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Reproductive Capacity of Male Autotetraploid *Rana nigromaculata* and Male and Female Amphidiploids Produced from them by Mating with Female Diploid *Rana brevipoda*

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(With 12 Text-figures)

CONTENTS

INTRODUCTION

Four amphidiploid frogs were first produced by KAWAMURA and NISHIOKA (1960) between *Rana nigromaculata* and *Rana brevipoda*. Three of them were males which attained sexual maturity. The fact that one of these male amphidiploids was not inferior to the control diploid male *Rana nigromaculata* and *Rana brevipoda* in reproductive capacity was reported by the same authors (1963a). This male amphidiploid was a 2-year-old frog raised from a fertilized *Rana nigromaculata* egg transplanted with a blastula nucleus of *Rana brevipoda*. KAWAMURA and NISHIOKA (1963b) also reported that 19 autotetraploid and seven amphidiploid frogs were respectively produced from cold-treated *Rana nigromaculata* and *Rana brevipoda* eggs inseminated with sperm of a male autotetraploid *Rana nigromaculata*. Of these autotetraploids, three were females and 16 were males, while one of the seven amphidiploids was a female and the other six were males.

In 1964, four of the above 16 male autotetraploids obtained in 1962 were mated with field-caught female *Rana nigromaculata* and *Rana brevipoda*. A part of the eggs was refrigerated shortly after insemination in order to obtain autotetraploids and amphidiploids. In 1966, male and female amphidiploids obtained in 1964 were mated with amphidiploids and offspring of field-caught *Rana nigromaculata* and *Rana brevipoda* produced in the same year.

The results of a series of experiments performed in 1964 and 1966 by the present authors for the purpose of obtaining numerous amphidiploids by good use of male autotetraploids will be described here in detail. A preliminary report of their findings has been published by KAWAMURA and NISHIOKA (1967).

MATERIALS AND METHODS

Rana nigromaculata HALLOWELL collected from the suburbs of Hiroshima and *Rana brevipoda* ITO from Konko-cho near Okayama were used as materials. Suppression of the first cleavage in *Rana nigromaculata* eggs was done by subjecting the eggs to 42°C for 5~9 minutes, 5 minutes before the cleavage furrow appeared. By this treatment, a few autotetraploids were produced. Suppression of the extrusion of the second polar body in *Rana nigromaculata* eggs was done by subjecting the eggs to 1~2°C for 3 hours, 20~25 minutes after insemination. In *Rana brevipoda* eggs, the same was done by subjecting the eggs to 1~2°C for 2.5 hours, 20~25 minutes after insemination. By suppressing the extrusion of the second polar body, triploids were easily produced (NISHIOKA, 1971, 1972).

The chromosomes of tadpoles were observed in the preparations of their tail-tips which were made by the squash method with water-pretreatment (MAKINO and NISHIMURA, 1952; NISHIOKA, 1972). The chromosomes of adult frogs were examined by the blood culture method or the bone marrow method (VOLPE and GEBHARDT, 1968; OMURA, 1967).

Serum proteins, hemoglobin and enzymes extracted from skeletal muscles were

analyzed by the method of starch-gel electrophoresis, as utilized by BREWER (1970), NISHIOKA, OHTANI and SUMIDA (1980) and NISHIOKA, UEDA and SUMIDA (1981).

Tadpoles were fed on boiled spinach or chard. Froglets shortly after metamorphosis were fed on mosquitoes, while somewhat grown frogs were fed on domestic flies or bag-worms.

The testes of male frogs were fixed in NAVASHIN's fluid, sectioned at 12μ and stained with HEIDENHAIN's iron hematoxylin for histological observations.

The following abbreviations are used in this report.

N .....A set of *Rana nigromaculata* chromosomes
B .....A set of *Rana brevipoda* chromosomes
(N) .....*Rana nigromaculata* cytoplasm
(B) .....*Rana brevipoda* cytoplasm
(N)NN .....Diploid *Rana nigromaculata*
(B)BB .....Diploid *Rana brevipoda*
(B)BN.....Diploid hybrid, *Rana brevipoda*♀ × *Rana nigromaculata*♂
(N)NNN .....Autotriploid *Rana nigromaculata*
(B)BBB .....Autotriploid *Rana brevipoda*
(N)NNB .....Allotriploid consisting of two *Rana nigromaculata* genomes and one *Rana brevipoda* genome
(B)BBN .....Allotriploid consisting of two *Rana brevipoda* genomes and one *Rana nigromaculata* genome
(N)NNNN...Autotetraploid *Rana nigromaculata*
(B)BBNN ....Amphidiploid consisting of two *Rana brevipoda* genomes and two *Rana nigromaculata* genomes

OBSERVATION

I. Production of amphidiploids from autotetraploid male Rana nigromaculata

In order to produce amphidiploids, four autotetraploid male *Rana nigromaculata* were used in crossing with female *Rana brevipoda*. These autotetraploids were produced from a single autotetraploid which was obtained in 1960 from a fertilized egg by applying a heat shock 5 minutes before the appearance of the first cleavage furrow. In 1962, eggs of a field-caught female *Rana nigromaculata* were inseminated with sperm of this male autotetraploid (60N12H7) and subjected to 1~2°C for 3 hours, 25 minutes after insemination. Of 25 autotetraploids raised from these eggs (cf. KAWAMURA and NISHIOKA, 1963b: p. 95, Table 8), 19 metamorphosed normally. The latter consisted of 3 females and 16 males. Four, (N)NNNN 62-I♂, Nos. 1~4, of these 16 male autotetraploids were those used in crossing with female *Rana brevipoda* in the present study.

In the breeding season of 1964, the four autotetraploid *Rana nigromaculata* were 2 years old and 40.5~49.0 mm in body length. They were mated with two field-caught female *Rana nigromaculata* and three field-caught female *Rana brevipoda*

to produce auto- and allotetraploids. The results are presented in Table 1.

1. Autotetraploids

Eggs of the two diploid female *Rana nigromaculata,* (N)NN 64W♀, Nos. 1 and 2, were inseminated with sperm of the four autotetraploid male *Rana nigromaculata,* (N)NNNN 62-I♂, Nos. 1～4. It was found that 7.4～19.7% of the respective number of eggs, 75 (12.1%) of 620 eggs in total, cleaved normally. After 16 eggs became abnormal mainly by incomplete invagination at the gastrula stage, 7.4～17.1%, 59 (9.5%) in total, became normal tail-bud embryos. Thereafter, 3.5～17.1%, 51 (8.2%) in total, hatched normally and 2.8～17.1%, 49 (7.9%) in total, became normally feeding tadpoles which were more than 30 mm in total length. The other embryos and tadpoles died of edema, underdevelopment or some other abnormalities.

