chromosomes of 80 (B)BB, 80 (B)BP, 40 (P)PB and 20 (P)PP tadpoles by the squash technique on their tail-tips, it was confirmed that all these tadpoles were diploids, being 26 in chromosome number.

II. Life histories at the tadpole stage

The total lengths of the 22 nucleo-cytoplasmic hybrids, (B)PP were measured 20, 30 and 40 days after the nuclear transplantation and compared with those of the four kinds of control tadpoles. As shown in Table 3, the nucleo-cytoplasmic hybrids were smaller, in general, than all the four kinds of controls at the ages of 20 and 30 days. However, they were nearly equal in size to the (P)PB hybrids

TABLE 3

Life histories of diploid nucleo-cytoplasmic hybrids and the control tadpoles

Series	Kinds	Individual no.	No. of tadpoles	No. of analysed mitoses	Ploidy	Total length of 20-day-old tadpoles, St. V (mm.)	Total length of 30-day-old tadpoles, St. XII (mm.)	Total length of 40-day-old tadpoles, St. XVII (mm.)
Control	(B)BB		291	mean 5.1	2n	31.2±0.5	46.8±0.7	60.2±1.3
	(B)BP		476	mean 6.2	2n	32.0±0.4	46.4±0.6	59.7±0.6
	(P)PB		493	mean 7.0	2n	32.5±0.4	45.0±0.6	54.0±0.6
	(P)PP		185	mean 5.2	2n	32.4±0.4	46.5±0.5	57.2±0.6
Experimental	(B)PP	63PI(B)I-1	1	12	2n	30.2	43.0	52.1
	(B)PP	63PI(B)I-2	1	10	2n	32.5	44.2	55.3
	(B)PP	63PI(B)II-1	1	14	2n	31.0	43.3	54.2
	(B)PP	63PI(B)III-1	1	15	2n	28.5	40.5	52.0
	(B)PP	63PI(B)IV-1	1	15	2n	34.0	46.9	59.8
	(B)PP	63PI(B)IV-2	1	13	2n	32.6	45.0	55.4
	(B)PP	63PI(B)IV-3	1	15	2n	31.0	42.2	54.2
	(B)PP	63PI(B)IV-4	1	15	2n	31.3	40.4	50.0
	(B)PP	63PII(B)V-1	1	14	2n	36.8	48.7	63.2
	(B)PP	63PII(B)V-2	1	15	2n	30.5	45.0	57.3
	(B)PP	63PII(B)V-3	1	14	2n	34.2	46.0	60.0
	(B)PP	63PII(B)V-4	1	14	2n	26.9	40.2	53.0
	(B)PP	63PII(B)V-5	1	15	2n	32.0	44.5	63.7
	(B)PP	63PII(B)VII-1	1	12	2n	27.5	40.0	50.5
	(B)PP	63PII(B)VII-2	1	13	2n	30.0	41.6	54.6
	(B)PP	63PII(B)VII-3	1	14	2n	31.2	43.3	53.0
	(B)PP	63PII(B)VIII-1	1	14	2n	32.4	44.2	50.5
	(B)PP	63PII(B)VIII-2	1	15	2n	34.0	46.8	60.5
	(B)PP	63PII(B)VIII-3	1	14	2n	22.5	35.6	45.3
	(B)PP	63PII(B)VIII-4	1	10	2n	20.0	27.3	—
	(B)PP	63PII(B)VIII-5	1	11	2n	26.7	40.2	51.0
	(B)PP	63PII(B)VIII-6	1	10	2n	25.0	41.0	52.3
Total			22	mean 13.4	2n	30.0±0.9	42.3±1.1	54.7±1.1

TABLE 4

Dental formulae of diploid nucleo-cytoplasmic hybrids and the control tadpoles

Series	Kinds	No. of analysed tadpoles	Dental formula			
			0/2	1/2	1/3	2/3
Control	(B)BB	125	—	—	6	119
	(B)BP	476	95	381	—	—
	(P)PB	493	—	—	419	74
	(P)PP	185	—	—	28	157
Experimental	(B)PP	22	1	1	7	13

at the age of 40 days, although still smaller than the other three kinds of control tadpoles.

The dental formulae at the tadpole stage of the 22 nucleo-cytoplasmic hybrids were examined at the age of 30 days and compared with those of the four kinds of control tadpoles, that is 125 (B)BB, obtained from 4 out of 8 females, 476 (B)BP from 8 females, 493 (P)PB from 2 females and 185 (P)PP from 2 females, of the same age (Table 4). Of the (B)BB tadpoles, 119(95%) had two dental rows on the upper jaw and three on the lower, while six had one on the upper and three on the lower. On the other hand, 157 (85%) of the 185 (P)PP tadpoles had two dental rows on the upper jaw and three on the lower, and the other 28 had one on the upper and three on the lower. The hybrid (P)PB tadpoles were different from both (B)BB and (P)PP in dental formula; 74 had two dental rows on the upper jaw and three on the lower, while 419(85%) had one on the upper and three on the lower. The reciprocal hybrids were defective in the formation of dental rows; 381(80%) of the 476 (B)BP tadpoles had one on the upper jaw and two on the lower, and the remaining 95 had none on the upper and two on the lower. These 95 tadpoles were ill-developed and all died by the time of metamorphosis.

The nucleo-cytoplasmic (B)PP hybrids in the experimental series had a tendency to resemble the control (B)BB tadpoles in the dental formula, as 13 of them had two dental rows on the upper jaw and three on the lower, and seven others had one dental row on the upper and three on the lower. The remaining two had one or no dental row on the upper and two on the lower. The smallest tadpole, No. 63PII(B)VIII–4, among the 22 nucleo-cytoplasmic hybrids died 35 days after the nuclear transplantation. At this time it was 29.3 mm. in total length. The two tadpoles, Nos. 63PII(B)VIII–5 and 63PII(B)VIII–6, which had no or only one dental row on the upper jaw were bad in growth, owing to their defective dental rows. These two died eventually without attaining the time of metamorphosis. They were 47 days old and 52.0 and 52.5 mm. in total length, respectively.

