

Nucleo-cytoplasmic Hybrids between *Rana japonica* and *Rana temporaria temporaria*

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(With 1 Text-figure and 2 Plates)

INTRODUCTION

In amphibians various kinds of nucleo-cytoplasmic hybrids have been produced so far by SAMBUICHI (1957, '61), McKINNEL (1960), GURDON (1961, '62), KAWAMURA and NISHIOKA (1963a, b, '72), NISHIOKA (1971a, b, '72a, b, c), ORTOLANI, FISCHBERG and SLATKINE (1966), GALLIEN, C.-L. (1970), et al. Generally speaking, the nucleo-cytoplasmic hybrids between two subspecies or varieties are nearly the same with that which provided the nuclei to them, in morphological characters, while those between two different species are not always equal to the nuclear species. A small number of the nucleo-cytoplasmic hybrids between two Japanese brown frog species, *Rana japonica* and *Rana ornativentris*, were similar to the cytoplasmic species or intermediate between the two species in some external characters. The remaining majority were quite similar to the nuclear species in appearance (KAWAMURA and NISHIOKA, 1963b).

It was the present authoress' belief that when some nucleo-cytoplasmic hybrids would be produced from two brown frogs species which are more remotely related from each other than the two species stated above, they should certainly reveal the morphological characters of the cytoplasmic species or intermediate between the two. As such a combination of brown frogs, she selected *Rana temporaria temporaria* from Europe and *Rana japonica*. KAWAMURA and KOBAYASHI (1960) have reported that they obtained interspecific hybrids from female *japonica* and male *temporaria*. From their crossing experiments, it was found that these hybrids were far inferior to those of female *japonica* and male *ornativentris* in the viability of embryos. Moreover, only a matured hybrid of *japonica* and *temporaria* was clearly more degenerative than those of *japonica* and *ornativentris* in the inner structures of the testes, although both kinds of hybrids were the same with each other in that they became sterile males. These situations seemed to the present authoress to show that the relation between the former two species was rather remote than that between the latter.

It has been found by SAMBUICHI (1957, '61) and KAWAMURA and NISHIOKA (1963 a) that the nucleo-cytoplasmic hybrids somewhat resemble the cytoplasmic species in the body color or the distribution of tail iridophores at the tadpole stage. However, as they were meager in number, it was necessary to make further

observations on many individuals in order to generalize the interesting information. The present research may fulfil such a request to a certain extent, too.

MATERIAL AND METHODS

The Japanese brown frog, *Rana japonica* GUENTHER was collected in the suburbs of Hiroshima, while the European brown frog, *Rana temporaria temporaria* L. was from Luxemburg. By the courtesy of Mr. R. THORN two pairs of *Rana temporaria* caught in Luxemburg were shipped to Dr. KAWAMURA in 1961 and 1962. The pair sent in 1961 was a female and a hermaphrodite, while that of 1962 was a female and a male.

Nucleo-cytoplasmic hybrids were produced by transplantation of blastula nuclei of *Rana temporaria* into enucleated eggs of *Rana japonica* in the breeding seasons of the years 1962 and 1963. Eggs were obtained from females whose ovulation was accelerated by transplantation of frog pituitaries. The blastulae of *Rana temporaria* were developed from such eggs after artificial fertilization. The nuclear transplantation was performed by the method of BRIGGS and KING(1952, '53). In the control series enucleated eggs of *Rana japonica* were transplanted with blastula nuclei of the same species. The ploidy of tadpoles in experimental series was examined by the tail-tip method (cf. KAWAMURA and NISHIOKA, 1960).

The following abbreviations are used in the present paper.

- T—— A set of *Rana temporaria* chromosomes
- J—— A set of *Rana japonica* chromosomes
- (T)—— *Rana temporaria* cytoplasm
- (J)—— *Rana japonica* cytoplasm

OBSERVATION

I. Experiments with blastulae produced from a hermaphroditic *Rana temporaria* as the donors

1. Production of nucleo-cytoplasmic hybrids

Of 66 self-fertilized eggs obtained in 1962 from a hermaphroditic *Rana temporaria* No. (T)TT62W1, 37(56.1%) cleaved normally. However, 26 of them attained the hatching stage, while three, three, four and one eggs became abnormal and died at the gastrula, neurula, tail-bud and hatching stage, respectively (Table 1). Four of the hatched embryos became edematous and died at the feeding tadpole stage. The remaining 22 grew normally and completed their metamorphosis.

When *Rana japonica* eggs were inseminated with sperm of the hermaphroditic *Rana temporaria*, only 63 (12.3%) of 514 eggs cleaved normally, while 416 did abnormally, probably owing to polyspermy. Of the normally cleaved eggs three, four and seven became abnormal and died at the blastula, gastrula and neurula stage, respectively, and the remaining 49 hatched normally. Six, eight and three of

TABLE 1
Results of crosses in the control series

| Parents | | No. of eggs | No. of normally cleaved eggs | No. of normal neurulae | No. of normal tail-bud embryos | No. of normally hatched tadpoles |
|--------------|--------------|-------------|------------------------------|------------------------|--------------------------------|----------------------------------|
| Female | Male | | | | | |
| (T)TT62W 1 ♀ | (T)TT62W 1 ♂ | 66 | 37 (56.1%) | 31 (47.0%) | 27 (40.9%) | 26 (39.4%) |
| (J)JJ62W 1~3 | (T)TT62W 1 ♂ | 514 | 63 (12.3%) | 49 (9.5%) | 49 (9.5%) | 49 (9.5%) |
| (J)JJ62W 1~3 | (J)JJ62W 1 | 143 | 143 (100%) | 143 (100%) | 143 (100%) | 143 (100%) |
| (T)TT63W 2 | (T)TT63W 2 | 74 | 74 (100%) | 74 (100%) | 74 (100%) | 74 (100%) |
| (J)JJ63W 1~2 | (T)TT63W 2 | 253 | 79 (31.2%) | 72 (28.5%) | 70 (27.7%) | 64 (25.3%) |
| (J)JJ63W 1~2 | (J)JJ63W 2 | 365 | 314 (86.0%) | 314 (86.0%) | 307 (84.1%) | 307 (84.1%) |

the latter died before and during the feeding tadpole and metamorphosing stage, respectively. The other 32 tadpoles completed their metamorphosis.

Concerning *Rana japonica* in the control series, 143 eggs obtained from three females were all fertilized with sperm of a male and grew into normal tadpoles. All these tadpoles completed their metamorphosis without becoming abnormal.

Diploid cell nuclei obtained from blastulae which developed from eggs of the hermaphroditic *Rana temporaria* after self-fertilization were transferred to enucleated eggs of *Rana japonica*. The recipient eggs were 640 in number; they were obtained from 11 females. The results of transplantation experiments are presented in Table 2.

Of 199 normally cleaved eggs only 15 grew normally and hatched. However, two of them became abnormal and died at the early tadpole stage. The remaining 13 tadpoles were examined in terms of the ploidy. As a result it was found that there were eleven diploids and two triploids. The diploids were considered to be possibly nucleo-cytoplasmic hybrids, while the triploids seemed to have developed from eggs in which the enucleation was not successful. All of them were produced from among eggs of four females, and it was especially notable that six of them were obtained from a single female No. 62W9.

2. Life history

When the three kinds of 50-day-old tadpoles in the control series were compared with one another in total length, it was found that hybrids were the largest, *japonica* the next and *temporaria* the smallest (Table 3). The *temporaria* began their landing earliest among the three kinds of tadpoles: all of them finished their landing 56 to 58, mean 57.5 days, after the development, while the *japonica* did latest, being 87 to 91, mean 89.4 days. The hybrids were intermediate, being 80 to 85, mean 83.2 days. At the time of the completion of metamorphosis the

TABLE 2
Synthesis of nucleo-cytoplasmic hybrids from *Rana japonica*

| 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. |
| (J)62W 1 | TT62W 1 ♀ | 120 | 62 (51.7%) | 50 (41.7%) | 36 (30.0%) | 26 (21.7%) | 26 (21.7%) | 10 (8.3%) |
| (J)62W 2 | " | 20 | 8 (40.0%) | 6 (30.0%) | 6 (30.0%) | 2 (10.0%) | 4 (20.0%) | 2 (10.0%) |
| (J)62W 3 | " | 20 | 4 (20.0%) | 8 (40.0%) | 2 (10.0%) | 2 (10.0%) | 0 | 2 (10.0%) |
| (J)62W 4 | " | 20 | 6 (30.0%) | 8 (40.0%) | 2 (10.0%) | 4 (20.0%) | 0 | 2 (10.0%) |
| (J)62W 5 | " | 20 | 4 (20.0%) | 16 (80.0%) | 2 (10.0%) | 2 (10.0%) | 2 (10.0%) | 0 |
| (J)62W 6 | " | 20 | 2 (10.0%) | 14 (70.0%) | 1 (5.0%) | 1 (5.0%) | 1 (5.0%) | 0 |
| (J)62W 7 | " | 120 | 56 (46.7%) | 64 (53.3%) | 38 (31.7%) | 18 (15.0%) | 28 (23.3%) | 10 (8.3%) |
| (J)62W 8 | " | 100 | 8 (8.0%) | 29 (29.0%) | 8 (8.0%) | 0 | 4 (4.0%) | 0 |
| (J)62W 9 | " | 100 | 35 (35.0%) | 25 (25.0%) | 35 (35.0%) | 0 | 33 (33.0%) | 2 (2.0%) |
| (J)62W 10 | " | 50 | 7 (14.0%) | 18 (36.0%) | 6 (12.0%) | 1 (2.0%) | 3 (6.0%) | 3 (6.0%) |
| (J)62W 11 | " | 50 | 7 (14.0%) | 26 (52.0%) | 7 (14.0%) | 0 | 7 (14.0%) | 0 |
| Total | | 640 | 199 (31.1%) | 264 (41.3%) | 143 (22.3%) | 56 (8.8%) | 108 (16.9%) | 31 (4.8%) |

TABLE 3
Life histories of diploid nucleo-cytoplasmic hybrids with nuclei derived from

| Kind or individual no. | 50-day-old tadpoles | | | | Age at the time of landing (days) | No. of metamorphosed frogs | Period of metamorphosing stage (days) |
|------------------------|---------------------|--------------------|-------------------|------------------|-----------------------------------|----------------------------|---------------------------------------|
| | No. | Total length (mm.) | Body length (mm.) | Body width (mm.) | | | |
| (1) (T)TT62W 1 ♀ | 22 | 43.5±0.3 | 13.0±0.2 | 8.0±0.1 | 56~58 (57.5) | 22 (33.3%) | 3~4 |
| (2) (J)JT62W | 40 | 47.1±0.5 | 14.2±0.2 | 8.5±0.1 | 80~85 (83.2) | 32 (6.2%) | 3~6 |
| (3) (J)JJ62W | 143 | 44.2±0.5 | 13.4±0.2 | 8.3±0.1 | 87~91 (89.4) | 143 (100%) | 3~5 |
| (1) (J)TT62W 1.1 | | 40.6 | 13.2 | 8.2 | 77 | | 5 |
| (2) (J)TT62W 1.2 | | 41.5 | 13.1 | 9.5 | 72 | | 4 |
| (3) (J)TT62W 7.1 | | 39.7 | 13.4 | 9.6 | 93 | | 8 |
| (4) (J)TT62W 7.2 | | 38.5 | 13.0 | 9.0 | 91 | | |
| (5) (J)TT62W 9.1 | | 39.0 | 13.6 | 8.2 | 93 | | 6 |
| (6) (J)TT62W 9.2 | | 33.5 | 13.1 | 7.0 | 115 | | |
| (7) (J)TT62W 9.3 | | 36.5 | 11.7 | 6.1 | 116 | | 7 |
| (8) (J)TT62W 9.4 | | 33.5 | 11.0 | 7.0 | | | |
| (9) (J)TT62W 9.5 | | 37.5 | 14.0 | 8.2 | | | |
| (10) (J)TT62W 9.6 | | 38.2 | 13.0 | 8.6 | 126 | | 5 |
| (11) (J)TT62W10.1 | | 39.6 | 13.2 | 9.3 | 95 | | 5 |