Chromosome numbers were determined in 46 feeding tadpoles which had

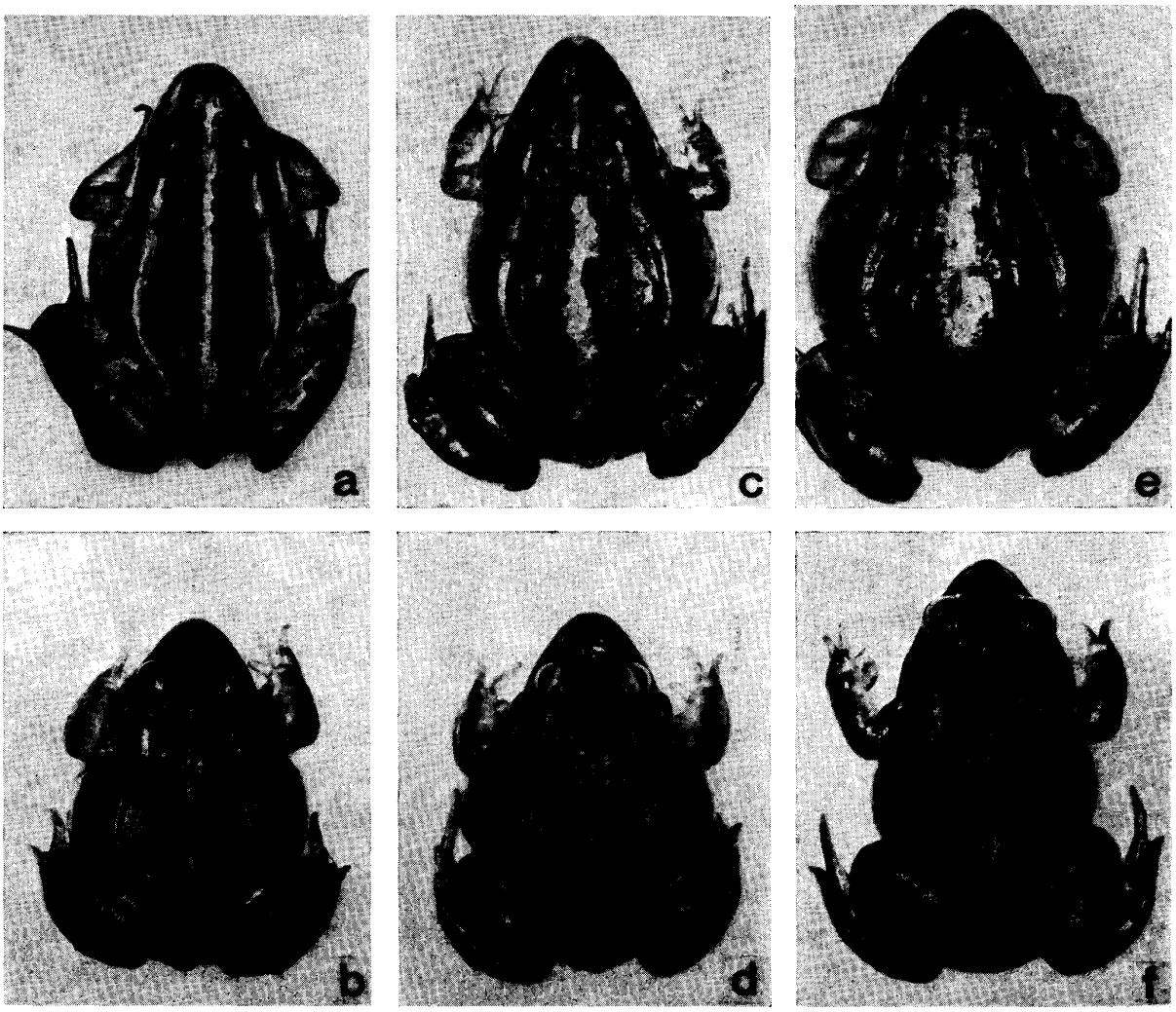

Fig. 1. Three-year-old diploid, triploid and tetraploid *Rana nigromaculata.* ×0.7

a. Diploid female (N)NN 64♀, No. 1
b. Diploid male (N)NN 64♂, No. 1
c. Triploid female (N)NNN 64♀, No. 1
d. Triploid male (N)NNN 64♂, No. 1
e. Tetraploid female (N)NNNN 64♀, No. 1
f. Tetraploid male (N)NNNN 64♂, No. 1

TABLE 1

Developmental capacity and ploidy of the offspring between diploid female *Rana nigromaculata* or *Rana brevipoda* and autotetraploid male *Rana nigromaculata*

Parents		Treatment of fertilized eggs	No. of eggs	No. of normal cleavages	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of analyzed tadpoles	Kind of ploidy			
Female	Male								2n	3n	4n	Mosaics
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 1	None	105	18 (17.1%)	18 (17.1%)	18 (17.1%)	18 (17.1%)	18	0	18	0	0
		Refrigeration	201	18 (9.0%)	18 (9.0%)	17 (8.5%)	12 (6.0%)	10	2	0	7	1
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 1	None	307	234 (76.2%)	137 (44.6%)	116 (37.8%)	112 (36.5%)	112	0	112	0	0
		Refrigeration	969	546 (56.3%)	260 (26.8%)	174 (18.0%)	152 (15.7%)	143	5	30	101	7
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 2	None	203	15 (7.4%)	15 (7.4%)	15 (7.4%)	14 (6.9%)	14	0	14	0	0
		Refrigeration	778	48 (6.2%)	29 (3.7%)	17 (2.2%)	11 (1.4%)	11	0	0	9	2
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 2	None	193	50 (25.9%)	46 (23.8%)	46 (23.8%)	40 (20.7%)	31	0	31	0	0
		Refrigeration	1672	171 (10.2%)	39 (2.3%)	26 (1.6%)	26 (1.6%)	26	2	8	16	0
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 3	None	170	14 (8.2%)	13 (7.6%)	13 (7.6%)	13 (7.6%)	10	0	9	0	1
		Refrigeration	951	49 (5.2%)	26 (2.7%)	19 (2.0%)	8 (0.8%)	8	2	2	3	1
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 3	None	384	112 (29.2%)	86 (22.4%)	85 (22.1%)	74 (19.3%)	74	0	74	0	0
		Refrigeration	1585	246 (15.5%)	45 (2.8%)	27 (1.7%)	25 (1.6%)	24	1	3	19	1
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 4	None	142	28 (19.7%)	13 (9.2%)	5 (3.5%)	4 (2.8%)	4	0	4	0	0
		Refrigeration	169	3 (1.8%)	3 (1.8%)	2 (1.2%)	2 (1.2%)	2	0	0	1	1
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 4	None	357	20 (5.6%)	17 (4.8%)	13 (3.6%)	9 (2.5%)	5	0	5	0	0
		Refrigeration	561	43 (7.7%)	11 (2.0%)	10 (1.8%)	10 (1.8%)	8	3	1	4	0

more than 4 analyzable mitotic figures in the tail-tip preparation. The results showed that 45 (97.8%) were triploids, (N)NNN, and the remainder was a 2n-3n mosaic (Table 1).