III. Life histories at the frog stage

Nineteen nucleo-cytoplasmic hybrids completed their metamorphosis. Their life histories are briefly shown in Table 5. They went ashore remarkably later than the four kinds of control tadpoles did, except for three, Nos. 63PI(B)IV–1, 63PII(B)V–3 and 63PII(B)VII–3, as shown in the table. However, they were nearly equal in body length to the controls when the comparison was made immediately after the completion of metamorphosis. One of them, No. 63PII(B)VIII–3, went ashore 64 days after the nuclear transplantation and died on the next day. Another tadpole, No. 63PII(B)V–1, died immediately after landing. The other 17 nucleo-cytoplasmic hybrids lived for more than 100 days after the nuclear transplantation, although one died at the age of 101 days. Seven of them died during a period of time from 148 to 196 days, and five others

TABLE 5

Life histories of diploid nucleo-cytoplasmic hybrids and the control frogs

Series	Kinds	Individual no.	No. of frogs	Age at the time of landing (days)	Body length immediately after metamorphosis (mm.)	Age at the time of death or preservation (days)	Body length at the time of death or preservation (mm.)
Control	(B)BB		230	44～54 mean 49.7	16.8±0.2	182(>100) 354(> 50)	20.2±0.3 25.7±0.4
	(B)BP		235	46～52 mean 48.5	15.2±0.2	182(>100) 354(6)	20.7±0.3 26.0～27.0
	(P)PB		376	44～53 mean 47.4	16.4±0.2	182(>100) 354(>100)	21.0±0.3 26.8±0.3
	(P)PP		132	44～47 mean 45.5	15.5±0.2	182(>100) 354(5)	20.3±0.3 26.5～27.9
Experimental	(B)PP	63PI(B)I-1	1	58	14.5	354	26.5
	(B)PP	63PI(B)I-2	1	58	14.2	369	25.4
	(B)PP	63PI(B)II-1	1	60	15.0	360	24.6
	(B)PP	63PI(B)III-1	1	57	14.0	196	22.3
	(B)PP	63PI(B)IV-1	1	50	14.5	315	24.2
	(B)PP	63PI(B)IV-2	1	60	14.0	207	23.0
	(B)PP	63PI(B)IV-3	1	61	14.5	101	18.0
	(B)PP	63PI(B)IV-4	1	63	15.0	182	20.5
	(B)PP	63PII(B)V-1	1	80	18.0	80	18.0
	(B)PP	63PII(B)V-2	1	73	15.5	214	22.8
	(B)PP	63PII(B)V-3	1	51	17.0	148	21.0
	(B)PP	63PII(B)V-4	1	75	14.3	177	21.5
	(B)PP	63PII(B)V-5	1	80	17.5	280	23.0
	(B)PP	63PII(B)VII-1	1	81	14.7	202	21.7
	(B)PP	63PII(B)VII-2	1	63	16.5	150	20.5
	(B)PP	63PII(B)VII-3	1	54	15.8	152	19.5
	(B)PP	63PII(B)VIII-1	1	60	16.0	223	24.7
	(B)PP	63PII(B)VIII-2	1	75	17.3	176	22.0
	(B)PP	63PII(B)VIII-3	1	64	14.0	65	14.0
Total			19	50～81 mean 64.4	15.4±0.3		

( ) ··· No. of frogs

died from 202 to 280 days after the nuclear transplantation. The remaining four frogs were 315 to 369 days old. These four were very similar in body length to the four kinds of control frogs.

IV. External characters of frogs

1. Control series

Rana brevipoda were very similar to *Rana plancyi chosenica* in shape, although the heads were wider and the dorso-lateral folds of the latter were distinctly more smooth and roundish in a cross section than those of the former. In these, the

two species were quite different. The backs of *Rana brevipoda* were dark brown in ground color and had several large roundish black spots which were clear-cut in outline, while those of *Rana plancyi chosenica* were bright green and had several small black spots which were not clear-cut and usually more numerous. The dorsal glandular tubercle situated in the center of each black spot was a short rod in the former species, while it was a small dot in the latter. The dorsal surfaces of the hind limbs of *Rana brevipoda* had distinct black cross-bars, while those of *Rana plancyi chosenica* had irregular black spots or flecks. The ventral body surfaces of *Rana brevipoda* had dark marble-like marking on white, while those of *Rana plancyi chosenica* were white or light orange and had no marking.

The two kinds of hybrids were very similar to each other in external characters. The heads, dorso-lateral folds, dorsal glandular tubercles and dorsal black spots were intermediate in specific characters between those of the two species. The bright green of the back, the white or light orange of the ventral surface and the cross-bars on the hind limbs found in *Rana brevipoda* were dominant and revealed themselves in both kinds of hybrids (Plate I).

2. Experimental series

Seventeen nucleo-cytoplasmic hybrids which were more than 100 days old were all quite similar to the nuclear species, *Rana plancyi chosenica,* in external characters (Plate II). Their heads were wide, the dorsal ground colors were bright green, and the ventral body surfaces were white or light orange. The dorsal black spots were small and not clear-cut in outline. The dorsal glandular tubercles situated in the centers of the black sopts were dots in shape. There were no cross-bars on the dorsal surfaces of the hind limbs. However, the dorso-lateral folds of 16 out of 17 frogs did not resemble those of the nuclear species. They were intermediate between those of the two species in the shape of rising. In the remaining one frog the shape of the dorso-lateral folds was unclear, owing to the post-mortem change.

V. Sex and structures of gonads

1. Control series

a. *Rana brevipoda* and *Rana plancyi chosenica*

In the control series there were 33 *Rana brevipoda* and 17 *Rana plancyi chosenica,* both of which were preserved within about one year after the fertilization. Among the former there were 17 males and 16 females, while the latter consisted of 10 males and 7 females (Table 6). Nine *Rana brevipoda* preserved within one month after their metamorphosis consisted of four males and five females. The ovaries of these five females were filled with growing auxocytes. Among six frogs of *Rana plancyi chosenica* which died within one month after their metamorphosis there were three males and three females. The ovaries of the later were filled with growing auxocytes, just as those of the above female *Rana brevipoda* (Plate

TABLE 6

Sex of diploid nucleo-cytoplasmic hybrids and the control frogs

Series	Kinds	Immediately after metamorphosis		3-month-old frogs		6-month-old frogs		one-year-old frogs		Total frogs	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Control	(B)BB	4	5	2	2	6	4	5	5	17	16
	(B)BP	2	3	1	1	—	—	—	2	3	6
	(P)PB	3	7	1	4	5	12	17	32	26	55
	(P)PP	3	3	1	—	2	3	4	1	10	7
Experimental	(B)PP	2	0	3	0	4	0	3	7	12	7

III, 17~18, 21).

b. Hybrids between female *Rana brevipoda* and male *Rana plancyi chosenica*

Nine hybrids were preserved within about one year after the fertilization; there were three males and six females. The testes of two males preserved immediately after the metamorphosis were of the same structure as those of the control *Rana brevipoda* and *Rana plancyi chosenica*. The ovaries of three females preserved simultaneously with the above males had no growing auxocytes, differing from those of the control females of both species. In their ovaries there were distinct ovarian cavities and numerous oogonia or oocytes of the earliest stage which were actively increasing in number.