and a hermaphroditic *Rana temporaria temporaria*

| 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 |
| 12 (10.0%) | 14 (11.7%) | 5 (4.2%) | 9 (7.5%) | 3 (2.5%) | 2 (1.7%) | 3 (2.5%) | 2 (1.7%) | 1 (0.8%) |
| 0 | 4 (20.0%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 (5.0%) | 1 (5.0%) | 1 (5.0%) | 0 | 1 (5.0%) | 0 | 0 | 0 | 0 |
| 0 | 1 (5.0%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 (13.3%) | 12 (10.0%) | 14 (11.7%) | 2 (1.7%) | 3 (2.5%) | 11 (9.2%) | 3 (2.5%) | 2 (1.7%) | 1 (0.8%) |
| 2 (2.0%) | 2 (2.0%) | 2 (2.0%) | 0 | 0 | 2 (2.0%) | 0 | 0 | 0 |
| 14 (14.0%) | 19 (19.0%) | 7 (7.0%) | 7 (7.0%) | 6 (6.0%) | 1 (1.0%) | 6 (6.0%) | 6 (6.0%) | 0 |
| 2 (4.0%) | 1 (2.0%) | 1 (2.0%) | 1 (2.0%) | 1 (2.0%) | 0 | 1 (2.0%) | 1 (2.0%) | 0 |
| 3 (6.0%) | 4 (8.0%) | 2 (4.0%) | 1 (2.0%) | 1 (2.0%) | 1 (2.0%) | 0 | 0 | 0 |
| 50 (7.8%) | 58 (9.1%) | 32 (5.0%) | 20 (3.1%) | 15 (2.3%) | 17 (2.7%) | 13 (2.0%) | 11 (1.7%) | 2 (0.3%) |

a hermaphroditic *Rana temporaria temporaria* and the control individuals

| Mean body length soon after metamorphosis | | Age at the time of death or observation (days) | Measurements at the time of death or observation | | | |
|---|------------------------|--|--|-------------------|------------------------|--------------|
| Body length (mm.) | Hind-limb length (mm.) | | No. | Body length (mm.) | Hind-limb length (mm.) | H.L. B.L. |
| 13.7±0.2 | 15.9±0.3 | 370 | 8 | 51.2±3.4 | 85.5±4.2 | 1.67 |
| 16.5±0.3 | 20.1±0.3 | 370 | 20 | 45.5±3.3 | 73.6±4.3 | 1.62 |
| 15.1±0.2 | 19.3±0.2 | 370 | 35 | 44.5±3.0 | 80.5±4.1 | 1.81 |
| 17.5 | 21.5 | 370 | 1 | 47.0 | 72.5 | 1.54 |
| 16.9 | 20.7 | 554 | 1 | 56.5 | 98.0 | 1.73 |
| 17.8 | 22.0 | 255 | 1 | 27.0 | 62.5 | 1.69 |
| | | 93 | | | | |
| 13.2 | 14.9 | 101 | | | | |
| | | 116 | | | | |
| 12.5 | 14.5 | 126 | | | | |
| | | 109 | 1 | (41.5)* | | |
| | | 102 | 1 | (46.0)* | | |
| 15.5 | 12.2 | 131 | | | | |
| 14.5 | 19.0 | 100 | | | | |

* Total length of the tadpole

hybrids were the largest, the *japonica* the next and the *temporaria* the smallest. After one year, however, the *temporaria* were the largest, the hybrids the next and the *japonica* the smallest.

The nuclear transfers at the 50-day-old tadpole stage were 38.0 mm. in total length on an average, being smaller than both *japonica* and *temporaria* controls. Nine of 11 nuclear transfers attained the metamorphosing stage; they began their landing 72~126, mean 97.6 days, after development. However, seven of them completed their metamorphosis. The period of time from their landing to the sufficient absorption of their tails was 4~8 days, in contrast to 3~6 days in the three kinds of frogs in the control series. The nuclear transfer frogs at the stage immediately after the completion of metamorphosis were 15.4 mm. on an average in body length. Four frogs died within 10 days after metamorphosis, and one died about four months later. The remaining two attained their sexual maturity; both of them were males.

TABLE 4
Synthesis of nucleo-cytoplasmic hybrids from *Rana japonica* and

| Individual no. of parents | | No. of eggs | No. of cleaved eggs | | No. of blastulae | | No. of gastrulae | |
|---------------------------|------------------|-------------|---------------------|----------------|------------------|---------------|------------------|---------------|
| Enu-cleaved egg | Blastula nucleus | | Norm. | Abnorm. | Norm. | Abnorm. | Norm. | Abnorm. |
| (J) 63W 1 | TT 63W 2 | 100 | 25 (25.0%) | 13 (13.0%) | 19 (19.0%) | 6 (6.0%) | 13 (13.0%) | 6 (6.0%) |
| (J) 63W 2 | " | 100 | 31 (31.0%) | 9 (9.0%) | 26 (26.0%) | 5 (5.0%) | 17 (17.0%) | 9 (9.0%) |
| (J) 63W 3 | " | 100 | 46 (46.0%) | 2 (2.0%) | 35 (35.0%) | 11 (11.0%) | 30 (30.0%) | 5 (5.0%) |
| (J) 63W 4 | " | 100 | 21 (21.0%) | 30 (30.0%) | 18 (18.0%) | 3 (3.0%) | 14 (14.0%) | 4 (4.0%) |
| (J) 63W 5 | " | 100 | 24 (24.0%) | 19 (19.0%) | 19 (19.0%) | 5 (5.0%) | 7 (7.0%) | 12 (12.0%) |
| (J) 63W 6 | " | 100 | 35 (35.0%) | 11 (11.0%) | 26 (26.0%) | 9 (9.0%) | 19 (19.0%) | 7 (7.0%) |
| (J) 63W 7 | " | 100 | 17 (17.0%) | 17 (17.0%) | 9 (9.0%) | 8 (8.0%) | 4 (4.0%) | 5 (5.0%) |
| Total | | 700 | 199 (28.4%) | 101 (14.4%) | 152 (21.7%) | 47 (6.7%) | 104 (14.9%) | 48 (6.9%) |
| (J) 63W 1 | JJ 63W 1 | 40 | 24 (60.0%) | 11 (27.5%) | 20 (50.0%) | 4 (10.0%) | 17 (42.5%) | 3 (7.5%) |
| (J) 63W 2 | " | 40 | 28 (70.0%) | 6 (15.0%) | 23 (57.5%) | 5 (12.5%) | 19 (47.5%) | 4 (10.0%) |
| (J) 63W 3 | " | 30 | 20 (66.7%) | 6 (20.0%) | 18 (60.0%) | 2 (6.7%) | 17 (56.7%) | 1 (3.3%) |
| (J) 63W 4 | " | 32 | 17 (53.1%) | 12 (37.5%) | 16 (50.0%) | 1 (3.1%) | 12 (37.5%) | 4 (12.5%) |
| Total | | 142 | 89 (62.7%) | 35 (24.6%) | 77 (54.2%) | 12 (8.5%) | 65 (45.8%) | 12 (8.5%) |

II. Experiments with blastulae produced from a pair of normal *Rana temporaria* as the donors

1. Production of nucleo-cytoplasmic hybrids

Eggs of the female *temporaria* No. (T)TT63W2 inseminated with sperm of the male *temporaria* No. (T)TT63W2 all cleaved regularly and grew into normal tadpoles. All these tadpoles completed their metamorphosis.

In the cross, *Rana japonica* ♀ × *Rana temporaria* ♂, 103(40.7%) of 253 eggs obtained from two females cleaved irregularly, probably owing to polyspermy. Although there were 79(31.2%) normally cleaved eggs, four, three, two, six and seven of them became abnormal and died at the gastrula, neurula, tail-bud, hatching and feeding stage, respectively. The remaining 57 eggs grew into normal tadpoles and attained the completion of metamorphosis.

Of 365 eggs of the same female *japonica* inseminated with sperm of a male of

normal *Rana temporaria temporaria*

| 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 |
| 11 (11.0%) | 2 (2.0%) | 7 (7.0%) | 4 (4.0%) | 6 (6.0%) | 1 (1.0%) | 3 (3.0%) | 2 (2.0%) | 1 (1.0%) |
| 15 (15.0%) | 2 (2.0%) | 9 (9.0%) | 6 (6.0%) | 8 (8.0%) | 1 (1.0%) | 4 (4.0%) | 4 (4.0%) | 0 |
| 26 (26.0%) | 4 (4.0%) | 16 (16.0%) | 10 (10.0%) | 13 (13.0%) | 3 (3.0%) | 7 (7.0%) | 6 (6.0%) | 1 (1.0%) |
| 11 (11.0%) | 3 (3.0%) | 8 (8.0%) | 3 (3.0%) | 7 (7.0%) | 1 (1.0%) | 2 (2.0%) | 2 (2.0%) | 0 |
| 6 (6.0%) | 1 (1.0%) | 4 (4.0%) | 2 (2.0%) | 2 (2.0%) | 2 (2.0%) | 1 (1.0%) | 1 (1.0%) | 0 |
| 14 (14.0%) | 5 (5.0%) | 5 (5.0%) | 9 (9.0%) | 5 (5.0%) | 0 | 2 (2.0%) | 1 (1.0%) | 1 (1.0%) |
| 3 (3.0%) | 1 (1.0%) | 2 (2.0%) | 1 (1.0%) | 2 (2.0%) | 0 | 1 (1.0%) | 0 | 1 (1.0%) |
| 86 (12.3%) | 18 (2.6%) | 51 (7.3%) | 35 (5.0%) | 43 (6.1%) | 8 (1.1%) | 20 (2.9%) | 16 (2.3%) | 4 (0.6%) |
| 9 (22.5%) | 8 (20.0%) | 4 (10.0%) | 5 (12.5%) | 3 (7.5%) | 1 (2.5%) | 3 (7.5%) | 2 (5.0%) | 1 (2.5%) |
| 12 (30.0%) | 7 (17.5%) | 6 (15.0%) | 6 (15.0%) | 4 (10.0%) | 2 (5.0%) | 2 (5.0%) | 2 (5.0%) | 0 |
| 10 (33.3%) | 7 (23.3%) | 6 (20.0%) | 4 (13.3%) | 4 (13.3%) | 2 (6.7%) | 4 (13.3%) | 4 (13.3%) | 0 |
| 7 (21.9%) | 5 (15.6%) | 6 (18.8%) | 1 (3.1%) | 4 (12.5%) | 2 (6.3%) | 4 (12.5%) | 2 (6.3%) | 2 (6.3%) |
| 38 (26.8%) | 27 (19.0%) | 22 (15.5%) | 16 (11.3%) | 15 (10.6%) | 7 (4.9%) | 13 (9.2%) | 10 (7.0%) | 3 (2.1%) |

the same species 314(86%) cleaved normally and grew into normal tadpoles, except seven which died at the tail-bud stage. While two tadpoles died at the feeding stage, the other 305 completed their metamorphosis (Table 1).

As the control of nuclear transplantation experiments, diploid blastula nuclei of *japonica* were transplanted into 142 enucleated eggs obtained from four female *japonica* Nos. 63W1, 63W2, 63W3 and 63W4. Of these eggs 89(62.7%) cleaved regularly and 15(10.6%) hatched normally. Within 40 days after nuclear transplantation two tadpoles became abnormal and died. The other 13 were examined in terms of the ploidy; there were ten diploids and three triploids. The most numerous tadpoles, though only four (13.3%) in number, were produced from the eggs of the female No. 63W3 (Table 4).

Diploid cell nuclei were obtained from blastulae which developed from eggs of a female *Rana temporaria* by artificial insemination with sperm of a male of the same species and transplanted into 700 enucleated eggs of seven female *Rana japonica*. Of these eggs 199(28.4%) cleaved normally and only 43(6.1%) hatched. Twenty-three of the latter died within 40 days after nuclear transplantation of various abnormalities, such as edema, curvature or distortion of the body, microcephaly, blister and atrophy. The remaining 20 tadpoles were examined in terms of the ploidy and it was found that there were 16 diploids and four triploids. The latter seemed to have developed from eggs in which the enucleation was unsuccessful. The number of diploids produced from one female significantly differed from female to female. Six, the largest number of such diploids, were obtained from a single female No. 63W3 as in the above experimental series. The ratio of interspecific nuclear transfers was generally lower than that of intraspecific nuclear transfers.

2. Life history

At the 50-day-old tadpole stage the hybrids in the control series were the largest, being 46.7 mm. on an average in total length, while the *temporaria* were 44.3 mm. and the *japonica* 43.4 mm. (Table 5). On the other hand, the intraspecific nuclear transfers of *japonica* were 40.5 mm., while the interspecific nuclear transfers were 38.5 mm., being the smallest.

The *temporaria* began their landing earliest, the hybrids next and the *japonica* last among the three kinds of frogs in the control series. The intraspecific transfers of *japonica* were mostly later in landing than the *japonica* controls. Most interspecific nuclear transfers were intermediate in landing between the *temporaria* and the hybrids in the control series, while a small number of them were even much later than the *japonica* controls. Moreover, in two of 14 interspecific nuclear transfers the tail was very slowly absorbed during metamorphosis.