Eggs of the same two females were subjected to 1~2°C for 3 hours, 25 minutes after insemination with sperm of the four male autotetraploids. It was found that 1.8~9.0% of the respective number of eggs, 118 (5.6%) of 2099 eggs in total, cleaved normally. After many of the normally cleaved eggs became abnormal by incomplete invagination at the gastrula stage and died of edema, underdevelopment or some other abnormalities, 1.8~9.0%, 76 (3.6%) in total, became normal tail-bud embryos, 1.2~8.5%, 55 (2.6%) in total, hatched normally and 0.8~6.0%, 33 (1.6%) in total, became normally feeding tadpoles which were more than 30 mm in total length.

Chromosome numbers were determined in 31 feeding tadpoles which had more than 4 analyzable mitotic figures in the tail-tip preparation. It was found that 20 (64.5%) were tetraploids, (N)NNNN, four were diploids, (N)NN, two were triploids, (N)NNN, and the remaining five were 2n-4n mosaics (Table 1). In these mosaics, the resting nuclei of the tail-tip were divided into two distinct

groups in size.

2. Amphidiploids

Eggs of the three *Rana brevipoda*, (B)BB 64W♀, Nos. 1～3, were inseminated with sperm of the four male autotetraploids, (N)NNNN 62-I♂, Nos. 1～4. It was found that 5.6～76.2% of the respective number of eggs, 416 (33.5%) of 1241 eggs in total, cleaved normally. At the late cleavage or blastula stage, 54 eggs became abnormal, and thereafter 76 other eggs showed incomplete invagination at the gastrula stage. Eventually, 3.6～37.8%, 286 (23.0%) eggs in total, became normal tail-bud embryos, 3.6～37.8%, 260 (21.0%) in total, hatched normally, and 2.5～36.5%, 235 (18.9%) in total, became normally feeding tadpoles which were more than 30 mm in total length.

Chromosome numbers were determined in 222 feeding tadpoles which had more than four analyzable mitotic figures in the tail-tip. It was found that all these tadpoles were triploids, (B)BNN (Table 1).

Eggs of the same three females were subjected to 1～2°C for 2.5 hours, 20～25 minutes after insemination with sperm of the four male autotetraploids. It was found that 7.7～56.3% of the respective number of eggs, 1006 (21.0%) of 4787 eggs in total, cleaved normally. After 216, 193 and 242 eggs became abnormal at the late cleavage or blastula, the gastrula and the tail-bud stage, respectively, 2.0～26.8%, 355 (7.4%) eggs in total, became normal tail-bud embryos and 1.6～18.0%, 237 (5.0%) in total, hatched normally. At the hatching stage, 24 became abnormal and died of edema, blisters or underdevelopment. Eventually, 1.6～15.7%, 213 (4.4%) in total, became normally feeding tadpoles which were more than 30 mm in total length.

Chromosome numbers were determined in the tail-tips of 201 feeding tadpoles which had more than four analyzable mitotic figures. The results showed that 140 (69.7%) were tetraploids, (B)BBNN, 11 were diploids, (B)BB or (B)NN, 42 were triploids, (B)BNN, and the remaining eight were 2n-4n mosaics, the resting nuclei being divided into two distinct groups in size (Table 1).

II. Growth and sex of amphidiploids

1. Autotriploids, (N)NNN

Forty-five autotriploids, (N)NNN, produced from matings between the two female *Rana nigromaculata*, (N)NN 64W♀, Nos. 1 and 2, and four male autotetraploids, (N)NNNN 62-I♂, Nos. 1～4, were continuously reared in four series derived from the four males. In these four series, autotriploids were 65.4 mm, 74.9 mm, 67.1 mm and 67.4 mm in mean total length at the age of 50 days. At the age of 55～64 days, 27 tadpoles completed metamorphosis. The froglets were 16.0～20.0 mm in body length. In the four series, the froglets were 18.3 mm, 17.3 mm, 16.7 mm and 18.0 mm in mean body length immediately after metamorphosis.

Two autotriploids, (N)NNN, produced from *Rana nigromaculata* eggs which had been refrigerated after insemination with sperm of male autotetraploids for the purpose of producing autotetraploids were tadpoles, which were 65.0 mm and 65.5 mm in total length at the age of 50 days. They completed metamorphosis at the age of 57 days and 59 days. The froglets were 17.0 mm and 18.0 mm in body length immediately after metamorphosis (Table 2).

Of the above 29 autotriploids in total, 17 died within three months after meta-

TABLE 2

Growth of diploids, triploids and tetraploids produced from matings between diploid female *Rana nigromaculata* or *Rana brevipoda* and autotetraploid male *Rana nigromaculata*