The remaining male preserved three months after the fertilization had testes which were abnormal in inner structure; there were a small number of large abnormal spermatozoa and pycnotic nuclei among abundant spermatogonia or first spermatocytes. Although there were some meiotic figures, reduction of the chromosome number did not seem to occur, owing to abortive divisions. A female preserved simultaneously with the above male had ovaries, in which there were distinct ovarian cavities and no growing auxocytes.

The remaining two females which died about one year after the fertilization had no growing auxocytes in the ovaries yet, although the latter were fairly large in size, owing to active multiplication of oogonia or oocytes of the earliest stage.

c. Hybrids between female *Rana plancyi chosenica* and male *Rana brevipoda*

There were 26 males and 55 females among the hybrids which were preserved within one year after the fertilization. Three males preserved immediately after the metamorphosis were quite similar to the control males of both species in size and inner structure of testes. Seven females of the same age, however, were considerably under-developed; six of them had no growing auxocytes and the other had only a few. Except for this point, all the ovaries seemed normal in structure.

One 3-month-old male had testes which contained no spermatozoa, except for a small number of abnormal ones. The ovaries of four females of the same

age were somewhat smaller than those of the control females of both species. One of the female hybrids had only four growing auxocytes in the ovaries, while the other three had none. These ovaries were filled with oogonia or oocytes of the earliest stage. Five 6-month-old males had no normal spermatozoa and 12 females of the same age had also no growing auxocytes. However, the ovaries of these females were comparatively large, owing to the active multiplication of oogonia or oocytes of the earliest stage, althoguh they were much smaller than those of the females of both species.

Of 17 one-year-old males three had a few oocyte-like cells which were abnormal, hypertrophied spermatocytes in their testes. Eleven other males had abnormal spermatozoa and no normal ones, while the remaining three had no abnormal as well as normal spermatozoa. In the ovaries of 13 of 32 one-year-old females there were one to three growing auxocytes surrounded by numerous oogonia or oocytes of the earliest stage. The other 19 females had no growing auxocytes in their ovaries. The ovaries of eight of these females, were especially small and thin (Plate III, 19~20, 22).

2. Experimental series

As shown in Table 6, there were 12 males and 7 females among the 19 metamorphosed nucleo-cytoplasmic hybrids.

a. Structures of testes

(i) *Immature testes* Five males, Nos. 63PI(B)IV–3, 63PII(B)V–1, 63PII(B)VII–2, 63PII(B)VII–3 and 63PII(B)VIII–3, which were 65~152 days old, had immature testes of nearly normal structures. The germ cells contained in their testes were mostly primary or secondary spermatogonia which had no sign of degeneration. The two oldest males, Nos. 63PII(B)VII–2 and 63PII(B)VII–3, had many spermatocytes at the prophase, besides spermatogonia.

(ii) *Testes with normal spermatozoa* Two males, Nos. 63PI(B)I–2 (Plate IV, 23) and 63PII(B)VIII–1, which were 369 and 223 days old, had a few small bundles of normal spermatozoa in some seminal tubules. The seminal tubules of the former male were filled for the most part with secondary spermatogonia, spermatocytes, prespermatids and spermatids. Along the walls of their cavities there were some primary spermatogonia. Besides these germ cells of various stages, a small number of abnormal spermatozoa and degenerating germ cells having pycnotic nuclei were distributed here and there in the cavities. On the other hand, the seminal tubules of the other male were filled with secondary spermatogonia and spermatocytes for the most part and had some germ cells of the other stages, together with a small number of abnormal spermatozoa and degenerating germ cells with pycnotic nuclei.

(iii) *Testes with abnormal spermatozoa* Three males, Nos. 63PI(B)I–1 (Plate IV, 24), 63PI(B)III–1 and 63PI(B)IV–4, which were 354, 196 and 182 days old, respectively, had a few abnormal spermatozoa. The testes were filled for the most part with abundant secondary spermatogonia and spermatocytes, and a small number of primary spermatogonia and degenerating germ cells

having pycnotic nuclei. There were no normal spermatozoa.

(iv) *Testes with degenerating germ cells* One male, No. 63PII(B)V–4 (Plate IV, 25), 177 days old, had testes in which most of the germ cells were degenerating or had degenerated. The degeneration usually seemed to occur at the prophase of first spermatocytes. The seminal tubules contained wide spaces in places where germ cells had degenerated. Primary spermatogonia were very distinct along the walls of the tubules.

(v) *Testes with abnormal appearance* A male, No. 63PII(B)V–3, 148 days old, had gonads which were in principle of testicular structures, although appeared as if they were ill-developed ovaries. Their ovarian cavities were completely filled with multiplied rete cells. It was very clear that the testes of this male were those transformed from ovaries. The germ cells were mostly primary spermatogonia surrounded with rete cells.

b. Structures of ovaries

(i) *Ovaries filled with growing auxocytes* Two females, Nos. 63PI(B)II–1 and 63PI(B)IV–1 (Plate IV, 26), 360 and 315 days old, respectively, had ovaries which were nearly normal, except that they were smaller in size and especially thinner than those of the control females of both species. These ovaries were filled with growing auxocytes of various sizes. In each cross section of the ovaries the number of growing auxocytes was remarkably smaller than that of the control females. There were a small number of degenerating auxocytes.

(ii) *Ovaries without growing auxocytes* Three females, Nos. 63PI(B)IV–2, 63PII(B)V–2 (Plate IV, 27) and 63PII(B)VIII–2, which were respectively 207, 214 and 176 days old, had abnormal ovaries which were quite similar to those of reciprocal hybrids. No growing auxocytes were found in their ovaries, which were filled with abundant oogonia and oocytes of the earliest stage and were considerably large in size. The oocytes seemed to degenerate soon after they began to grow. The ovarian cavities were clearly found as narrow spaces surrounded with thin walls. No hypertrophy was found in the walls.