In the body length immediately after the completion of metamorphosis the hybrids were 16.8 ± 0.4 mm., the *japonica*, 16.3 ± 0.2 mm. and the *temporaria*, 14.1 ± 0.2 mm. The smaller size of the *temporaria* seemed to be mainly due to the fact that their metamorphosis occurred extremely earlier than those of the other two kinds of frogs. The intraspecific nuclear transfers of *japonica* were

somewhat inferior in growth to the *japonica* controls; their body length was 15.7 mm. on an average. The interspecific nuclear transfers were also 15.7 mm. on an average in body length. The matter that the interspecific nuclear transfers were larger than the *temporaria* seemed to be due to their later metamorphosis. At the age of one year the *temporaria* were the largest, being 54.3 ± 3.0 mm. in body length, and the hybrids and the *japonica* were 49.0 ± 4.5 mm. and 47.5 ± 3.2 mm., respectively. The intraspecific and interspecific nuclear transfers were 44.8 mm. and 47.7 mm. on an average.

Eight of the intraspecific nuclear transfers completed their metamorphosis, although two of them died within 4 months after metamorphosis. The other six reached sexual maturity. Of 16 nuclear transfers, 14 completed their metamorphosis. Three died immediately and two within 3 weeks after metamorphosis, respectively. Two others died 4.5 and 5.5 months after metamorphosis, while the remaining seven reached sexual maturity.

III. External characters of tadpoles

1. Eight external characters

Observations were made about the following eight external characters which were useful in distinguishing between the two species at the tadpole stage.

a. The dorsal ground color

The *japonica* control tadpoles were pale grey, while the *temporaria* yellow-green. The interspecific hybrids were intermediate between both species, that is, light yellow-green.

Eleven nuclear transfers (J)TT with nuclei of the hermaphroditic *temporaria* strain were not uniform in color. One tadpole No. 62W9.3 was perfectly of the *japonica* type, three Nos. 62W1.1, 62W7.2 and 62W9.5 were intermediate between the *japonica* and the interspecific hybrids and two Nos. 62W7.1 and 62W9.1 were very similar to the interspecific hybrids, that is, light yellow-green. Three tadpoles Nos. 62W9.2, 62W9.4 and 62W10.1 were rather of the *temporaria* type, as they had yellow-green tone, although they were extremely melanistic. Another tadpole No. 62W1.2 was intermediate between the interspecific hybrids and the *temporaria* control, and the remaining one No. 62W9.6 was perfectly of the *temporaria* type.

Of the sixteen nuclear transfer tadpoles which were produced by the combination of *japonica* cytoplasm and nuclei of the normal *temporaria* strain, two Nos. 63W2.3 and 63W4.2 were perfectly of the *japonica* type, ten Nos. 63W1.1, 63W2.4, 63W3.1, 63W3.3, 63W3.4, 63W3.5, 63W3.6, 63W4.1, 63W5.1 and 63W6.1 were quite the same as the interspecific hybrids and three Nos. 63W1.2, 63W2.1 and 63W3.2 were intermediate between the interspecific hybrids and the *temporaria* control. The remaining one No. 63W2.2 was perfectly of the *temporaria* type.

b. Distribution of iridophores

In the *japonica* control tadpoles whitish iridophores distributed on the dorsal surface of the body showed an arrangement like pavement stones, owing to ag-

TABLE 5
Life histories of diploid nucleo-cytoplasmic hybrids with nuclei derived

| Kind or individual no. | 50-day-old tadpoles | | | | Age at the time of landing (days) | No. of metamorphosed frogs | Period of metamorphosing stage (days) |
|------------------------|---------------------|--------------------|-------------------|------------------|-----------------------------------|----------------------------|---------------------------------------|
| | No. | Total length (mm.) | Body length (mm.) | Body width (mm.) | | | |
| (1) (T)TT63W2 | 74 | 44.3±0.3 | 13.2±0.2 | 8.1±0.1 | 57~60 (58.2) | 74 (100%) | 3~5 |
| (2) (J)JT63W | 61 | 46.7±0.4 | 14.0±0.2 | 8.5±0.1 | 82~86 (84.0) | 57 (22.5%) | 3~7 |
| (3) (J)JJ63W | 305 | 43.4±0.5 | 13.5±0.1 | 8.4±0.1 | 90~95 (93.3) | 305 (83.6%) | 3~5 |
| (1) (J)+JJ63W 1.1 | | 42.5 | 13.5 | 8.0 | 94 | | 3 |
| (2) (J)+JJ63W 1.2 | | 43.0 | 13.5 | 8.5 | 97 | | 5 |
| (3) (J)+JJ63W 2.1 | | 40.5 | 13.0 | 8.0 | 94 | | 3 |
| (4) (J)+JJ63W 2.2 | | 41.0 | 14.0 | 8.5 | 102 | | 4 |
| (5) (J)+JJ63W 3.1 | | 39.0 | 14.0 | 8.0 | | | |
| (6) (J)+JJ63W 3.2 | | 39.5 | 13.5 | 8.0 | 105 | | 6 |
| (7) (J)+JJ63W 3.3 | | 40.0 | 13.0 | 8.5 | 97 | | 4 |
| (8) (J)+JJ63W 3.4 | | 40.0 | 13.5 | 7.5 | 103 | | 5 |
| (9) (J)+JJ63W 4.1 | | 41.0 | 13.0 | 8.5 | 95 | | 3 |
| (10) (J)+JJ63W 4.2 | | 38.5 | 12.5 | 7.5 | | | |
| (1) (J)TT63W 1.1 | | 41.0 | 13.0 | 8.0 | 72 | | 4 |
| (2) (J)TT63W 1.2 | | 37.0 | 13.0 | 7.0 | 72 | | 10 |
| (3) (J)TT63W 2.1 | | 40.5 | 13.0 | 8.5 | 73 | | 4 |
| (4) (J)TT63W 2.2 | | 42.5 | 13.5 | 9.5 | 75 | | 4 |
| (5) (J)TT63W 2.3 | | 36.0 | 12.0 | 7.0 | | | |
| (6) (J)TT63W 2.4 | | 34.5 | 11.5 | 7.0 | 82 | | 6 |
| (7) (J)TT63W 3.1 | | 38.0 | 12.5 | 8.5 | 74 | | 4 |
| (8) (J)TT63W 3.2 | | 41.5 | 13.5 | 9.0 | 80 | | 5 |
| (9) (J)TT63W 3.3 | | 38.0 | 13.0 | 8.0 | 80 | | 5 |
| (10) (J)TT63W 3.4 | | 39.0 | 13.0 | 8.5 | 95 | | 7 |
| (11) (J)TT63W 3.5 | | 41.5 | 13.5 | 9.0 | 102 | | 4 |
| (12) (J)TT63W 3.6 | | 40.5 | 14.0 | 10.0 | 114 | | 10 |
| (13) (J)TT63W 4.1 | | 37.0 | 13.5 | 9.0 | 70 | | 6 |
| (14) (J)TT63W 4.2 | | 32.0 | 11.0 | 7.0 | | | |
| (15) (J)TT63W 5.1 | | 35.5 | 12.0 | 7.5 | 83 | | 4 |
| (16) (J)TT63W 6.1 | | 42.0 | 14.5 | 9.0 | 105 | | 6 |

gregation into numerous small masses. In contrast with this, iridophores of the *temporaria* control were scatteringly distributed on the dorsal surface. They appeared as if minute golden spots were sprinkled on black ground. In the interspecific hybrids iridophores on the dorsal surface were intermediate in color, amount and arrangement between those of the two species.

Of the eleven nuclear transfer tadpoles with nuclei of the hermaphroditic *temporaria* strain, three Nos. 62W7.1, 62W7.2 and 62W9.1 were of the *japonica* type, one No. 62W9.6 was intermediate between the *japonica* and interspecific hybrid

from a pair of normal *Rana temporaria temporaria* and the control individuals

| Mean body length soon after metamorphosis | | Age at the time of death or observation (days) | Measurements at the time of death or observation | | | |
|---|------------------------|--|--|-------------------|------------------------|-------------|
| Body length (mm.) | Hind-limb length (mm.) | | No. | Body length (mm.) | Hind-limb length (mm.) | H.L. / B.L. |
| 14.1±0.2 | 16.0±0.3 | 374 | 45 | 54.3±3.0 | 95.2±4.3 | 1.75 |
| 16.8±0.4 | 20.4±0.3 | 374 | 35 | 49.0±4.5 | 85.6±4.3 | 1.75 |
| 16.3±0.2 | 20.2±0.2 | 374 | 48 | 47.5±3.2 | 86.7±4.4 | 1.83 |
| 16.5 | 20.5 | 374 | 1 | 45.5 | 86.0 | 1.89 |
| 16.5 | 20.0 | 374 | 1 | 47.0 | 87.5 | 1.86 |
| 16.0 | 18.5 | 109 | | | | |
| 16.0 | 20.5 | 215 | 1 | 32.5 | 65.0 | 2.00 |
| | | 84 | 1 | (57.0)* | | |
| 15.5 | 19.5 | 734 | 1 | 55.0 | 110.0 | 2.00 |
| 15.0 | 19.0 | 734 | 1 | 53.5 | 104.0 | 1.94 |
| 14.5 | 18.5 | 735 | 1 | 52.0 | 98.5 | 1.89 |
| 15.5 | 20.0 | 374 | 1 | 42.0 | 85.0 | 2.02 |
| | | 90 | 1 | (56.0)* | | |
| 15.0 | 17.5 | 239 | 1 | 34.5 | 57.0 | 1.65 |
| 14.0 | 17.0 | 220 | 1 | 32.5 | 55.5 | 1.71 |
| 16.5 | 20.5 | 734 | 1 | 74.0 | 114.5 | 1.55 |
| 17.5 | 21.5 | 374 | 1 | 50.0 | 90.0 | 1.80 |
| | | 90 | 1 | (41.0)* | | |
| 14.5 | 18.5 | 735 | 1 | 79.5 | 128.0 | 1.61 |
| 16.0 | 18.0 | 359 | 1 | 47.5 | 76.0 | 1.60 |
| 16.5 | 19.0 | 85 | | | | |
| 13.5 | 15.0 | 374 | 1 | 44.5 | 65.5 | 1.47 |
| 17.5 | 22.0 | 125 | 1 | 20.0 | 27.5 | 1.37 |
| 17.0 | 20.5 | 106 | | | | |
| 17.5 | 22.0 | 126 | | | | |
| 14.0 | 15.5 | 374 | 1 | 45.0 | 67.5 | 1.50 |
| | | 106 | 1 | (45.5)* | | |
| 13.5 | 14.5 | 87 | | | | |
| 17.0 | 20.0 | 374 | 1 | 51.5 | 80.0 | 1.55 |

* Total length of the tadpole

tadpoles, and one No. 62W1.1 was very similar to the interspecific hybrid tadpoles, being intermediate between the *japonica* and *temporaria* controls. Two others Nos. 62W1.2 and 62W9.5 were intermediate between the interspecific hybrids and *temporaria* controls. Three Nos. 62W9.2, 62W9.4 and 62W10.1 which were extremely dark in dorsal color, had a few minute golden iridophores sprinkled on the black ground, being rather of *temporaria* type. The remaining one tadpole No. 62W9.3 had a specific pattern of iridophores, that is, abundant minute golden iridophores were so closely distributed that they were hardly dis-

tinguishable from one another at a glance.

Of sixteen nuclear transfers with nuclei of the normal *temporaria* strain, five Nos. 63W2.3, 63W3.3, 63W3.6, 63W4.2 and 63W5.1 were perfectly of the *japonica* type. Seven tadpoles Nos. 63W1.1, 63W1.2, 63W3.1, 63W3.2, 63W3.4, 63W3.5 and 63W6.1 were very similar to the interspecific hybrids. While three others Nos. 63W2.1, 63W2.2 and 63W2.4 were quite similar to the *temporaria* controls, the remaining one No. 63W4.1 was intermediate between the interspecific hybrids and *temporaria* controls.

c. Dorso-lateral black spots

The *japonica* control tadpoles had a pair of remarkable black spots on the dorsal surface of the body, while the *temporaria* had no such spots. In all the interspecific hybrids there were a pair of distinguishable black spots (Table 6).

Five Nos. 62W1.2, 62W7.1, 62W9.3, 62W9.4 and 62W10.1 of the 11 nuclear transfers with nuclei of the hermaphroditic *temporaria* strain had no black spots, being similar to the *temporaria* controls. In three others Nos. 62W1.1, 62W9.1 and 62W9.6, a pair of faint black spots were observed at the early tadpole stage, and later on they eventually disappeared. In another tadpole No. 62W7.2 there were a pair of faint spots at first, which did not disappear later, although they became much fainter. The remaining two tadpoles Nos. 62W9.2 and 62W9.5 had a pair of clear dorso-lateral spots, which were quite similar to those of the *japonica* controls.