Parents		Treatment of fertilized eggs	No. of mitoses	Ploidy	Constitution	No. of tadpoles	Mean total length of 50-day-old tadpoles (mm)	Period of tadpole stage (days)	No. of metamorphosed frogs	Mean body length soon after metamorphosis (mm)
Female	Male									
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 1	None	5~15	3n	(N)NNN	18	65.4± 0.4	57~61 (58.4)	14	18.3± 0.1
		Refrigeration	5~15	2n	(N)NN	2	65.0, 67.0	58	1	17.0
			5~20	4n	(N)NNNN	7	73.5~76.0 (74.9)	57~59 (58.0)	6	18.5~20.0 (19.4)
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 1	None	5~15	3n	(B)BNN	112	69.0± 0.03	57~73 (61.6)	98	19.5± 0.2
		Refrigeration	5~15	2n	(B)BB or (B)NN	5	70.5~75.5 (74.3)	54~64 (59.5)	4	17.0~19.0 (17.9)
			5~15	3n	(B)BNN	30	72.3± 0.5	58~64 (60.0)	17	19.7± 0.2
			5~20	4n	(B)BBNN	101	74.2± 0.4	54~65 (60.3)	74	20.5± 0.3
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 2	None	5~15	3n	(N)NNN	14	74.9± 0.5	55~64 (59.5)	6	17.0~17.5 (17.3)
		Refrigeration	5~15	4n	(N)NNNN	9	70.5~76.0 (74.7)	57~75 (65.5)	6	17.5~18.0 (17.8)
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 2	None	5~15	3n	(B)BNN	31	73.4± 0.2	57~65 (61.0)	23	17.5± 0.2
		Refrigeration	7, 9	2n	(B)BB	2	64.0, 69.5	57, 59	2	17.5, 18.0
			5~15	3n	(B)BNN	8	73.5~75.5 (74.3)	55~60 (56.4)	5	17.0~19.5 (18.2)
			10~20	4n	(B)BBNN	16	76.3± 0.7	66~73 (69.5)	8	17.5~20.5 (19.1)
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 3	None	5~11	3n	(N)NNN	9	66.5~67.5 (67.1)	55~58 (56.0)	5	16.7± 0.2
		Refrigeration	4, 5	2n	(N)NN	2	64.0, 65.0	60	1	17.5
			5, 6	3n	(N)NNN	2	65.0, 65.5	57, 59	2	17.0, 18.0
			7~10	4n	(N)NNNN	3	65.5~66.0 (65.8)	55	1	16.5
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 3	None	5~10	3n	(B)BNN	74	69.0± 0.4	59~64 (62.2)	71	17.1± 0.3
		Refrigeration	5	2n	(B)NN	1	62.5	68	1	16.5
			7~12	3n	(B)BNN	3	69.0~72.0 (70.3)	—	—	—
			6~16	4n	(B)BBNN	19	75.6± 0.9	61~68 (63.8)	14	19.2± 0.4
(N)NN 64W, Nos. 1, 2	(N)NNNN 62-I, No. 4	None	5~7	3n	(N)NNN	4	60.0~72.5 (67.4)	57, 60	2	17.5, 18.5
		Refrigeration	5	4n	(N)NNNN	1	65	57	1	17.0
(B)BB 64W, Nos. 1~3	(N)NNNN 62-I, No. 4	None	6~13	3n	(B)BNN	5	70.5~72.0 (71.2)	57~62 (59.5)	4	18.0~18.5 (18.3)
		Refrigeration	4~6	2n	(B)BB or (B)NN	3	65.0~73.5 (69.5)	59, 64	2	17.5, 18.0
			6	3n	(B)BNN	1	—	—	—	—
			6~10	4n	(B)BBNN	4	69.5~72.5 (71.0)	60, 63	2	18.0

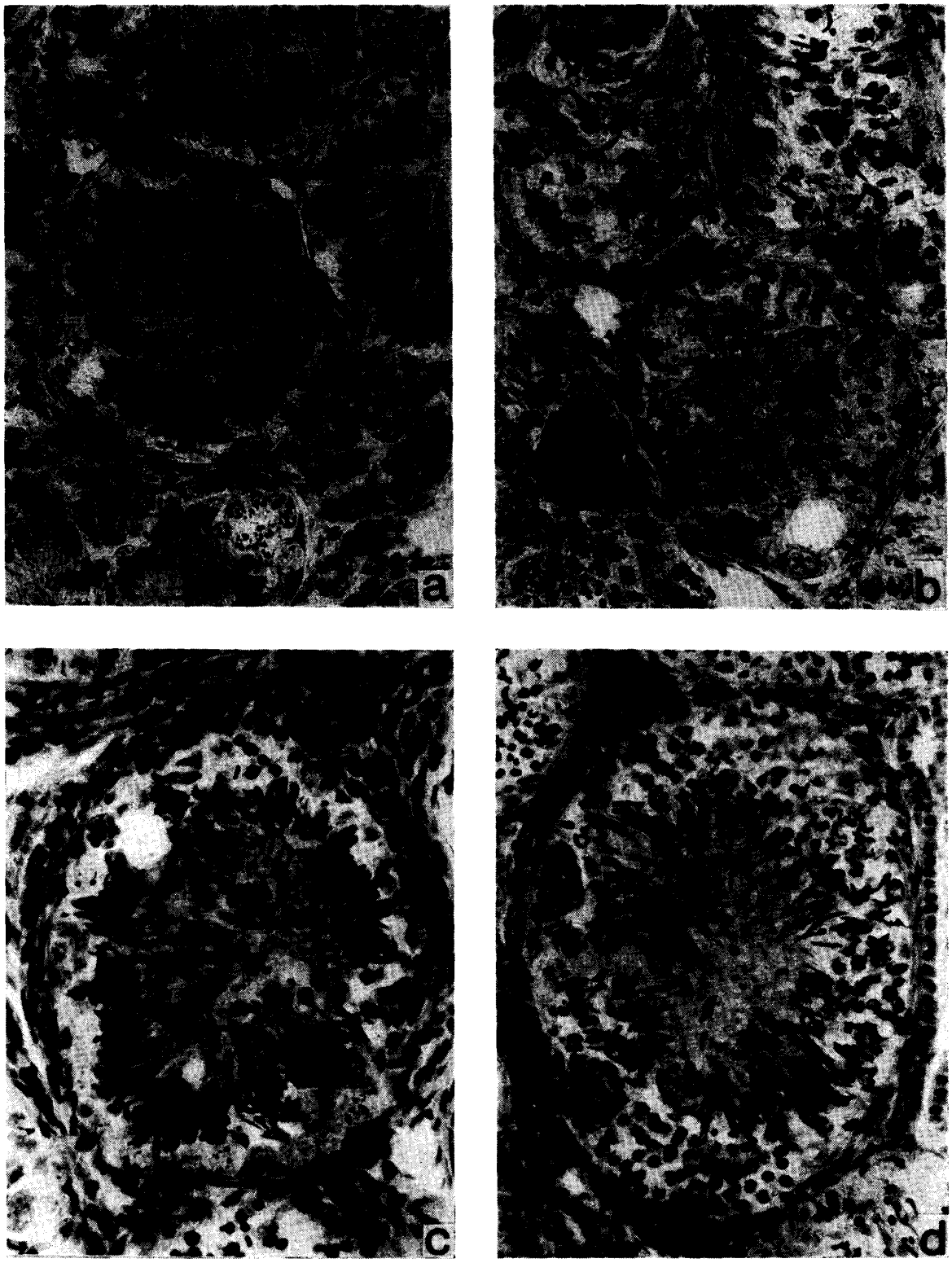

Fig. 2. Cross-sections of the testes of diploid, triploid and tetraploid male *Rana nigromaculata*. ×260

a. Control diploid male (N)NN 64♂, No. 1
b. Triploid male (N)NNN 64♂, No. 1
c. Tetraploid male (N)NNNN 64♂, No. 1
d. Tetraploid male (N)NNNN 64♂, No. 2

morphosis. Five of them were females with normal ovaries, five were females with underdeveloped ovaries, one was a hermaphrodite and the remaining six were males. The other 12 living autotriploids attained sexual maturity. Seven of them were females and five were males. When the hermaphrodite was counted as a male, 17 of 29 autotriploids in total were females and 12 (41.4%) were males.