(iii) *Ovaries with degenerating germ cells* Two females, Nos. 63PII(B)V–5 and 63PII(B)VII–1, 280 and 202 days old, respectively, had extremely degenerative ovaries. In these ovaries there were wide ovarian cavities surrounded with thin walls, and most of the germ cells had degenerated, although a few degenerating auxocytes were found in the ovaries of one of the two females, No. 63PII(B)V–5 (Plate IV, 28).

DISCUSSION

By using the nuclear transplantation method devised by BRIGGS and KING (1952), SAMBUICHI (1957, '61) obtained reciprocal nucleo-cytoplasmic hybrids between *Rana nigromaculata* and *R. brevipoda*. According to him, a nucleo-cytoplasmic hybrid composed of *nigromaculata* cytoplasm and *brevipoda* nuclei revealed a color pattern similar to that of the cytoplasmic species at the early tadpole stage. However, at the metamorphosing stage this individual became quite

similar to the nuclear species in the points that there was no median stripe on the dorsal surface and there were dark marble marks on the ventral surface. Another nucleo-cytoplasmic hybrid constructed of *brevipoda* cytoplasm and *nigromaculata* nuclei had some characters similar to those of the cytoplasmic species at the stage of completion of its metamorphosis, although this frog was of the nuclear species type on the whole in the color patterns of the body and hind limbs. KAWAMURA and NISHIOKA (1963a) and NISHIOKA (1971a, b, 1972a) repeated the experiments performed by SAMBUICHI and obtained 13 nucleo-cytoplasmic hybrid frogs composed of *nigromaculata* cytoplasm and *brevipoda* nuclei and five of the reciprocal combination. These frogs were very similar in the most external characters to the nuclear species. However, some characters of some frogs were similar to those of the cytoplasmic species or intermediate between those of the two species. Moreover, a matured female nucleo-cytoplasmic hybrid constructed of *nigromaculata* cytoplasm and *brevipoda* nuclei transmitted an effect from the cytoplasmic parent to about one-third of the offspring, although the nucleo-cytoplasmic hybrids behaved themselves on the whole like the nuclear species in inheritance.

KAWAMURA and NISHIOKA (1963b) produced reciprocal nucleo-cytoplasmic hybrids between *Rana japonica* and *R. ornativentris,* too. Of 14 metamorphosed frogs constructed of *ornativentris* cytoplasm and *japonica* nuclei, 11 closely resembled the nuclear species in external characters throughout the larval and frog stages. The remaining three were similar to the cytoplasmic species in some characters, although they were of the nuclear species type in the main. One frog composed of *japonica* cytoplasm and *ornativentris* nuclei was also similar to the cytoplasmic species or the ordinary hybrids of the two species in some external characters. By mating with females of the nuclear species, three male nucleo-cytoplasmic hybrids constructed of *ornativentris* cytoplasm and *japonica* nuclei produced progenies which were not always of the nuclear species type in external characters. A small number of them were different from the nuclear species in color pattern or intermediate between the two species in the shape of the dorso-lateral folds. In contrast with the results obtained by these investigators, the adult reciprocal nucleo-cytoplasmic hybrids produced recently by GALLIEN (1970) from two distinct species of *Pleurodeles, waltlii* and *poireti*, were of the nuclear species type in morphological, physiological, serological and karyological characters, as far as they were examined.

As described in this paper, the adult nucleo-cytoplasmic hybrids produced from enucleated *Rana brevipoda* eggs transplanted with blastula nuclei of *R. plancyi chosenica* were very similar to the nuclear species in external characters, such as the shape of the head, the dorsal color patterns of the body and hind limbs, the ventral color of the body and the shape of dorsal tubercles. However, the width of the dorso-lateral folds appeared intermediate between those of the two species in the majority of the frogs.

Intraspecific nucleo-cytoplasmic hybrids were produced by MCKINNELL (1960) between the common *Rana pipiens* and *the kandiyohi mutant*, by GURDON

(1961) between *Xenopus l. laevis* and *X. l. victrianus* and by ORTOLANI, FISCHBERG and SLATKINE (1966) between *X. l. laevis* and *X. l. petersi*. All these nucleo-cytoplasmic hybrids were nearly the same as the nuclear forms in various morphological characters. It may be surmised from the same angle that interspecific nucleo-cytoplasmic hybrids resemble the nuclear species in morphological characters, when the two species are very close in systematic relationship. In this case the genetical elements of the nucleus may function almost normally in the foreign cytoplasm. According to GALLIEN (1969, '70), *Pleurodeles waltlii* and *P. poireti* produced viable and fertile hybrids by reciprocal crosses, although a reproductive barrier appeared in the hybrids of the second generation. This fact seems to indicate a close relationship of the two *Pleurodeles* species.

The relation of *R. brevipoda* with *R. plancyi chosenica* in reproductive intimacy seems to be more remote than that with *R. nigromaculata,* since the former two species are almost completely isolated from each other by hybrid sterility, while the isolation of the latter two is incomplete, that is, only male hybrids are nearly perfectly sterile. This remote relationship between *Rana brevipoda* and *R. plancyi chosenica* seems to be supported by the inferiority of their nucleo-cytoplasmic hybrids in reproductive ability, too. While two of the seven females had nearly normal ovaries, the others had very abnormal ones, which contained no growing auxocytes. In this point these females also differed from the female nucleo-cytoplasmic hybrids between *Rana brevipoda* and *R. nigromaculata,* since the latter were all fertile to a certain degree: 7~67 percent of eggs of each female developed into normally metamorphosed frogs (KAWAMURA and NISHIOKA, 1963a). Each of the two female nucleo-cytoplasmic hybrids constructed of *Rana ornativentris* cytoplasm and *japonica* nuclei produced likewise many mature eggs which developed normally and reached the late embryonal stage after fertilized with *japonica* or *ornativentris* sperm, although these embryos revealed severe abnormalities and died at the hatching stage (KAWAMURA and NISHIOKA, 1963b).