Of the 16 nucleo-cytoplasmic hybrids with nuclei of the normal *temporaria* strain, seven Nos. 63W2.1, 63W2.2, 63W3.2, 63W3.6, 63W3.5, 63W5.1 and 63W6.1 had no dorso-lateral spots at their tadpole stage, being the same as the *temporaria* controls, while two others Nos. 63W2.3 and 63W4.2 had a pair of clearly marked dorso-lateral spots, being quite similar to the *japonica* controls. The

TABLE 6
External characters of nucleo-cytoplasmic

| Series | Kind | No. of tadpoles | No. of tadpoles with a pair of black spots on the back | | |
|--------------|-------------|-----------------|--|-----------------------|--------|
| | | | Present | Gradually fading away | Absent |
| Control | (T)TT62W1 ♀ | 22 | 0 | 0 | 22 |
| | (J)JT62W | 40 | 40 | 0 | 0 |
| | (J)JJ62W | 143 | 143 | 0 | 0 |
| | (T)TT63W2 | 74 | 0 | 0 | 74 |
| | (J)JT63W | 61 | 61 | 0 | 0 |
| | (J)JJ63W | 305 | 305 | 0 | 0 |
| Experimental | (J)TT62W1 ♀ | 11 | 3 | 3 | 5 |
| | (J)TT63W2 | 16 | 4 | 5 | 7 |
| | (J)+JJ63W | 10 | 10 | 0 | 0 |

All the tadpoles are 40 days old

remaining seven tadpoles were intermediate in this respect; they had a pair of spots which were clear at first, and then became faint. In five of these tadpoles, Nos. 63W1.1, 63W2.4, 63W3.3, 63W3.4 and 63W4.1, the spots eventually disappeared, while in the other two Nos. 63W1.2 and 63W3.1, the spots did not completely disappear.

d. Dorso-median stripe

The *temporaria* control tadpoles had a black median stripe on the back, owing to the lack of iridophores, while in the *japonica* such a stripe was not found. All the hybrids between these two species had a black dorso-median stripe without exception, although it was not so clear as in the *temporaria* controls.

Of the 11 nuclear transfer tadpoles with nuclei of the hermaphroditic *temporaria* strain, only two Nos. 62W1.2 and 62W10.1 had clearly a black dorso-median stripe which was quite similar to that in the *temporaria* controls. In the remaining nine, there was a black stripe which was very similar to that of the hybrids.

Four Nos. 63W2.1, 63W2.4, 63W3.5 and 63W4.1 of the 16 nuclear transfer tadpoles with nuclei of the normal *temporaria* strain had a clear black dorso-median stripe which was quite the same with that in the *temporaria* controls, while two others Nos. 63W2.3 and 63W4.2 had no dorso-median stripe. The remaining ten tadpoles had a faint, black median stripe which was very similar to that in the hybrids between the two species.

e. Dorso-lateral ridges

The *temporaria* control tadpoles had no lateral ridges on the back. However, there were silver lateral stripes along the black median stripe from the head to the one-third of the tail. These silver stripes were composed of closely packed iridophores. Differing from the *temporaria* control tadpoles, the *japonica* had

hybrid tadpoles and the controls

| No. of tadpoles with a pale median stripe on the back | | No. of tadpoles with each type of pigment pattern on the tail fin | | |
|---|--------|---|-------------------|------------|
| Present | Absent | (T)TT type | Intermediate type | (J)JJ type |
| 22 | 0 | 22 | 0 | 0 |
| 40 | 0 | 0 | 26 | 14 |
| 0 | 143 | 0 | 0 | 143 |
| 74 | 0 | 74 | 0 | 0 |
| 61 | 0 | 0 | 41 | 20 |
| 0 | 305 | 0 | 0 | 305 |
| 11 | 0 | 2 | 7 | 2 |
| 14 | 2 | 1 | 12 | 3 |
| 0 | 10 | 0 | 0 | 10 |

TABLE 7
External characters of each of 27 nuclear transfers

| Individual no. | Tadpole stage | | | | | | | | Frog stage | | |
|----------------|---------------|-----|-----|-----|-----|-----|-----|-----|------------|---------------------|-----------------------|
| | (a) | (b) | (c) | (d) | (e) | (f) | (g) | (h) | Snout | Dorso-lateral folds | Markings of underside |
| (J)TT62W1.1 | M | M | M | M | M | M | M | T | M | T | T |
| (J)TT62W1.2 | M | M | T | T | T | M | M | J | M | T | T |
| (J)TT62W7.1 | M | J | T | M | M | M | M | J | M | T | T |
| (J)TT62W7.2 | M | J | J | M | M | M | J | M | M | T | (Young) |
| (J)TT62W9.1 | M | J | M | M | M | M | M | T | M | T | (Young) |
| (J)TT62W9.2 | T | T | J | M | M | M | M | M | — | T | (Young) |
| (J)TT62W9.3 | J | S | T | M | M | M | M | J | J | M | (Young) |
| (J)TT62W9.4 | T | T | T | M | M | M | T | J | — | — | — |
| (J)TT62W9.5 | M | M | J | M | M | M | J | M | — | — | — |
| (J)TT62W9.6 | T | M | M | M | M | M | M | M | J | T | (Young) |
| (J)TT62W10.1 | T | T | T | T | M | M | T | M | T | T | (Young) |
| (J)TT63W1.1 | M | M | M | M | M | M | M | T | J | T | T |
| (J)TT63W1.2 | M | M | M | M | M | M | J | J | T | T | T |
| (J)TT63W2.1 | M | T | T | T | T | T | M | T | T | T | T |
| (J)TT63W2.2 | T | T | T | M | M | M | T | M | T | T | T |
| (J)TT63W2.3 | J | J | J | J | J | J | J | J | — | — | — |
| (J)TT63W2.4 | M | T | M | T | T | T | M | T | T | T | T |
| (J)TT63W3.1 | M | M | M | M | M | M | M | J | M | T | T |
| (J)TT63W3.2 | M | M | T | M | M | M | M | M | T | T | (Young) |
| (J)TT63W3.3 | M | J | M | M | M | M | M | M | T | T | T |
| (J)TT63W3.4 | M | M | M | M | M | M | M | M | T | T | (Young) |
| (J)TT63W3.5 | M | M | T | T | T | M | M | M | J | T | (Young) |
| (J)TT63W3.6 | M | J | T | M | M | M | M | T | M | T | (Young) |
| (J)TT63W4.1 | M | M | M | T | T | M | M | M | T | T | T |
| (J)TT63W4.2 | J | J | J | J | J | J | J | J | — | — | — |
| (J)TT63W5.1 | M | J | T | M | M | M | M | M | J | T | (Young) |
| (J)TT63W6.1 | M | M | T | M | M | M | M | M | T | T | T |

J, *japonica* type T, *temporaria* type M, intermediate type S, special type (a)~(h), in text

lateral ridges on the back. Between these ridges abundant iridophores were crowded into masses arranged like paving-stones on the back. All the inter-specific hybrids between *japonica* and *temporaria* had no lateral ridges on the back. They had silver lateral stripes without exceptions along the black median stripe from the head to the one-third of the tail, although the stripes were not so distinct as those of the *temporaria* tadpoles.

Of the 11 nuclear transfer tadpoles with nuclei of the hermaphroditic *temporaria* strain, only one No. 62W1.2 was similar to the *temporaria* controls, having distinct silver lateral stripes of iridophores. The other ten tadpoles were nearly the same as the hybrids between the two species in the distinctness of silver lateral stripes on the back.

Of the 16 nuclear transfers with nuclei of the normal *temporaria* strain, two Nos. 63W2.3 and 63W4.2 were quite of the *japonica* type, four Nos. 63W2.1, 63W2.4, 63W3.5 and 63W4.1 were quite of the *temporaria* type, and the remaining

ten were very similar to the hybrids.

f. Glitter of the irises

The irises of the *temporaria* control tadpoles were brightly glittery without exceptions, while those of the *japonica* control were not glittery. The irises of the interspecific hybrids were intermediate between the two species, that is, feebly glittery.

The irises of the 11 nuclear transfers with nuclei of the hermaphroditic *temporaria* strain were very similar to the interspecific hybrids, being feebly glittery. Two Nos. 63W2.1 and 63W2.4 of the 16 nuclear transfers with nuclei of the normal *temporaria* strain were quite the same with the *temporaria* controls; their irises were brightly glittery, while two others Nos. 63W2.3 and 63W4.2 were quite the same with the *japonica* control. The irises of the remaining 12 tadpoles were intermediate between the two species; they were feebly glittery.

g. Color pattern of the tail

In the *japonica* control tadpoles, melanophores were distributed in minute groups over the tail and there were several small black spots on both membranous and muscular portions. These black spots consisted of closely crowded melanophores. Such black spots were not found on the tails of the *temporaria* control tadpoles; melanophores were uniformly dispersed without forming groups on both membranous and muscular portions.

The tails of 14 of 40 interspecific hybrid tadpoles produced as the controls of nuclear transfers with nuclei of the hermaphroditic *temporaria* strain were quite of the *japonica* type in color pattern, while those of the remaining 26 were intermediate, although there was a wide range of variation, from a type near the *temporaria* to a type near the *japonica*. Seven of these tadpoles were very similar to the *temporaria* controls in the distribution of melanophores, except for the presence of a few small black spots on the muscular portion. Thirteen others were the same as the *japonica* controls in the distribution of melanophores, except for the absence of small black spots. In the remaining six, melanophores were distributed in minute groups, as found in the *japonica* controls, and there were one or two small black spots on the muscular portion alone.

Among 61 interspecific hybrids produced as the controls of nuclear transfers with nuclei of the normal *temporaria* strain, there were 20 of the *japonica* type and 41 of the intermediate. Of the latter, five were very similar to the *temporaria* controls, except for the presence of 3~5 small black spots on the muscular portion. Eleven others were of the *japonica* type in the distribution of melanophores, except for the absence of small black spots. The remaining 25 were of the *japonica* type in the distribution of melanophores, except for small black spots. A few of the latter were found on the muscular portion alone.

In the 11 nuclear transfer tadpoles with nuclei of the hermaphroditic *temporaria* strain, the distribution of melanophores on the tail was very similar to the situation found in the interspecific hybrids stated above. Two tadpoles Nos. 62W9.4

and 62W10.1 were of the *temporaria* type, while two others Nos. 62W7.2 and 62W9.5 of the *japonica* type. The remaining seven were intermediate between the two species; in three Nos. 62W1.2, 62W9.2 and 62W9.3 of them melanophores were uniformly dispersed on the membranous portion and there were two to five small black spots on the muscular portion. In three others Nos. 62W1.1, 62W7.1 and 62W9.1, there were no small black spots on both portions, although melanophores were distributed in minute groups on the membranous portion. In the remaining one tadpole No. 62W9.6, there were a few small black spots on the muscular portion alone as well as minute groups of melanophores on the membranous portion.

Among the 16 nuclear transfers with nuclei of the normal *temporaria* strain, there were one tadpole No. 63W2.2 of the *temporaria* type, three Nos. 63W1.2, 63W2.3 and 63W4.2 of the *japonica* and 12 of the intermediate.

In five Nos. 63W2.1, 63W3.2, 63W3.4, 63W3.5 and 63W4.1 of the latter, melanophores were uniformly dispersed on the membranous portion and there were three to five small black spots on the muscular portion. In four others Nos. 63W1.1, 63W2.4, 63W5.1 and 63W6.1, melanophores were distributed in minute groups on the membranous portion and no black spots were found on both portions. In the remaining three tadpoles Nos. 63W3.1, 63W3.3 and 63W3.6, melanophores were distributed in minute groups on the membranous portion and one to four small black spots were found on the muscular portion.

h. Dental formula

As shown in Table 8, the dental formula of 96 *temporaria* tadpoles was 4/4 with one exception which was 3/4, while that of 448 *japonica* tadpoles was 3/3 with three exceptions which were 2/3. The dental formula of interspecific hybrids between the two species was variable; one of 101 tadpoles was 2/3, five were 3/3, 41 were 4/3, 11 were 2/4, 38 were 3/4 and five were 4/4.