Of these autotriploids, seven females and five males attained sexual maturity. These mature autotriploids were indistinguishable in appearance from normal diploids (Fig. 1). Their chromosome number was confirmed by the blood culture method (Fig. 3).

The testes of two mature male autotriploids were sectioned in order to examine their inner structure. It was found that the seminiferous tubules contained a few abnormal spermatozoa of various sizes and abundant pycnotic nuclei. Besides, there were many spermatocytes and some spermatogonia situated in the peripheral region. No normal spermatozoa were observed in the testes (Fig. 2).

2. Autotetraploids, (N)NNNN

Twenty autotetraploids, (N)NNNN, produced from eggs of the two female *Rana nigromaculata*, (N)NN 64W♀, Nos. 1 and 2, by subjecting to cold after insemination with sperm of the four autotetraploid male *Rana nigromaculata* were continuously reared. They were tadpoles which were 65.0~76.0 mm in

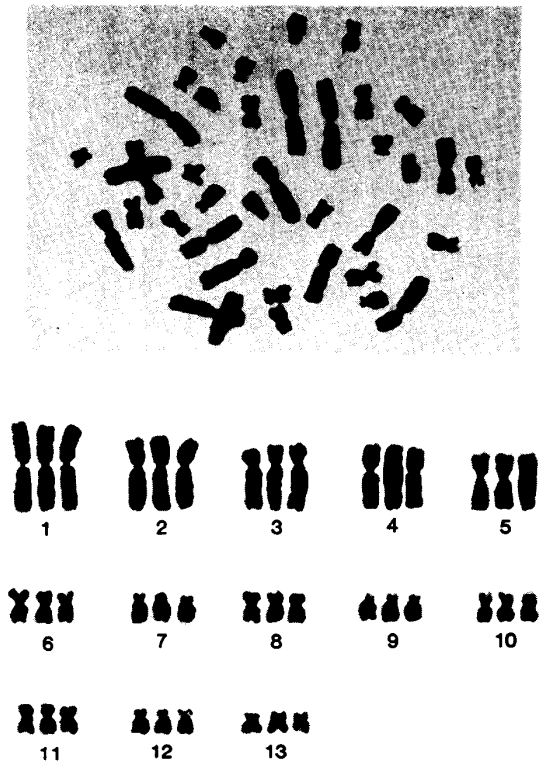

Fig. 3. Metaphase plate and the karyotype of a triploid male *Rana nigromaculata*. ×900

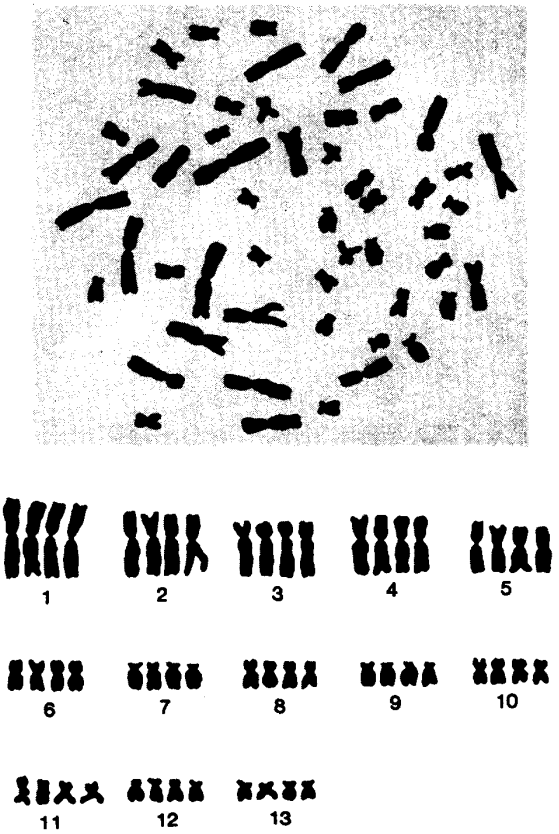

Fig. 4. Metaphase plate and the karyotype of a tetraploid male *Rana nigromaculata*. ×900

TABLE 3

Sex of diploids, triploids and tetraploids produced from matings between diploid

Parents		Treatment of fertilized eggs	Ploidy	Constitution	No. of metamorphosed frogs
Female	Male				
(N)NN 64W, Nos. 1, 2	(N)NNNN 62–I, No. 1	None	3n	(N)NNN	14
		Refrigeration	2n	(N)NN	1
			4n	(N)NNNN	6
(B)BB 64W, Nos. 1～3	(N)NNNN 62–I, No. 1	None	3n	(B)BNN	98
		Refrigeration	2n	(B)NN	4
			3n	(B)BNN	17
			4n	(B)BBNN	74
(N)NN 64W, Nos. 1, 2	(N)NNNN 62–I, No. 2	None	3n	(N)NNN	6
		Refrigeration	4n	(N)NNNN	6
(B)BB 64W, Nos. 1～3	(N)NNNN 62–I, No. 2	None	3n	(B)BNN	23
		Refrigeration	2n	(B)BB	2
			3n	(B)BNN	5
			4n	(B)BBNN	8
(N)NN 64W, Nos. 1, 2	(N)NNNN 62–I, No. 3	None	3n	(N)NNN	5
		Refrigeration	2n	(N)NN	1
			3n	(N)NNN	2
			4n	(N)NNNN	1
(B)BB 64W, Nos. 1～3	(N)NNNN 62–I, No. 3	None	3n	(B)BNN	71
		Refrigeration	2n	(B)NN	1
			4n	(B)BBNN	14
(N)NN 64W, Nos. 1, 2	(N)NNNN 62–I, No. 4	None	3n	(N)NNN	2
		Refrigeration	4n	(N)NNNN	1
(B)BB 64W, Nos. 1～3	(N)NNNN 62–I, No. 4	None	3n	(B)BNN	4
		Refrigeration	2n	(B)BB or (B)NN	2
			4n	(B)BBNN	2

total length at the age of 50 days. In the four series, they were 74.9 mm, 74.7 mm, 65.8 mm and 65.0 mm in mean total length. While six of them died before or during metamorphosis, the other 14 completed metamorphosis at the age of 55～75 days. These froglets were 16.5～20.0 mm in body length. In the four series, the autotetraploids were 19.4 mm, 17.8 mm, 16.5 mm and 17.0 mm in mean body length (Table 2).