Such a remote relationship was also found in the male nucleo-cytoplasmic hybrids between *Rana brevipoda* and *R. plancyi chosenica*. Except for five males whose germ cells were mostly spermatogonia, five out of seven males had abnormal testes which did not seem to produce normal spermatozoa, while the remaining two had normal spermatozoa, together with abnormal spermatozoa and degenerating germ cells. In spite of such a remote relationship of the two species, the adult nucleo-cytoplasmic hybrids constructed of *brevipoda* cytoplasm and *plancyi chosenica* nuclei were very similar to the nuclear species in the expression of external characters. This seems to indicate that the function of genes of the nuclear species was not affected by the foreign cytoplasm in this respect. The width of the dorso-lateral folds intermediate between those of the two species in the majority of the nucleo-cytoplasmic hybrids seems to be such a character as not always attributable to a genetic effect of the foreign cytoplasm. It may be a manifestation of general under-development of the nucleo-cytoplasmic hybrids. The ill-formation of the dental rows of some tadpoles as well as the ill-development of the gonads of most of the adults may equally be explained by such general

under-development, which is due to an inconsistency between the nuclei and cytoplasm of the nucleo-cytoplasmic hybrids.

SUMMARY

1. Twenty-two nucleo-cytoplasmic hybrids were produced by transferring blastula nuclei of *Rana plancyi chosenica* to enucleated eggs of *Rana brevipoda.* Their diploidy was ascertained at the tadpole stage by the squash technique applied on their tail-tips. They were compared with *Rana brevipoda*, *R. plancyi chosenica* and reciprocal hybrids of these two species in life history, external characters, sex and the structures of gonads.

2. The nucleo-cytoplasmic hybrids were similar to both the nuclear and cytoplasmic species and different from reciprocal hybrids in the formation of dental rows at the tadpole stage.

3. Nineteen nucleo-cytoplasmic hybrids completed their metamorphosis, which occurred remarkably later than that of the control tadpoles did, except three. Seventeen of them lived for more than 100 days after the nuclear transplantation. Twelve died during the period from 148 to 280 days after the nuclear transplantation. Four others lived for more than 315 days. These four were very similar in body length to the control frogs.

4. The nucleo-cytoplasmic hybrids which lived for more than 100 days were quite similar to the nuclear species in external characters, excepting the dorsolateral folds, which were intermediate between the two species in the shape of rising.

5. Of the 19 metamorphosed nucleo-cytoplasmic hybrids 12 were males and 7 females. Five of the males had immature testes of nearly normal structures. Two had a few small bundles of spermatozoa in some seminal tubules, three had a few abnormal spermatozoa, one had testes in which most of the germ cells were degenerating and one had abnormal testes which appeared to have been transformed from ovaries.

Two of the 7 females had nearly normal ovaries with growing auxocytes of various sizes, three had abnormal ovaries which contained no growing auxocytes, and two had ovaries with degenerating germ cells.

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EXPLANATION OF PLATES

PLATE I

Four kinds of control female frogs, 354 days old. $\times 1.5$

1, 2. *Rana brevipoda,* (B)BB.
3, 4. Diploid hybrid, (B)BP, between *Rana brevipoda* ♀ and *Rana plancyi chosenica* ♂.
5, 6. Diploid hybrid, (P)PB, between *Rana plancyi chosenica* ♀ and *Rana brevipoda* ♂.
7, 8. *Rana plancyi chosenica,* (P)PP.