Of all the 27 nuclear transfers, two Nos. 62W9.4 and 63W4.2, were 2/3, six

TABLE 8
Dental formulae of nucleo-cytoplasmic hybrid tadpoles and the controls

| Series | Kind | No. of tadpoles | No. of rows of teeth in upper and lower lips | | | | | |
|--------------|-------------|-----------------|--|-----|-----|-----|-----|-----|
| | | | 2/3 | 3/3 | 4/3 | 2/4 | 3/4 | 4/4 |
| Control | (T)TT62W1 ♀ | 22 | 0 | 0 | 0 | 0 | 1 | 21 |
| | (J)JT62W | 40 | 0 | 2 | 17 | 5 | 15 | 1 |
| | (J)JJ62W | 143 | 1 | 142 | 0 | 0 | 0 | 0 |
| | (T)TT63W2 | 74 | 0 | 0 | 0 | 0 | 0 | 74 |
| | (J)JT63W | 61 | 1 | 3 | 24 | 6 | 23 | 4 |
| | (J)JJ63W | 305 | 2 | 303 | 0 | 0 | 0 | 0 |
| Experimental | (J)TT62W1 ♀ | 11 | 1 | 3 | 2 | 1 | 2 | 2 |
| | (J)TT63W2 | 16 | 1 | 3 | 1 | 3 | 4 | 4 |
| | (J)+JJ63W | 10 | 3 | 7 | 0 | 0 | 0 | 0 |

All the tadpoles are 40 days old

Nos. 62W1.2, 62W7.1, 62W9.3, 63W1.2, 63W2.3 and 63W3.1 were 3/3, three Nos. 62W7.2, 62W9.6 and 63W6.1 were 4/3, four Nos. 62W9.2, 63W3.2, 63W3.5 and 63W5.1 were 2/4, six Nos. 62W9.5, 62W10.1, 63W2.2, 63W3.3, 63W3.4 and 63W4.1 were 3/4 and six Nos. 62W1.1, 62W9.1, 63W1.1, 63W2.1, 63W2.4 and 63W3.6 were 4/4. Although the dental formula of the nuclear transfer was variable just as found in the interspecific hybrids, the tadpoles of the *temporaria* type were comparatively numerous, differing from those of the interspecific hybrids. There was a possibility that the number of tadpoles of the *temporaria* type might be more numerous if they were examined at nearly the same body length with the other kinds of tadpoles, because the nuclear transfers were most inferior in the growth of teeth among all kinds of tadpoles examined at the same age of 40 days.

2. Summary of the external characters of the nuclear transfer tadpoles

As stated above, the morphology of the 27 nuclear transfer tadpoles in total were observed in terms of eight external characters, (a) dorsal ground color, (b) distribution of iridophores, (c) dorso-lateral black spots, (d) dorso-median stripe, (e) dorso-lateral ridges, (f) glitter of the irises, (g) color pattern of the tail and (h) dental formula. The results of these observations are summarized in Table 7. From this Table, it was noticeable that there were no tadpoles which were quite similar to those of the nuclear species in all the eight external characters. Only one of the 27 tadpoles was of the *temporaria* type in six of the eight external characters. This tadpole was the one which most resembled the nuclear species. The others bore a resemblance to those of the latter in less than five characters; there were two, two and two tadpoles which resembled the nuclear species in three, four and five of the above eight external characters, respectively. Nine and three tadpoles were of the *temporaria* type in one and two characters, respectively. Eight tadpoles had no characters of the *temporaria* type.

On the other hand, 25 of the 27 tadpoles were intermediate between *temporaria* and *japonica* in more than two external characters. One tadpole was of the intermediate type in all the eight. Five, five and seven tadpoles were also intermediate in five, six and seven, respectively, while one, three and three were intermediate in two, three and four, respectively. If the tadpoles which had external characters of either *temporaria* or intermediate type were counted, there were one, four, eight and twelve tadpoles, having five, six, seven and eight characters, respectively.

There were a goodly number of tadpoles which were of the *japonica* type in some external characters. Four and one tadpoles were similar to *japonica* in two and three characters, respectively, while eight were in only one. Twelve tadpoles had no external characters of the *japonica* type. Exceptionally, two tadpoles Nos. (J)TT63W2.3 and (J)TT63W4.2 were quite of the *japonica* type in all the eight characters. These two were so feeble that they died at the late tadpole stage. They were considered to be parthenogenetically developed diploids, due to failure of enucleation and doubling of haploid chromosomes at the egg stage.

Two other tadpoles Nos. (J)TT62W9.4 and (J)TT62W9.5 died at the late tadpole stage, too, although they were of the *temporaria* or intermediate type in seven or six of the eight external characters. The remaining 23 tadpoles completed their metamorphosis and with one exception revealed distinctly a specific character of *temporaria* in the shape of dorso-lateral ridges, as described later. Accordingly, there seemed to be no doubt but that the 25 tadpoles including two of the four which died at the late tadpole stage were nucleo-cytoplasmic hybrids. At the same time, it was very interesting that all of them were not the same with the nuclear species, *Rana temporaria*. To be brief, the nucleo-cytoplasmic hybrids were generally intermediate between the two species in external morphology at the tadpole stage.

IV. *External characters, sex and reproductive ability of nucleo-cytoplasmic hybrid frogs*

1. External characters of control frogs

a. *Rana japonica*, about 1-year-old frogs

The snout of the head was comparatively long and slender. The dorso-lateral folds were parallel with each other without interruptions. There were no black spots on the lateral sides below the dorso-lateral folds. The upper surface of the body was brown in ground color; some of the frogs had no spots on the back, while the others had large grey ones. There was no dorso-median stripe. The underside was white, yellowish white or rarely orange and always had no marking. The dorsal skin was smooth and had no protuberances.

b. *Rana temporaria temporaria*, about 1-year-old frogs.

The snout was comparatively short and stout. The anterior parts of the dorso-lateral folds were curved outwards at the tympanum. There were clearly or faintly distinguishable black spots on the lateral sides below the dorso-lateral folds in some frogs, while such spots were scarcely distinguishable in the others. The upper surface of the body was brown, ochreous brown or light brown; some of the frogs had no spots on the back, while the other had black ones. Some frogs had a pale dorso-median stripe, while the others had none. The lower surface was white or yellowish in ground color and had orange or brown markings. The dorsal skin was rough and had many tubercles. In each black spot on the back there was usually a tubercle.

c. Interspecific hybrids, *japonica* ♀ × *temporaria* ♂, about 1-year-old frogs

The snout was long and slender in most hybrids, while it was somewhat shorter in a small number of them. The anterior parts of the dorso-lateral folds were parallel with each other in some frogs, while they were curved outwards in the others. Concerning the black spots on the lateral sides below the dorso-lateral folds, the hybrid frogs were very similar to the *temporaria* controls: they were clearly, faintly or scarcely distinguishable. The upper surface of the body was brown,

yellowish brown or light brown with clearly defined black spots. In the center of each black spot there was a protuberance. Some hybrid frogs had a pale median stripe on the back, while the others had none. The under surface was white or pale yellow, mottled with pale brown, pink or dark brown markings.

2. Experiments with blastulae produced from a hermaphroditic
Rana temporaria as the donors

a. Sex and reproductive ability of control frogs

- i) Frogs from the crosses between three female *Rana japonica* Nos. (J)JJ62W1~3 and a male *Rana japonica* No. (J)JJ62W1

Normally metamorphosed frogs were 143 in total number. Ninety-three of them were killed for preservation or died within one month after metamorphosis. There were 43 females and 47 males among them, besides three whose sex was undetermined, owing to postmortem change. Among 15 frogs preserved one year after metamorphosis there were 7 females and 8 males. Their gonads were normal and filled with eggs or bundles of spermatozoa. Two years after metamorphosis nine frogs were preserved; there were five females and four males. The ovaries and testes were quite normal. All the frogs which were examined in terms of reproductive ability produced eggs, of which more than 85% were normally fertilized.

- ii) Frogs from a hermaphroditic *Rana temporaria* No. (T)TT62W by self-fertilization

In this self-fertilization series 22 tadpoles completed their metamorphosis. Thirteen of them were killed for preservation or died naturally immediately after their metamorphosis. Ten of these young frogs were all females. The sex of the other three could not be determined, owing to their postmortem change. The remaining nine frogs of this series were killed by two years after their metamorphosis; one at eight months, four at one year, and four at two years. All these frogs were perfect males, whose testes were normal in inner structure and filled with bundles of mature spermatozoa.

- iii) Frogs from normal female *Rana japonica* Nos. (J)JJ62W1~3 mated with a hermaphroditic *Rana temporaria* No. (T)TT62W1 ♂

Thirty-two tadpoles of this series completed their metamorphosis, but afterwards seven died within one month. Three of the latter were perfect females and three others hermaphrodites transforming from females to males. In the gonads of these hermaphrodites there were distinctly multiplied rete cells and a few isolated germ cells, besides ovarian cavities. The sex of the remaining one was undetermined, owing to their postmortem change.

Within five to seven months after metamorphosis four frogs were preserved. No secondary sexual characters were found in these frogs. Their gonads were apparently testes, although they were slender and unsymmetrical in shape. Some germ cells were degenerating here and there in the gonads.

Twelve frogs were preserved one year after metamorphosis and eight others,

two years after. All these frogs did not show the secondary sexual characters, although they were males. The testes were remarkably underdeveloped; these were nearly the same in size and shape as those of the above 5- to 7-month-old frogs. In the testes there were only a small number of germ cells, which had escaped degeneration. The testes were mostly filled with stroma.

b. Nucleo-cytoplasmic hybrid frogs

While two and two of the 11 nuclear transfer tadpoles died at the late tadpole and metamorphosing stage, respectively, the other seven completed their metamorphosis. Three of them lived to the age of 255, 370 or 554 days. The one-year-old frog was killed to examine the reproductive ability.

i) No. (J)TT62W1.1, 370-day-old male.

The body and extremities were quite normal in shape, although somewhat smaller than the *temporaria* controls. This frog was a male with distinct secondary sexual characters. The snout was intermediate in shape between those of the two species, though more similar to those of the *temporaria* controls. The dorso-lateral folds were of the *temporaria* type: their anterior parts were curved outwards at the tympanum. The upper surface of the body was yellowish brown with irregularly shaped brown spots. The underside was reddish yellow, mottled with purplish brown.

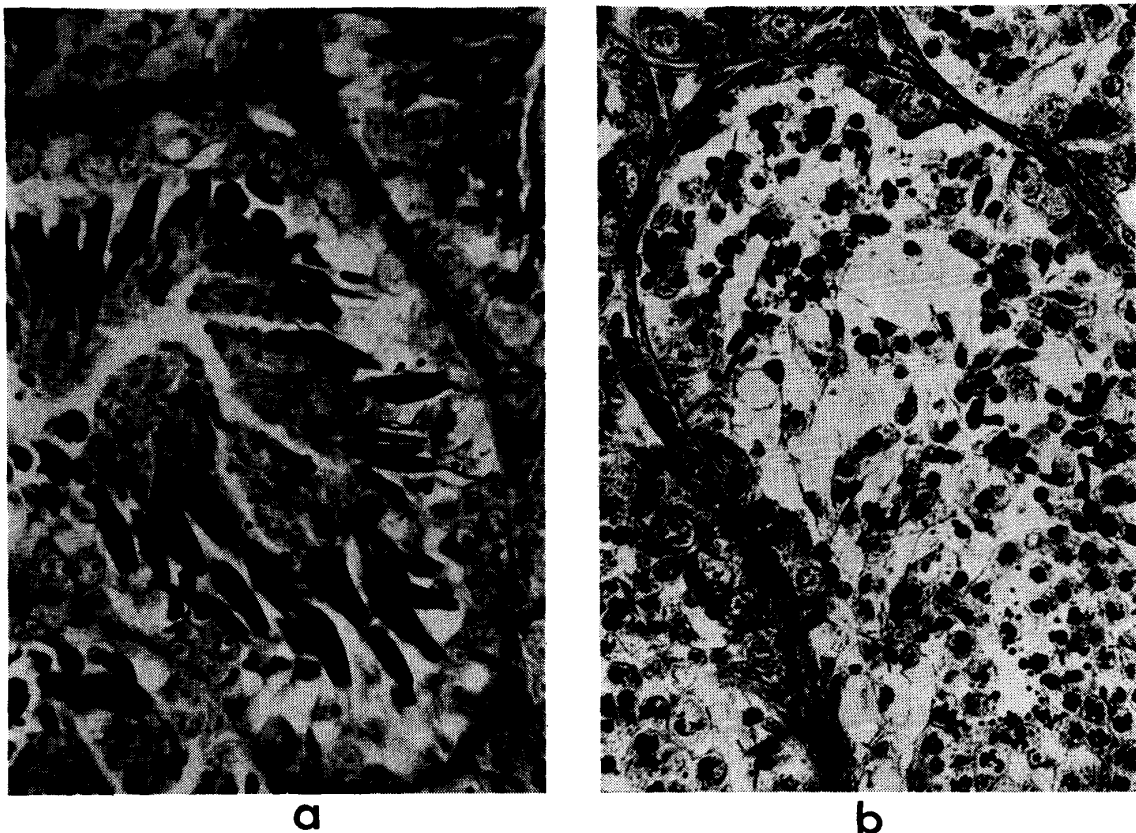


Fig. 1. Cross-sections of seminal tubules of the testes of a male diploid nucleocytoplasmic hybrid and the control. × 250.
 a, Male *Rana temporaria*, (T)TT. b, Nucleocytoplasmic hybrid, No. (J)TT62W1.1, 370 days old.