Ten of the metamorphosed autotetraploids died within three months after metamorphosis. Two were females with normal ovaries, four others were females with underdeveloped ovaries and the remaining four were males. The other four living autotetraploids attained sexual maturity. Two of them were females and the other two were males. Of a total of 14 autotetraploid frogs, eight were females and six (42.9%) were males (Table 3).

female *Rana nigromaculata* or *Rana brevipoda* and autotetraploid male *Rana nigromaculata*

Sex of frogs dead or killed within three months after metamorphosis						Sex of frogs, one or two years old			Sex of all frogs examined		
Total	♀N1	♀N2	♀U	⚥	♂	Total	♀	♂	Total	♀	♂ (%)
7	0	5	0	0	2	7	3	4	14	8	6(42.9)
1	0	0	0	0	1	—	—	—	1	0	1
2	0	1	0	0	1	4	2	2	6	3	3(50.0)
55	1	1	47	0	6	43	40	3	98	89	9(9.2)
1	0	0	1	0	0	3	3	0	4	4	0
7	0	0	5	0	2	10	8	2	17	13	4(23.5)
60	26	10	9	0	15	14	12	2	74	57	17(23.0)
3	0	0	1	0	2	3	3	0	6	4	2(33.3)
6	0	1	4	0	1	—	—	—	6	5	1(16.7)
10	0	0	6	0	4	13	7	6	23	13	10(43.5)
2	2	0	0	0	0	—	—	—	2	2	0
5	0	0	4	0	1	—	—	—	5	4	1(20.0)
3	0	0	2	0	1	5	3	2	8	5	3(37.5)
5	0	0	3	1	1	—	—	—	5	3	2(40.0)
1	1	0	0	0	0	—	—	—	1	1	0
2	0	0	1	0	1	—	—	—	2	1	1
1	0	0	0	0	1	—	—	—	1	0	1
56	0	6	22	1	27	15	7	8	71	35	36(50.7)
1	0	0	0	0	1	—	—	—	1	0	1
10	0	0	5	0	5	4	2	2	14	7	7(50.0)
—	—	—	—	—	—	2	1	1	2	1	1
1	0	0	0	0	1	—	—	—	1	0	1
4	0	1	2	1	0	—	—	—	4	3	1(25.0)
2	1	0	0	0	1	—	—	—	2	1	1
2	0	0	0	1	1	—	—	—	2	0	2

Of these autotetraploids, two females and two males attained sexual maturity. These mature autotetraploids were indistinguishable from normal diploids and autotriploids in appearance (Fig. 1). Their chromosome number was confirmed by the blood culture method (Fig. 4). The two female autotetraploids, (N)NNNN♀, Nos. 1 and 2, had a small number of well-grown ova in their ovaries, although no ovulation occurred after pituitary injection.

The testes of one (No. 1) of the two male autotetraploids, (N)NNNN♂, Nos. 1 and 2, were slightly abnormal. Although there were loose bundles of large spermatozoa in the large central part surrounded by spermatocytes and spermatogonia in the seminiferous tubules, the spermatozoa were far fewer than those of male amphidiploids and, moreover, were not uniform in size. There were many spermatozoa which were abnormal in size or shape. Many pycnotic nuclei of

various sizes were also found. In the testes of the other male autotetraploid, (No. 2), the spermatozoa were fewer than those of the above autotetraploid (No. 1). However, a considerable number of normally shaped diploid spermatozoa were found. (Fig. 2).

3. Gynogenetic or androgenetic diploids, (N)NN, (B)BB or (B)NN

Four diploids, (N)NN, which were produced from *Rana nigromaculata* eggs subjected to cold after insemination with sperm of two autotetraploid male *Rana nigromaculata*, (N)NNNN 62-I♂, Nos. 1 and 3, were continuously reared. They were tadpoles which were 64.0～67.0 mm, 65.3 mm on the average, in total length at the age of 50 days. Two of them completed metamorphosis at the age of 58 or 60 days, when they were 17.0 mm and 17.5 mm in body length (Table 2). These two froglets died two and three weeks after metamorphosis. One of them was a female with normal ovaries, while the other was a male (Table 3).

Eleven diploid feeding tadpoles, produced from the eggs of the two female *Rana brevipoda* by subjecting to cold after insemination with sperm of four male autotetraploids, (N)NNNN 62-I♂, Nos. 1～4, were continuously reared. They were 62.5～75.5 mm in total length at the age of 50 days. One tadpoles derived from male No. 1 and another derived from male No. 4 died immediately before or during metamorphosis. These two individuals were of *Rana brevipoda* type in external character. The other nine completed metamorphosis at the age of 54～68 days. Four diploids derived from male No. 1 were 17.0～19.0 mm, 17.9 mm on the average, in body length. All of them were of *Rana nigromaculata* type in external character. Two diploids derived from male No. 2 were 17.5 mm and 18.0 mm in body length and of *Rana brevipoda* type. One diploid derived from male No. 3 was 16.5 mm in body length and of *Rana nigromaculata* type. One of two diploids derived from male No. 4 was 18.0 mm in body length and of *Rana nigromaculata* type, while the other was 17.5 mm in body length and of *Rana brevipoda* type. Thus, it was evident that the six diploids of *Rana nigromaculata* type were androgenetically produced frogs, (B)NN, while the three of *Rana brevipoda* type were gynogenetically produced ones, (B)BB (Table 2).

Three of the six (B)NN diploids died within three months after metamorphosis. One of these three diploids was a female with underdeveloped ovaries, while the other two were males. The remaining three diploids attained sexual maturity and were females. The three (B)BB diploids all died within three months after metamorphosis. They were females with normal ovaries (Table 3).

4. Allotriploids, (B)BNN

Two hundred and twenty-two allotriploids, (B)BNN, produced from the three female *Rana brevipoda*, (B)BB 64W♀, Nos. 1～3, by mating with the four autotetraploid male *Rana nigromaculata*, (N)NNNN 62-I♂, Nos. 1～4, were continuously reared. In four series derived from the four males, tadploes were 69.0 mm, 73.4 mm, 69.0 mm and 71.2 mm in mean total length at the age of 50 days. Thereafter, 98, 23, 71 and 4 tadpoles completed metamorphosis at the age of

61.6 days, 61.0 days, 62.2 days and 59.5 days on the average, respectively, 57～73 days on the whole. The froglets in the four series were 19.5 mm, 17.5 mm, 17.1 mm and 18.3 mm in mean body length immediately after metamorphosis (Table 2).