NUCLEO-CYTOPLASMIC HYBRIDS BETWEEN *R. BREV.* AND *R. PLAN. CHOSEN.*

M. NISHIOKA

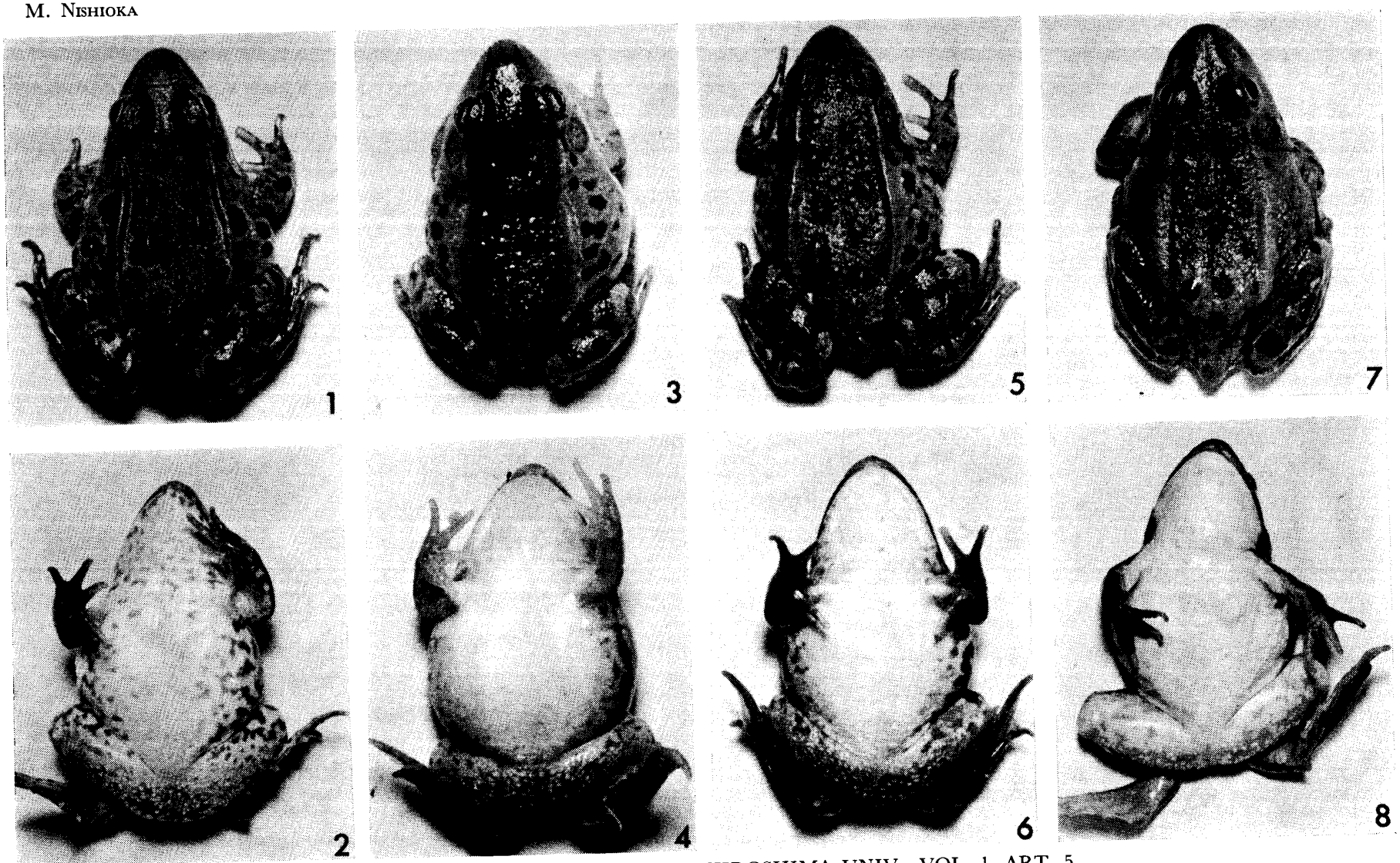

PLATE II

Diploid nucleo-cytoplasmic hybrid frogs consisting of *Rana brevipoda* cytoplasm and *Rana plancyi chosenica* nuclei, (B)PP. $\times 1.5$

9, 10. Male nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)I–1, 354 days old.
11, 12. Male nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)I–2, 369 days old.
13, 14. Female nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)II–1, 360 days old.
15, 16. Female nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)IV–1, 315 days old.

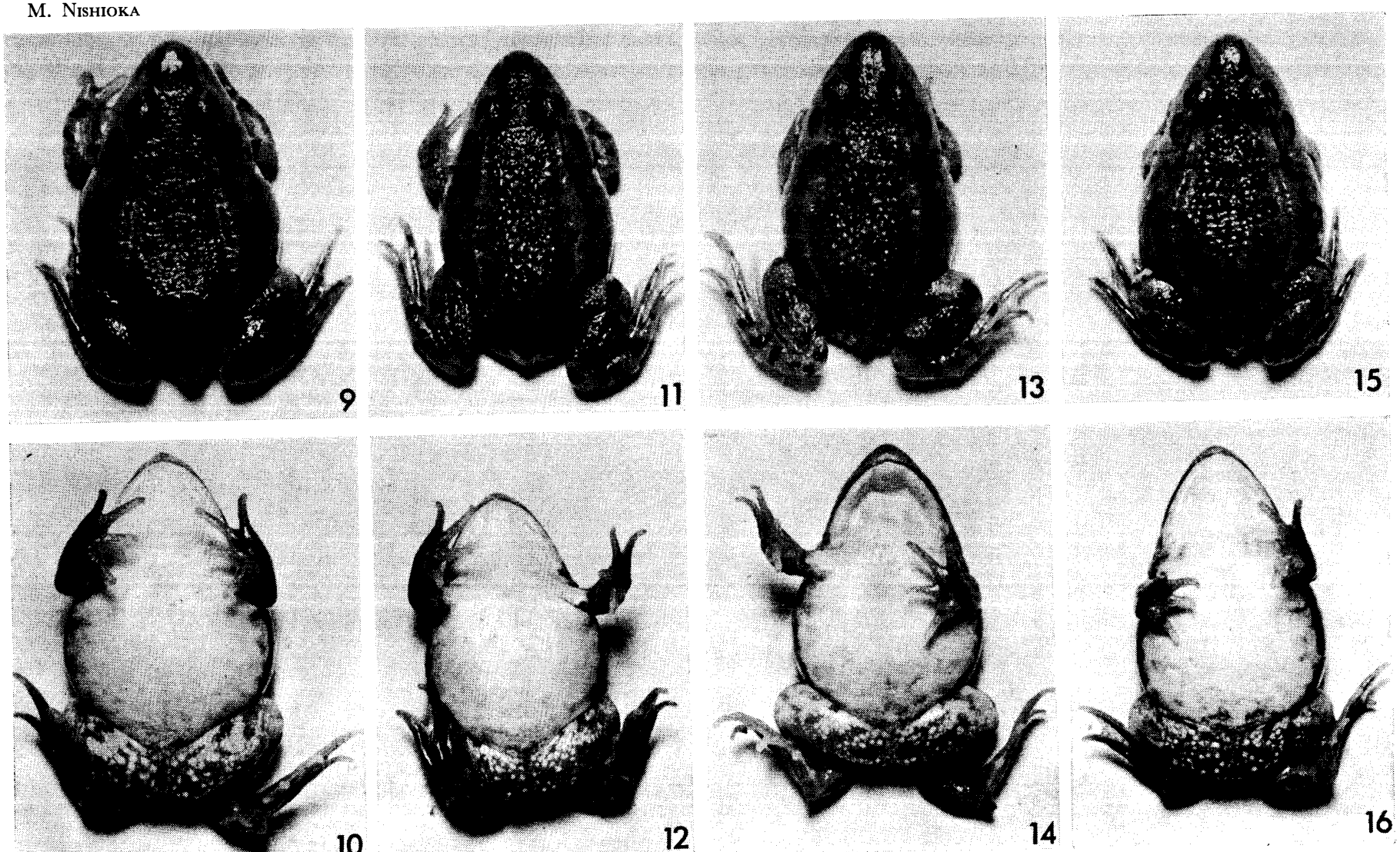
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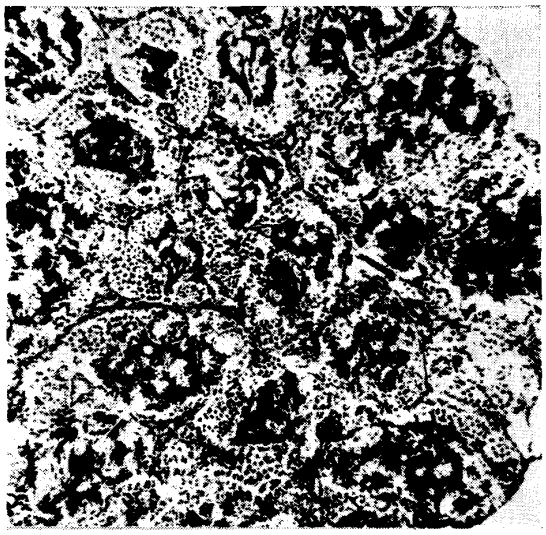
PLATE III

Cross-sections of the gonads of control frogs, 1 year old.