This male was killed at the age of one year, for examining the reproductive ability. After autopsy it was found that the kidneys were normal and the testes were nearly the same in size as those of the *temporaria* controls; the left was 8.5 mm. \times 4.5 mm. and the right 9.0 mm. \times 5.0 mm. in size (Table 9). The reproductive ability of this male was examined by the method of artificial fertilization. As a result, it was found that this frog was perfectly sterile. In the testes there were no normal spermatozoa (Fig. 1). In place of the latter a small number of pycnotic or abnormal spermatozoa were sparsely distributed.

ii) No. (J)TT62W1.2, 554-day-old male.

This frog was rather better in growth than the *temporaria* controls. It was a male with distinct secondary sexual characters. When this frog died in August, there were many white blisters on the whole surface of the body and extremities. The snout and the dorso-lateral folds were similar to those of No. 62W1.1. The upper surface of the body was yellowish brown tinged with blue and had well defined black spots, each of which had a protuberance in the center. The lower surface was yellow, mottled with purplish brown.

The testes were very abnormal in inner structure. They contained no normal spermatozoa, although there were a small number of pycnotic nuclei. The kidneys were normal in size and shape.

iii) No. (J)TT62W7.1, 255-day-old male.

In spite of the good growth, this frog died at the age of 8.5 months. It was a male with remarkable secondary sexual characters. The snout and the dorso-lateral folds were similar to those of No. 62W1.1. The upper surface of the body was light orange, covered with distinct black spots. The dorsal skin was

TABLE 9
Sex of nucleo-cytoplasmic hybrids with nuclei derived from a hermaphroditic
temporaria and the controls

| Kind or individual no. | Age (days) | Body length (mm.) | Sex | Size of testes of matured male frogs | | Remarks |
|------------------------|------------|-------------------|--------|--------------------------------------|-------------------|------------|
| | | | | Left (mm.) | Right (mm.) | |
| (1) (T)TT62W1 ♀ | 370 | 51.0~52.5 | ♀0 ♂4 | 11.2 \times 6.0 | 11.3 \times 5.7 | Fertile |
| (2) (J)JT62W | 370 | 44.5~50.0 | ♀0 ♂12 | 1.3 \times 0.3 | 1.4 \times 0.3 | Sterile |
| (3) (J)JJ62W | 370 | 42.0~46.5 | ♀7 ♂8 | 3.4 \times 3.0 | 3.4 \times 2.7 | Fertile |
| (1) (J)TT62W1.1 | 370 | 47.0 | ♂ | 8.5 \times 4.5 | 9.0 \times 5.0 | Sterile |
| (2) (J)TT62W1.2 | 554 | 56.5 | ♂ | 5.5 \times 3.5 | 5.4 \times 3.5 | (Sterile)* |
| (3) (J)TT62W7.1 | 255 | 27.0 | ♂ | 4.4 \times 3.3 | 4.4 \times 3.3 | (Sterile)* |
| (4) (J)TT62W7.2 | 93 | — | ♀ | | | |
| (5) (J)TT62W9.1 | 101 | 13.2 | ♂ | | | |
| (6) (J)TT62W9.2 | 116 | — | ♂ | | | |
| (7) (J)TT62W9.3 | 126 | 12.5 | ♀ | | | |
| (8) (J)TT62W9.4 | 109 | — | | | | |
| (9) (J)TT62W9.5 | 102 | — | | | | |
| (10) (J)TT62W9.6 | 131 | 15.5 | ♂ | | | |
| (11) (J)TT62W10.1 | 100 | 14.5 | ♀ | | | |

* Sterility determined by the inner structures of testes

smooth. The lower surface was white, marked with irregular orange patches.

The testes were very abnormal in inner structure. There were no normal spermatozoa and a few pycnotic nuclei.

iv) No. (J)TT62W7.2, 93-day-old hermaphrodite.

This individual died during metamorphosis. The tail was half absorbed. The snout and the dorso-lateral folds were similar to those of No. 62W1.1. The upper surface of the body was brown without dark spots. The dorsal skin was smooth. The underside had no markings.

This individual was a hermaphrodite transforming from a female to a male. Although there were still ovarian cavities in the gonads, nearly all the germ cells were surrounded by multiplied rete cells.

v) No. (J)TT62W9.1, 101-day-old male.

This frog completed its metamorphosis by absorption of the whole tail, without protruding the right limb which was so bad in growth. The other left limb was abnormally slender, too. It died two days after the completion of metamorphosis. The snout and the dorso-lateral folds were similar to those of No. 62W1.1. The dorsal skin was smooth.

Although the body and hind limbs were nearly normal in growth, the kidneys were extremely atrophied. This frog was a male, whose gonads were nearly the same in inner structure as those of the *temporaria* controls at the same developmental stage.

vi) No. (J)TT62W9.2, 116-day-old male.

This individual was so bad in growth and development that it could not begin its metamorphosis until the age of 115 days. Moreover, it died one day later, without completing the metamorphosis. The dorso-lateral folds were of the *temporaria* type. The other external characters were obscure, owing to the death during metamorphosis.

The testes were quite normal in inner structure. The kidneys were distinctly bad in development.

vii) No. (J)TT62W9.3, 126-day-old female.

This individual was very similar in growth and development to the above 116-day-old male. It began its metamorphosis at the age of 116 days and completed seven days later. In this stage the body and hind limbs were comparatively small. This frog died at the age of 126 days. The snout was similar to that of *japonica*, while the dorso-lateral folds were intermediate between those of *japonica* and *temporaria*. The upper surface of the body was brown, having no markings. The dorsal skin was smooth.

The kidneys were atrophied and very small. This frog was a female with underdeveloped ovaries. The latter, however, seemed normal in structure, although there were not yet found auxocytes.

viii) No. (J)TT62W9.4, 109-day-old tadpole.

ix) No. (J)TT62W9.5, 102-day-old tadpole.

These two died at the late tadpole stage. The latter tadpole was rather good in growth until the death. It died of a tumor-like disease which appeared on

the back.

x) No. (J)TT62W9.6, 131-day-old male.

This was remarkably slow in development and began to metamorphose at the age of 126 days. Five days later it completed the metamorphosis and soon after died from drowning. At this time it was nearly normal in body length, although the extremities were distinctly underdeveloped. The snout of this frog was of the *japonica* type, while the dorso-lateral folds were of the *temporaria*. There were no distinct markings on both upper and lower surfaces of the body.

This frog was a male, whose testes were normal in inner structure. The kidneys were normal.

xi) No. (J)TT62W10.1, 100-day-old female.

The snout and the dorso-lateral folds were of the *temporaria* type. There were black spots on the upper surface of the body and brown markings on the lower. This individual was hardly distinguishable from the *temporaria* control in appearance. The hind limbs were normally developed, while the fore-limbs were abnormally slender, curved and paralyzed. Moreover, the latter were asymmetrical in the positions of their bases. Although this individual grew normally, it died immediately after the completion of metamorphosis.

This frog was a female with nearly normal ovaries. In the ovaries there were ovarian cavities and many auxocytes. Besides, there were a considerable number of degenerating auxocytes.

3. Experiments with blastulae produced from a pair of normal *Rana temporaria* as the donors

a. Sex and reproductive ability of control frogs

i) Frogs from two female *Rana japonica* Nos. (J)JJ63W1~2 mated with a male *Rana japonica* No. (J)JJ63W2

There were 305 metamorphosed frogs. Of these young frogs 255 were killed within two weeks after the metamorphosis. Among them there were 133 females and 122 males. Two other females died at the age of eight months. Five females and four males were made use of for artificial fertilization at the age of one year (Table 10). These frogs were quite normal in reproductive capacity; the rate of fertilization was more than 70% in each mating. Among the remaining 39 frogs there were 21 females and 18 males.

ii) Frogs from a pair of normal *Rana temporaria*, No. (T)TT63W2(♀) × No. (T)TT63W2(♂)

From this mating between a female and a male *temporaria*, 74 metamorphosed frogs were obtained. Twenty-five of them were preserved or died within two weeks after their metamorphosis; there were 12 females and 13 males. Among four frogs which died within two months there were one female and three males. Just one year after fertilization five females and five males were made use of for artificial fertilization, and it was found that all these frogs were quite normal in reproductive ability. One year later 13 other females and 12 other males

were utilized for artificial fertilization. As a result, it was clear that these 25 frogs were also normal in reproductive ability. The remaining ten frogs were preserved or died within three years after fertilization. They were quite normal and consisted of five females and five males (Table 10).

iii) Frogs from two female *Rana japonica* Nos. (J)JJ63W1~2 mated with a male *Rana temporaria* No. (T)TT63W2

There were 57 metamorphosed frogs obtained from matings between two female *japonica* and one male *temporaria*. Within two weeks after metamorphosis 17 frogs were preserved or died; 11 males and six hermaphrodites transforming from females to males. Five other frogs died within 10 months after fertilization;

TABLE 10
Sex of nucleo-cytoplasmic hybrids with nuclei derived from a pair
of normal *temporaria* and the controls

| Kind or individual no. | Age (days) | Body length (mm.) | Sex | Size of testes of matured male frogs | | Remarks |
|------------------------|------------|-------------------|------------------|--------------------------------------|--------------------|-----------------------------------|
| | | | | Left (mm.) | Right (mm.) | |
| (1) (T)TT63W 2 | 374 | 15.1~57.0 | ♀ 5 ♂ 5 | 12.2×7.1 | 12.4×7.2 | Fertile |
| (2) (J)JT63W | 374 | 34.5~54.0 | ♀ 0 ♂ 13 ♂ 12 | 7.0×3.5 1.2×0.3 | 7.5×3.6 1.1×0.3 | Sterile Rudimentary Fertile |
| (3) (J)JJ63W | 374 | 37.0~47.5 | ♀ 5 ♂ 4 | 3.9×2.7 | 3.8×2.5 | Fertile |
| (1) (J)+JJ63W1.1 | 374 | 45.5 | ♂ | 4.3×2.7 | 4.1×2.5 | Fertile |
| (2) (J)+JJ63W1.2 | 374 | 47.0 | ♀ | | | Fertile |
| (3) (J)+JJ63W2.1 | 109 | 14.5 | ♀ | | | |
| (4) (J)+JJ63W2.2 | 215 | 32.5 | ♂ | 3.5×2.5 | 3.4×2.5 | |
| (5) (J)+JJ63W3.1 | 84 | | ♂ | | | |
| (6) (J)+JJ63W3.2 | 734 | 55.0 | ♀ | | | Fertile |
| (7) (J)+JJ63W3.3 | 734 | 53.5 | ♂ | 4.5×3.0 | 4.2×3.0 | Fertile |
| (8) (J)+JJ63W3.4 | 735 | 52.0 | ♂ | 4.1×2.7 | 4.0×2.7 | Fertile |
| (9) (J)+JJ63W4.1 | 374 | 42.0 | ♀ | | | Fertile |
| (10) (J)+JJ63W4.2 | 90 | | ♂ | | | |
| (1) (J)TT63W1.1 | 239 | 34.5 | ♂ | 0.5×0.3 | 0.5×0.3 | (Sterile)* |
| (2) (J)TT63W1.2 | 220 | 32.5 | ♂ | 1.7×1.0 | 1.8×1.0 | (Sterile)* |
| (3) (J)TT63W2.1 | 734 | 74.0 | ♂ | 10.5×8.0 | 9.0×7.0 | Sterile |
| (4) (J)TT63W2.2 | 374 | 50.0 | ♂ | 11.0×5.5 | 11.5×5.9 | Sterile |
| (5) (J)TT63W2.3 | 90 | | | | | |
| (6) (J)TT63W2.4 | 735 | 79.5 | ♂ | 11.5×9.5 | 12.0×9.5 | Sterile |
| (7) (J)TT63W3.1 | 359 | 47.5 | ♂ | 5.7×3.0 | 6.0×3.5 | Sterile |
| (8) (J)TT63W3.2 | 85 | 16.5 | ♀ | | | |
| (9) (J)TT63W3.3 | 374 | 44.5 | ♂ | 9.5×5.0 | 9.5×4.5 | Sterile |
| (10) (J)TT63W3.4 | 125 | 20.0 | ♀ | | | |
| (11) (J)TT63W3.5 | 106 | 17.0 | ♂ | | | |
| (12) (J)TT63W3.6 | 126 | 20.0 | ♂ | | | |
| (13) (J)TT63W4.1 | 374 | 45.0 | ♂ | 10.0×5.5 | 9.5×5.5 | Sterile |
| (14) (J)TT63W4.2 | 106 | | | | | |
| (15) (J)TT63W5.1 | 87 | 13.5 | ♀ | | | |
| (16) (J)TT63W6.1 | 374 | 51.5 | ♂ | 7.5×3.7 | 7.5×3.5 | Sterile |

* Sterility determined by the inner structure of testes

all of them were males. Twenty-five frogs were preserved at the age of one year. All of them were males, too. Thirteen frogs had testes which were nearly equal to or slightly smaller than those of the *temporaria* controls (Table 10). While the testes of these hybrids were normal in appearance, they were very abnormal in inner structure. There were no normal spermatozoa in the testes, although there were abnormal spermatozoa or pycnotic nuclei. As the result of examining their reproductive ability, it was ascertained that the 13 males were all quite sterile.