Of 42 allotriploids, (B)BNN, produced from the eggs of the three female *Rana brevipoda* by subjecting to cold after insemination with sperm of the autotetraploid male *Rana nigromaculata*, 41 were continuously reared, while the remainder derived from a single male, (N)NNNN 62-I♂, No. 4, died soon after chromosomal examination. The allotriploid tadpoles in the three series derived from three males, (N)NNNN 62-I♂, Nos. 1～3, were 72.3 mm, 74.3 mm and 70.3 mm in mean total length at the age of 50 days. While all three tadpoles derived from male No. 3 died immediately before or during metamorphosis, 17 derived from male No. 1 and five derived from male No. 2 completed metamorphosis at the age of 60.0 days and 56.4 days on the average, respectively, 55～64 days on the whole. They were 19.7 mm and 18.2 mm in body length, respectively, immediately after metamorphosis (Table 2).

Of a total of 218 allotriploids, (B)BNN, 137 died or were killed within three months after metamorphosis. Of these young allotriploids, one was a female with normal ovaries, eight were females with somewhat underdeveloped ovaries, 86 were females with distinctly underdeveloped ovaries, two were hermaphrodites and the remaining 40 were males. The other 81 living allotriploids attained sexual maturity. Of these frogs, 62 were females and 19 were males. When the hermaphrodites were counted as males, 157 of the 218 allotriploids in total were females and 61 (28.0%) were males (Table 3).

5. Amphidiploids, (B)BBNN

From the eggs of the three female *Rana brevipoda*, (B)BB 64W♀, Nos. 1～3, by subjecting to cold after insemination with sperm of the four autotetraploid male *Rana nigromaculata*, (N)NNNN 62-I♂, Nos. 1～4, 101, 16, 19 and 4 amphidiploids, 140 in total, were produced in the four experimental series derived from the four males and were continuously reared. They were 74.2 mm, 76.3 mm, 75.6 mm and 71.0 mm in total length, respectively, at the age of 50 days. Thereafter, 74, 8, 14 and 2 tadpoles, 98 in total, completed metamorphosis at the age of 60.3 days, 69.5 days, 63.8 days and 61.5 days on the average, respectively. When measured immediately after metamorphosis, they were 20.5 mm, 19.1 mm, 19.2 mm and 18.0 mm in body length, respectively (Table 2).

Of the 98 metamorphosed amphidiploids, 75 died within three months after metamorphosis. When the sex of these dead frogs was examined, it was found that 26 were females with normal ovaries, 10 were females with somewhat underdeveloped ovaries, 16 were females with distinctly underdeveloped ovaries, one was a hermaphrodite and the remaining 22 were males. The other 23 living amphidiploids attained sexual maturity. Of these frogs, 17 were females and the other six were males. When the hermaphrodite was counted as a male, 69 of the 98 metamorphosed amphidiploids in total were females and the other 29 (29.6%) were males (Table 3).

III. Characters of amphidiploids

The 98 young metamorphosed amphidiploids produced in 1964 from the eggs of three *Rana brevipoda*, (B)BB 64W♀, Nos. 1～3, by subjecting to cold after insemination with sperm of four male autotetraploids, (N)NNNN 62-I♂, Nos. 1～4, were very similar to the diploid hybrids between *Rana nigromaculata* and *Rana brevipoda* in external character. The 23 mature amphidiploids including 17 females and six males were also very similar to the mature diploid hybrids between the two species. However, the skin of the amphidiploids was loose and coarse in texture. The body was somewhat inelastic as compared with that of diploid hybrids.

The chromosomes of mature amphidiploids were observed by the blood culture method together with those of control diploids, (N)NN and (B)BB, and allotriploids, (N)NNB (Figs. 5～8). They consisted of two sets of *Rana nigromaculata* chromosomes and two sets of *Rana brevipoda* chromosomes.

1. Female amphidiploids

In the control diploids at the age of two years, two mature female *Rana brevipoda* were 45.0 mm and 47.5 mm in body length, while two mature female *Rana nigromaculata* were 55.0 mm and 56.5 mm. Two 4-year-old female *Rana brevipoda* were 56.5 mm and 56.0 mm in body length, while two 4-year-old female *Rana nigromaculata* were 65.0 mm and 69.5 mm (Table 4). The eggs of *Rana brevipoda*

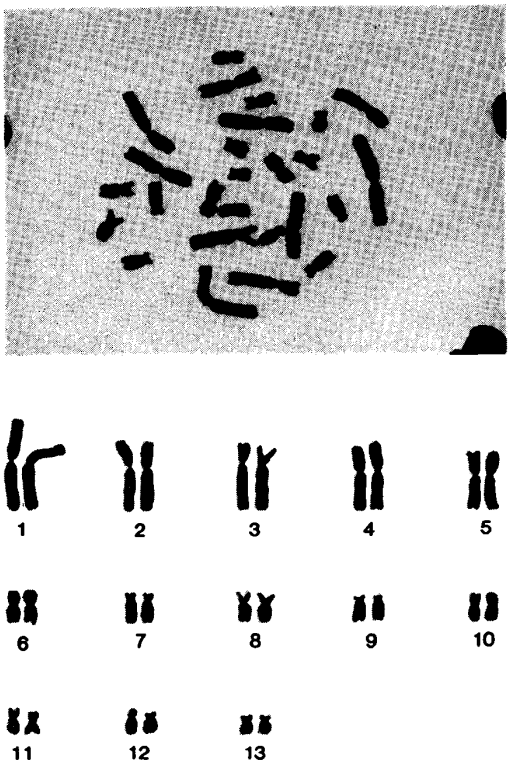

Fig. 5. Metaphase plate and the karyotype of a control diploid male *Rana nigromaculata*. ×900