17. Testis of a male *Rana plancyi chosenica*, (P)PP. ×100.
18. Ditto ×250.
19. Testis of a male hybrid, (P)PB. ×100.
20. Ditto ×250.
21. Ovary of a female *Rana plancyi chosenica*, (P)PP. ×100.
22. Ovary of a female hybrid, (P)PB. ×100.



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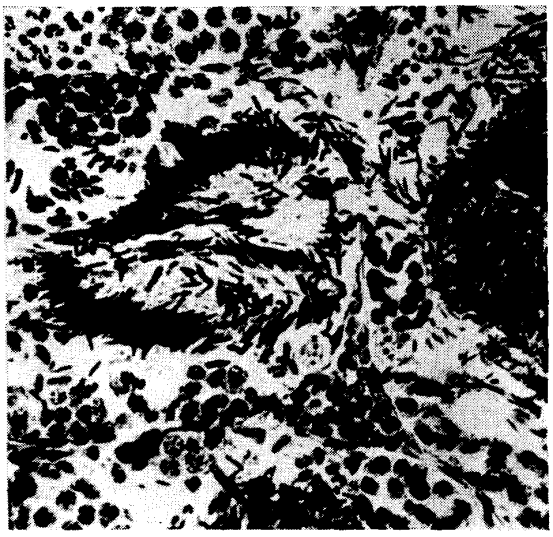

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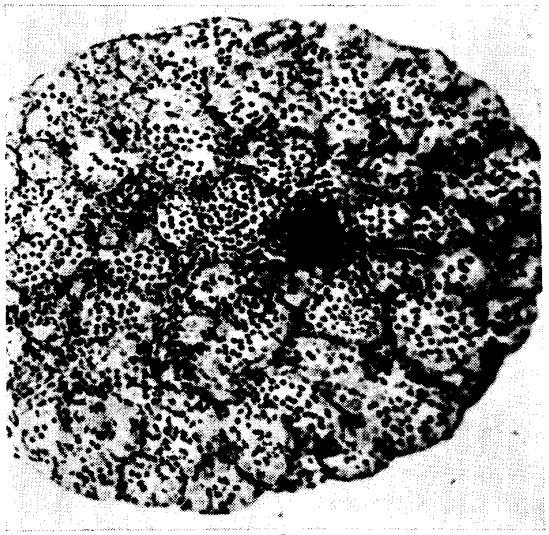

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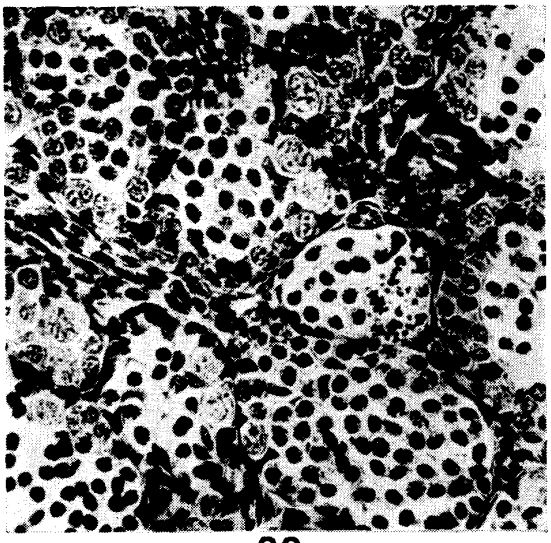

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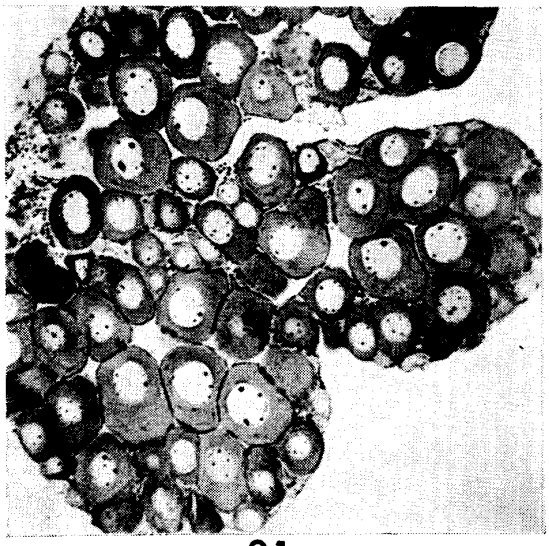

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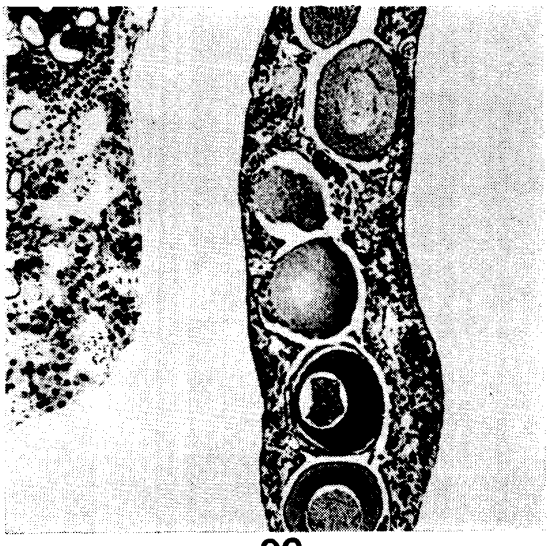