In the remaining 12 males the testes were very small and degenerative. Although they were of testicular structures, germ cells were very few or scarcely found, and in each testis there were abundant stroma cells or large cavities.

Ten frogs were preserved at the age of three years. All of them were male with very small and degenerative testes.

iv) Intraspecific nuclear transfers of *Rana japonica*, (J)+JJ

Eight individuals completed their metamorphosis (Table 10). One frog died 12 days after metamorphosis; it was a normal female. Another one died 110 days after metamorphosis; it was a normal male. At the age of one year two females and one male were utilized for artificial fertilization. The next year the remaining three frogs, one female and two males, were used for the same purpose. All the frogs which were utilized for artificial fertilization were normal in reproductive capacity; most of their eggs were normally fertilized with their spermatozoa and developed normally.

b. Nucleo-cytoplasmic hybrid frogs

Two nuclear transfers which died at the late tadpole stage were considered to be parthenogenetically developed diploids produced by failure of enucleation and nuclear transplantation. The others were identified as nucleo-cytoplasmic hybrids from their external characters. All of them completed their metamorphosis, although four died immediately after metamorphosis. Five and two frogs were killed at the ages of one and two years, respectively, for examining their reproductive ability (Table 10).

i) No. (J)TT63W1.1, 239-day-old male.

This frog died five and a half months after the metamorphosis. The snout was similar to that of *japonica*, while the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was yellowish brown, marbled with purplish brown and had several black spots. The dorsal skin was smooth except the center of each black spot, which was somewhat protruded. The underside of the body was white, covered with distinct patches of dark brown. The fore and hind limbs were slender and feeble, although they were nearly normal in length.

The testes were very small and rudimentary. Germ cells were scarcely found in the testes. Accordingly, this male was considered to be quite sterile. The kidneys were normal in size and structure.

ii) No. (J)TT63W1.2, 220-day-old male.

Although the body and fore and hind limbs were very good in growth, this frog died four and a half months after the metamorphosis. The snout and the

dorso-lateral folds were of the *temporaria* type. The upper surface of the body was yellowish brown, marbled with purplish brown. There were several black spots between the dorso-lateral folds. The underside was yellowish white, marbled with light purplish brown. The body and limbs were very good in growth.

The testes were very small and rudimentary and had no germ cells. The kidneys were normal in size and structure.

iii) No. (J)TT63W2.1, 734-day-old male.

This frog was very good in growth and development. The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface was light brown with several black spots. The underside was light yellow, marked with purplish brown spots.

This frog was killed at the age of about two years for examining the reproductive ability. The testes were somewhat smaller than those of the *temporaria* controls. By the use of the right testis, artificial insemination was attempted in vain for eggs of the female *japonica* and *temporaria* controls. The left testis was preserved and cut into microscopic sections. As the result of observations, it was found that the inner structure was very abnormal and had no normal spermatozoa. In the cavities of seminiferous ampullae there were abundant pycnotic nuclei. The kidneys were normal.

iv) No. (J)TT63W2.2, 374-day-old male.

This frog was very good in growth. The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was light brown and had a few black spots as well as a faint median stripe. The lower surface was nearly white with light purplish brown markings.

At the age of one year, this frog was killed for examining the reproductive ability. The testes were somewhat slender than those of the *temporaria* controls. By the use of the right testis, insemination of *japonica* eggs was attempted in vain, while the left one was preserved and sectioned. The inner structure of this testis was very abnormal and there were no normal spermatozoa. The seminal tubules were mostly filled up with pycnotic nuclei. The kidneys were normal in size and structure.

v) No. (J)TT63W2.3, 90-day-old tadpole.

This individual died at the late tadpole stage.

vi) No. (J)TT63W2.4, 735-day-old male.

The growth of this frog was very good. The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was brown with large patches of light grey. The underside was light yellow with markings of purplish brown tinged with orange.

This male was killed at the age of about two years for examining the reproductive ability. The testes were somewhat small for his large body size, as compared with those of the *temporaria* controls. The right testis was utilized for the insemination test, while the left one was preserved and sectioned for microscopic observations. The results of the insemination test for *temporaria* and *japonica* eggs and the microscopical observations of the inner structure

of the testis revealed that this male was quite sterile; no fertilized eggs were obtained, and no normal spermatozoa were found. In the testis were contained numerous pycnotic nuclei.

vii) No. (J)TT63W3.1, 359-day-old male.

The growth was quite normal. The snout was intermediate in shape, while the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was brown covered with darker brown spots. The lower surface was light yellow, marked with dark purplish brown.

This male was killed for examining the reproductive ability at the age of one year. The testes were distinctly smaller than those of the *temporaria* controls. An insemination test was made for *japonica* eggs in vain by the use of the right testis; no fertilized eggs were obtained. The left testis was preserved and sections for microscopic observations. There were a small number of pycnotic nuclei and no normal spermatozoa.

viii) No. (J)TT63W3.2, 85-day-old female.

Although the growth was good, this frog died immediately after the metamorphosis. The snout and the dorso-lateral folds were of the *temporaria* type. In the ovaries there were distinct ovarian cavities, and no auxocytes were found. There were still no symptoms of sex reversal to a male.

ix) No. (J)TT63W3.3, 374-day-old male.

The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was light brown with distinct markings of light orange. On the back there was a whitish wide median stripe. The under parts were light yellow marked with purplish brown spots.

This frog was killed at the age of one year to examine the reproductive ability. The testes were nearly the same in size as those of the *temporaria* controls. Insemination was attempted for *japonica* eggs in vain, by the use of the right testis; no cleaved eggs were obtained. The left testis was preserved and sectioned; there were a few pycnotic nuclei and no normal spermatozoa.

x) No. (J)TT63W3.4, 125-day-old female.

This frog suddenly died two weeks after the metamorphosis, in spite of good growth till that time. The snout and the dorso-lateral folds were of the *temporaria* type. In the ovaries there were distinct ovarian cavities. But there were no auxocytes as well as no symptoms of sex reversal were found.

xi) No. (J)TT63W3.5, 106-day-old male.

Although this individual had been very good in growth and development, it died immediately after the metamorphosis. The snout was of the *japonica* type, while the dorso-lateral folds were of the *temporaria*. The other characters were not clear. The testes were normal in inner structure.

xii) No. (J)TT63W3.6, 126-day-old male.

The growth was normal. However, the metamorphosis occurred late and slowly; it began at the age of 114 days and came to the end after ten days. The frog died two days after the completion of metamorphosis. The snout was intermediate in shape, while the dorso-lateral folds were of the *temporaria* type.

The testes were normal in shape and size as well as in inner structure.

xiii) No. (J)TT63W4.1, 374-day-old male.

The growth was normal. The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was light brown, speckled with many small black spots. A pale dorso-median stripe was clearly observed. The underside was white, marked with purplish brown spots.

This male was killed at the age of one year for examining the reproductive ability. The testes were nearly the same in size as those of the *temporaria* controls. Insemination was attempted for *japonica* eggs in vain, by the use of the right testis; no cleaved eggs were obtained. The left testis was preserved and sectioned; it was abnormal, containing numerous pycnotic nuclei and no normal spermatozoa.

xiv) No. (J)TT63W4.2, 106-day-old tadpole.

This individual died at the late tadpole stage.

xv) No. (J)TT63W5.1, 87-day-old female.

The growth was normal. This frog died immediately after metamorphosis. The snout was of the *japonica* type, while the dorso-lateral folds were of the *temporaria*. The other characters were not clear.

The ovaries were underdeveloped. Although there were distinct ovarian cavities, no auxocytes were found. There were no symptoms of sex reversal in the ovaries.

xvi) No. (J)TT63W6.1, 374-day-old male.

The growth was very good. The snout and the dorso-lateral folds were of the *temporaria* type. The upper surface of the body was light brown, covered with irregularly shaped brown spots. The lower surface was yellowish white, marked with purplish brown spots.

This male was killed at the age of one year to examine the reproductive ability. The testes were remarkably smaller than those of the *temporaria* controls. Insemination of *japonica* eggs was performed in vain, by the use of the right testis; no fertilized eggs were obtained. The left testis was preserved and sectioned. This was abnormal in inner structure, having no normal spermatozoa.

4. Summary of various characters of the nucleo-cytoplasmic hybrid frogs

a. External characters

The 23 metamorphosed nucleo-cytoplasmic hybrids were of the *temporaria* type in the shape of the dorso-lateral folds, with one exception (Table 7). The anterior parts of the dorso-lateral folds were curved outwards at the tympanum, differing from those of *japonica*, which were parallel with each other. The exceptional frog No. (J)TT62W9.3 was intermediate between the two species in this character. In contrast with the dorso-lateral folds, the shape of the snout of each nucleo-cytoplasmic hybrid was not always of the *temporaria* type. Although ten of 22 frogs were of the *temporaria* type, that is, short and stout, five were of

the *japonica* type, that is, long and slender, and the other seven were intermediate between the two species.

The existence of brown or orange markings on the underside of the body was a specific characteristic of grown *temporaria*. In this point, all of the 12 nucleo-cytoplasmic hybrids which lived for more than 220 days were of the *temporaria* type.

The hind limbs of the *japonica* controls were longer than those of the *temporaria*. This characteristic was rather exaggeratedly expressed in all the seven intra-specific nuclear transfers with *japonica* nuclei (Table 5). In all the 12 nucleo-cytoplasmic hybrids which lived for more than 220 days, the hind limbs were clearly shorter than those of the above stated intraspecific nuclear transfers, when compared by the ratios of hind-limb lengths to body lengths (Tables 3 and 5). However, it was remarkable that only four frogs Nos. (J)TT62W1.2, (J)TT62W7.1, (J)TT63W1.1 and (J)TT63W1.2 were nearly the same with the *temporaria* controls in the ratios of the hind-limb lengths. Seven others Nos. (J)TT62W1.1, (J)TT63W2.1, (J)TT63W2.4, (J)TT63W3.1, (J)TT63W3.3, (J)TT63W4.1 and (J)TT63W6.1 were considerably smaller than the *temporaria* controls in these ratios, while the remaining one No. (J)TT63W2.2 was similar to the *japonica*. One frog No. (J)TT63W3.4 which died at the age of 125 days had the shortest hind limbs (Table 5).

b. Sex and reproductive ability

Out of the nine nucleo-cytoplasmic hybrids with nuclei derived from a hermaphroditic *temporaria*, six frogs, 93 to 131 days old, consisted of two females, one juvenile hermaphrodite and three males. The remaining three, 255 to 554 days old, were all males, like the *temporaria* controls (Table 9). These three were quite sterile.

On the other hand, nine 220- to 735-day-old frogs of the 14 nucleo-cytoplasmic hybrids with nuclei derived from a pair of normal *temporaria* were all males, differing from the intraspecific nuclear transfers with *japonica* nuclei as well as the *temporaria* controls (Table 10). Among the seven intraspecific nuclear transfers, 215 to 735 days old, there were three females and four males. There were also an equal number of females and males among the ten *temporaria* controls. The remaining five nucleo-cytoplasmic hybrids, which were 85 to 126 days old, consisted of three females and two males. All the nine nucleo-cytoplasmic hybrids, which were 220 to 735 days old, were quite sterile differing from the intraspecific nuclear transfers.

DISCUSSION

The nucleo-cytoplasmic hybrids consisted of *Rana japonica* cytoplasm and *Rana temporaria temporaria* nuclei were not the same with the nuclear species in appearance, especially at the tadpole stage. All the 25 nucleo-cytoplasmic hybrids were intermediate between the nuclear and cytoplasmic species in two to all

of the eight external characters observed at the tadpole stage. Moreover, 13 tadpoles were of the cytoplasmic species type in one to three external characters, although there were 19 tadpoles which were of the nuclear type in one to six. However, they were very similar to the nuclear species in appearance after their metamorphosis. Nearly all the frogs were of the nuclear species type in the shape of the dorso-lateral folds and the existence of markings on the lower surface of the body, while they were similar to the nuclear or cytoplasmic species or intermediate between the two species in the shape of the snout.

A similar phenomenon has been observed in another kind of nucleo-cytoplasmic hybrids of Japanese brown frog species. The only two nucleo-cytoplasmic hybrids produced by KAWAMURA and NISHIOKA (1963b) from enucleated eggs of *Rana ornativentris* and blastula nuclei of *Rana japonica* were rather of the *ornativentris* type in the distribution of iridophores on the back at the tadpole stage, while they were of the *japonica* type in the existence of dorso-lateral black spots. After metamorphosis they were quite similar to the nuclear species in every respect.

The fact that nucleo-cytoplasmic hybrids show the characteristics of the cytoplasmic species to some extent was reported for the first time by SAMBUICHI (1957, '61) by the use of two Japanese pond frog species. While the single metamorphosing frog consisted of *Rana nigromaculata* cytoplasm and *Rana brevipoda* nuclei was of the nuclear species type at the tadpole and metamorphosing stage (SAMBUICHI, 1957), the two young frogs of the reciprocal combination appeared to be of the cytoplasmic species type in the distribution pattern of iridophores on the tail-fin at the tadpole stage, although they were generally similar to the nuclear species in body color, teeth rows and tail shape (SAMBUICHI, 1961). Moreover, one of the two frogs was generally intermediate between the two species in the color patterns on the upper surfaces of the body and hind limbs. The same kinds of nucleo-cytoplasmic hybrids were produced numerously and observed in detail by KAWAMURA and NISHIOKA (1963a). The 13 frogs produced from enucleated *Rana nigromaculata* eggs and *Rana brevipoda* nuclei, as well as the five frogs of the reciprocal combination were similar to the cytoplasmic species or intermediate between the two species in some external characters.

In this volume the present authoress reports on the morphological characters of two other kinds of nucleo-cytoplasmic hybrids. The two nucleo-cytoplasmic hybrid frogs produced from the egg cytoplasm of *Rana esculenta* and blastula nuclei of *Rana brevipoda* were not quite the same with the nuclear species in color pattern. One of them revealed the dorsal color of the cytoplasmic species in a degree, while the other was different from both species in dorsal color pattern (NISHIOKA, 1972b). The 18 nucleo-cytoplasmic hybrid frogs consisted of *Rana brevipoda* cytoplasm and *Rana plancyi chosenica* nuclei were very similar to the nuclear species in external characters, except the dorso-lateral folds, which were mostly of the cytoplasmic species type in the shape of projection (NISHIOKA, 1972c). No attention was paid to the external characters of these two kinds of nucleo-cytoplasmic hybrids at the tadpole stage, aside from the dental formulae in the

latter kind.

In urodeles, the ten reciprocal nucleo-cytoplasmic hybrids between *Pleurodeles waltlii* and *Pleurodeles poireti* obtained by GALLEIN, C.-L. (1970) were of the nuclear species type in morphological, physiological, serological and karyological characters. However, an effect of egg cytoplasm upon the appearance of specific characteristics in urodeles has been observed in androgenetic hybrids, in other words, haploid nucleo-cytoplasmic hybrids (HADORN, 1936 and DALTON, 1946). As these haploid animals could not live until a stage in which specific characteristics appear, pieces of their skin or neural crests were transplanted into normal diploid embryos and kept alive. As the results of these experiments, the androgenetic hybrid skin revealed the epidermal protuberances which were typical for the cytoplasmic species (HADORN), and the neural crest produced a tendency toward the pigment pattern of the cytoplasmic species (DALTON).

On the other hand, intraspecific nucleo-cytoplasmic hybrids, such as those produced by MCKINNEL (1960) between the common *Rana pipiens* and the kandi-yohi mutant, by GURDON (1961) between *Xenopus laevis laevis* and *Xenopus laevis victorianus*, and by ORTOLANI, FISCHBERG and SLATKINE (1966) between *Xenopus laevis laevis* and *Xenopus laevis petersi*, were nearly the same with the nuclear species in all the morphological characters. Accordingly, it is quite clear that the cytoplasmic influence found in interspecific nucleo-cytoplasmic hybrids is related to the biological differences between the two species.

Effects of cytoplasm on the external characters of nucleo-cytoplasmic hybrids have been discussed to some extent by KAWAMURA and NISHIOKA (1963a, b). The egg cytoplasm which has been elaborated by its own genome in the ovary, seems to give an effect upon the activity of the foreign genome introduced by nuclear transplantation. When the foreign genome can fully function in the egg cytoplasm, there is no room for the appearance of morphological characters of the cytoplasmic species in the nucleo-cytoplasmic hybrids. On the contrary, such characteristics may appear when the activity of the foreign genome is disturbed within the limits that nearly normal viability is guaranteed for the nucleo-cytoplasmic hybrid. The perfect sterility of the nucleo-cytoplasmic hybrids between *Rana japonica* and *Rana temporaria temporaria* seems to show that the compatibility of the genome with the cytoplasm is in a critical situation. Such a situation may permit the cytoplasmic properties determined in the ovary to become influential factors in the differentiation of specific external characters. Moreover, it is probable that the cytoplasmic properties are more active at the tadpole stage than they are after metamorphosis, although their nature is not yet known. It may be considered that mRNA accumulated in the egg cytoplasm interfere with the production of new mRNA from DNA of the foreign nuclei and play a role in the formation of morphological characters whose primordia have been established early in development.

SUMMARY

1. Nucleo-cytoplasmic hybrids were obtained from enucleated eggs of *Rana japonica* by transplanting blastula nuclei of *Rana temporaria temporaria*. The donor blastulae were produced from a hermaphrodite by self-fertilization or a pair of normal frogs. Twenty-seven diploid tadpoles developed from 1340 treated eggs. Twenty-five of them were identified as nucleo-cytoplasmic hybrids by their external characters, while the other two were considered to be parthenogenetic diploids, produced by the failure of enucleation and the doubling of haploid chromosomes.

2. There were no nucleo-cytoplasmic hybrids which were quite similar to the nuclear species in all the eight external characters observed at the tadpole stage, although they were of this type or intermediate between the nuclear and cytoplasmic species in more than five external characters. While about one half of the nucleo-cytoplasmic hybrids were of the cytoplasmic species type in none of the eight external characters, the other half were of this type in one to three.

3. Twenty-three nucleo-cytoplasmic hybrids completed their metamorphosis. They were of the nuclear species type in the shape of the dorso-lateral folds, with one exception which was intermediate between the nuclear and cytoplasmic species. However, their snouts were of the nuclear or cytoplasmic species type or intermediate between the two species.

4. Twelve nucleo-cytoplasmic hybrids lived for more than 220 days. All of them were of the nuclear species type in the color patterns of their undersides, having brown or orange markings. They were perfectly sterile males; their testes were very abnormal and had no normal spermatozoa. Although eight of them attained sexual maturity, no fertilized eggs were obtained between these males and normal *japonica* or *temporaria* females.

5. In nucleo-cytoplasmic hybrids there seems to be a close relationship between the revelation of some external characters of the cytoplasmic species and the biological differences between the two species.

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EXPLANATION OF PLATES

PLATE I

Three kinds of control diploid frogs, 1 year old. $\times 1.5$

1, 2. Female *Rana temporaria*, (T)TT.

3, 4. Male hybrid, (J)JT, produced by *Rana japonica* ♀ \times *Rana temporaria* ♂.

5, 6. Female *Rana japonica*, (J)JJ.

NUCLEO-CYTOPLASMIC HYBRIDS BETWEEN *R. JAP.* AND *R. TEMP.* TEMP.
M. NISHOKA

PLATE I

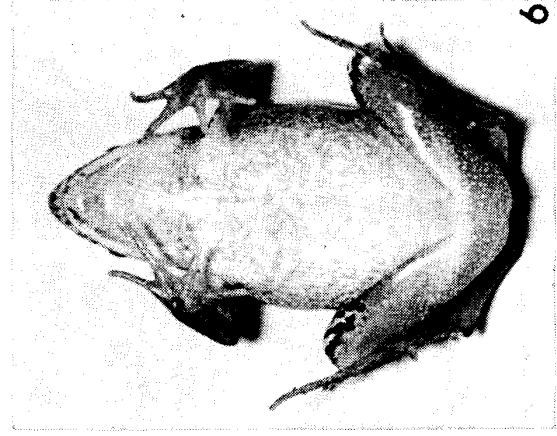
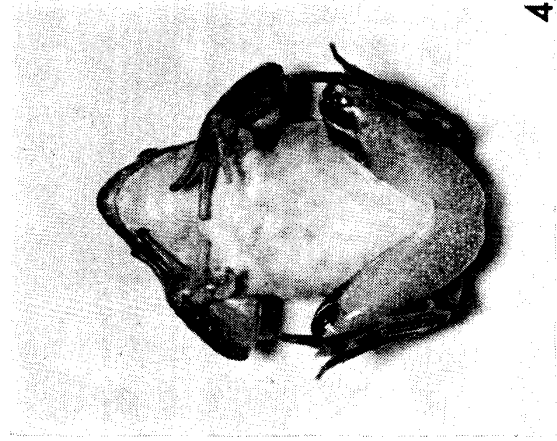
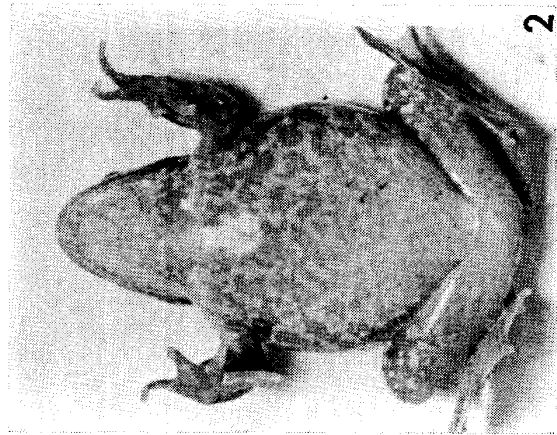
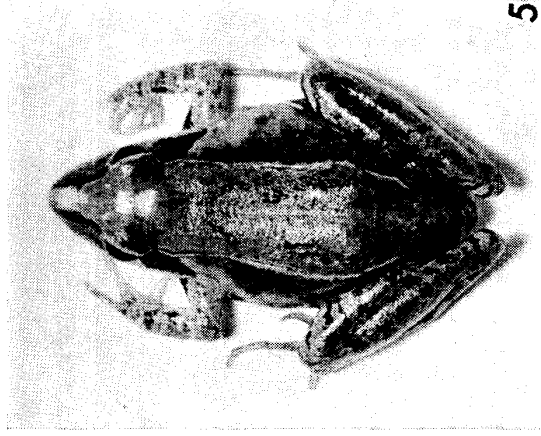
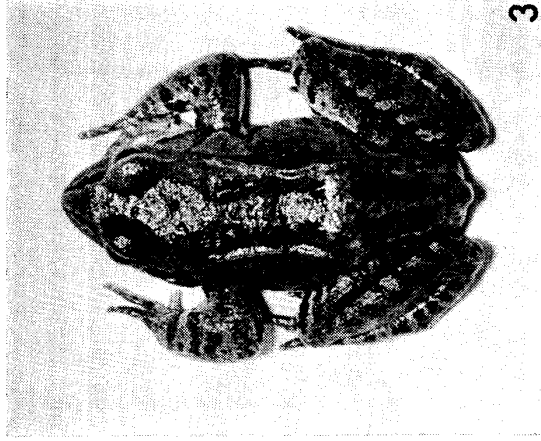
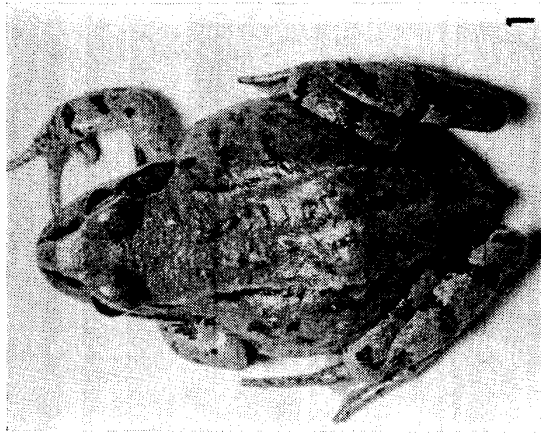


PLATE II

Male diploid nucleo-cytoplasmic hybrids consisting of *Rana japonica* cytoplasm and *Rana temporaria* nuclei, (J)TT, 250 days old. $\times 1.5$

- 7, 8. No. (J)TT62W1.2.
- 9, 10. No. (J)TT62W7.1.
- 11, 12. No. (J)TT63W4.1.
- 13, 14. No. (J)TT63W3.3.

NUCLEO-CYTOPLASMIC HYBRIDS BETWEEN *R. JAP.* AND *R. TEMP.* TEMP.
M. NISHIOKA

PLATE II