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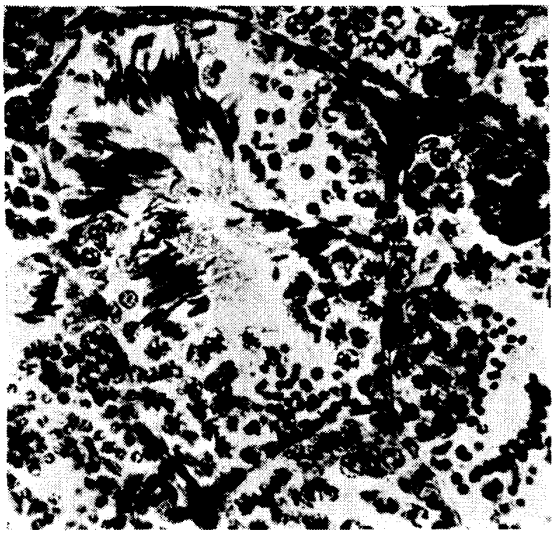
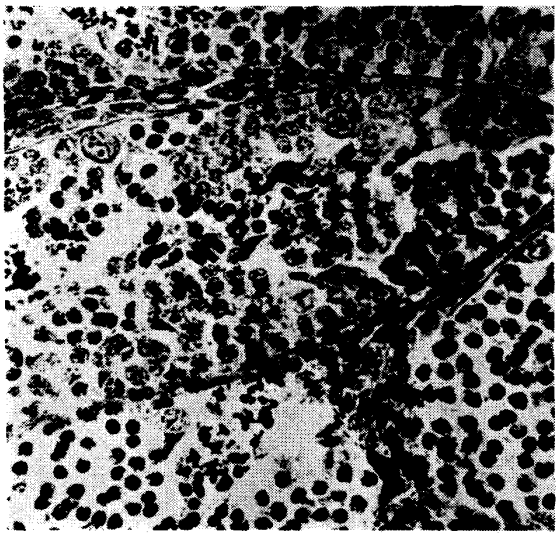
PLATE IV

Cross-sections of the gonads of diploid nucleo-cytoplasmic hybrid frogs consisting of *Rana brevipoda* cytoplasm and *Rana plancyi chosenica* nuclei, (B)PP.

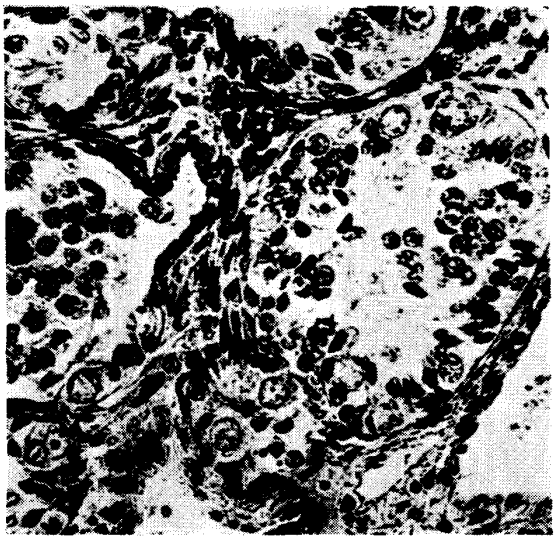
23. Testis of a male nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)I–2, 369 days old, containing a few small bundles of normal spermatozoa. ×250.
24. Testis of a male nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)I–1, 354 days old, containing a few abnormal spermatozoa. ×250.
25. Testis of a male nucleo-cytoplasmic hybrid, (B)PP, No. 63PII(B)V–4, 177 days old, containing a small number of degenerating germ cells. ×250.
26. Ovary of a female nucleo-cytoplasmic hybrid, (B)PP, No. 63PI(B)IV–1, 315 days old, containing many growing auxocytes. ×100.
27. Ovary of a female nucleo-cytoplasmic hybrid, (B)PP, No. 63PII(B)V–2, 214 days old, containing no growing auxocytes. ×100.
28. Ovary of a female nucleo-cytoplasmic hybrid, (B)PP, No. 63PII(B)V–5, 280 days old, containing a small number of degenerating germ cells. ×100.



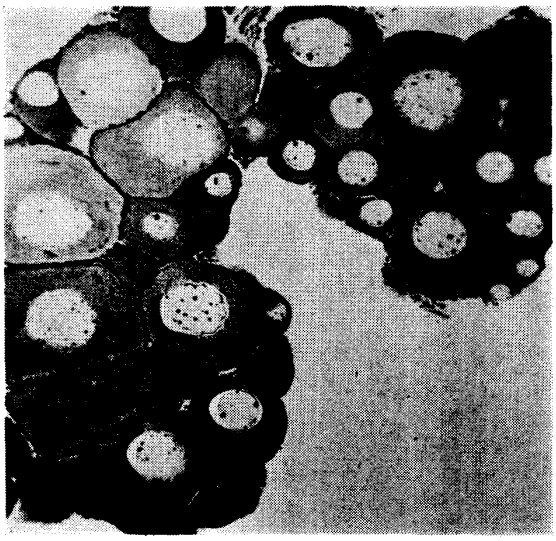
23



24



25



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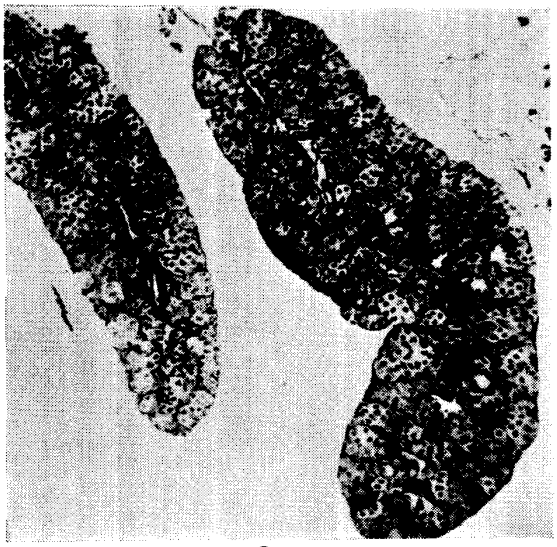
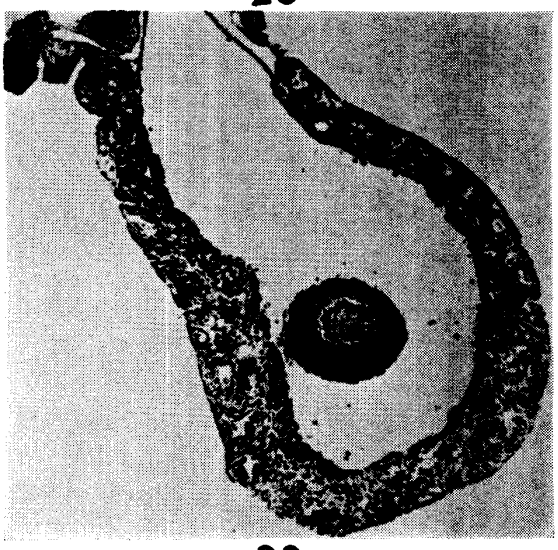


27



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