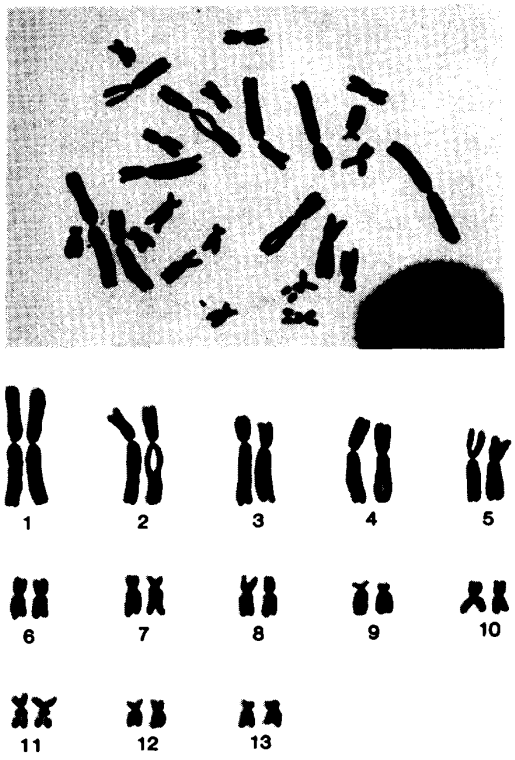

Fig. 6. Metaphase plate and the karyotype of a control diploid male *Rana brevipoda*. ×900

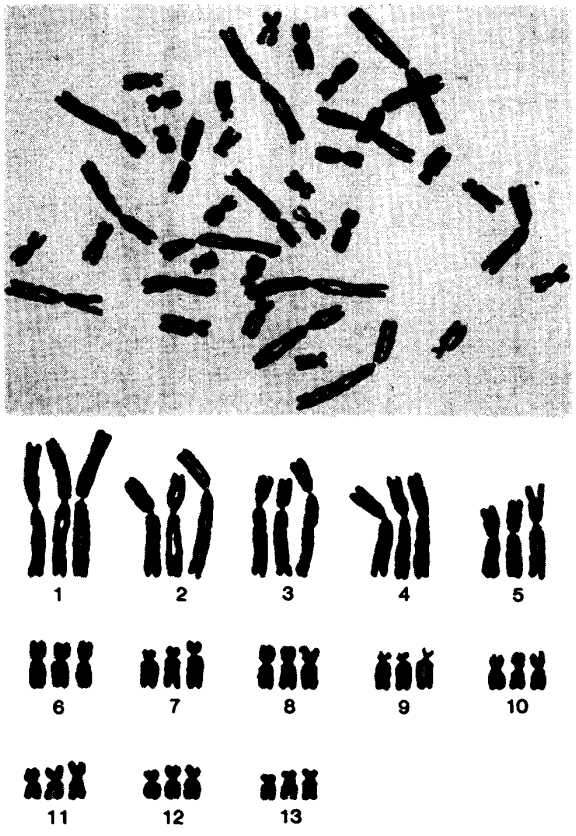

Fig. 7. Metaphase plate and the karyotype of a (N)NNB allotriploid female between *Rana nigromaculata* and *Rana brevipoda*. ×900

Fig. 8. Metaphase plate and the karyotype of a (B)BBNN amphidiploid female between *Rana brevipoda* and *Rana nigromaculata*. ×900

were also remarkably smaller than those of *Rana nigromaculata*. The eggs of the above four female *Rana brevipoda* were 1.47～1.58 mm, 1.53 mm on the average, in mean diameter of 50 eggs, while those of the above four female *Rana nigromaculata* were 1.95～2.16 mm, 2.01 mm on the average. The two 2-year-old female *Rana brevipoda* laid 1350 and 1417 eggs and the 4-year-old female *Rana brevipoda* laid 1854 and 1722 eggs, while the two 2-year-old female *Rana nigromaculata* laid 1204 and 1290 eggs and the 4-year-old female *Rana nigromaculata* laid 2063 and 2174 eggs. The jelly envelope surrounding eggs of *Rana brevipoda* was soft, while that of *Rana nigromaculata* was hard.

Three 2-year-old female hybrids, (B)BN, were 54.0～57.5 mm in body length and laid 646～1136 eggs, 959 eggs on the average, 50 eggs of which were 1.69～1.70 mm in mean diameter. Three 4-year-old female hybrids, (B)BN, were 60.0～63.5 mm in body length and laid 1234～1463 eggs, 1353 eggs on the average, 50 eggs of which were 1.74～1.81 mm in mean diameter. However, there were 3～13 exceptionally large eggs which were 2.2～2.6 mm in diameter among the normal-sized eggs in the six female hybrids. The jelly envelope of eggs was intermediate in hardness between those of the two species.

Four 2-year-old female allotriploids, (B)BNN, were 50.5～56.0 mm, 53.8 mm on the average, in body length. They laid 490～716 eggs, 585 eggs on the average,

TABLE 4

Number and mean diameter of eggs laid by females of diploid *Rana nigromaculata* or *Rana brevipoda*, diploid hybrids, allotriploids and amphidiploids

Kind	Individual no.	Ploidy	Age (years)	Body length (mm)	No. of eggs	Diameters or mean diameter of 50 eggs (mm)
(N)NN 64–I	1	2n	2	55.0	1204	2.03±0.01
	2	2n	2	56.5	1290	1.95±0.01
	3	2n	4	65.0	2063	1.88±0.01
	4	2n	4	69.5	2174	2.16±0.01
(B)BB 64–I	1	2n	2	45.0	1350	1.47±0.01
	2	2n	2	47.5	1417	1.53±0.01
	3	2n	4	56.5	1854	1.58±0.01
	4	2n	4	56.0	1722	1.54±0.1
(B)BN 64–I	1	2n	2	56.0	1136	1.70±0.02
					3	2.5～2.6
	2	2n	2	54.0	646	1.70±0.01
					4	2.3～2.4
	3	2n	2	57.5	1096	1.69±0.01
					13	2.2～2.3
	4	2n	4	60.0	1463	1.81±0.01
					9	2.4～2.5
	5	2n	4	63.5	1362	1.74±0.01
					7	2.3～2.4
	6	2n	4	61.5	1234	1.76±0.01
					5	2.3～2.5
(B)BNN 64–I	1	3n	2	56.0	716	1.4～3.2
	2	3n	2	55.5	515	1.3～2.7
(B)BNN 64–II	3	3n	2	50.5	620	1.3～2.7
(B)BNN 64–III	4	3n	2	53.0	490	1.3～2.7
(B)BBNN 64–I	1	4n	2	52.5	670	2.63±0.02
	2	4n	2	54.0	621	2.43±0.01
	3	4n	2	50.0	439	2.35±0.01
	4	4n	2	47.5	670	2.29±0.01
	5	4n	2	55.5	746	2.30±0.01
	6	4n	2	57.0	810	2.28±0.01
	7	4n	2	53.0	619	2.39±0.01
	8	4n	2	45.0	605	2.05±0.01
	9	4n	4	63.0	916	2.35±0.01
(B)BBNN 64–II	10	4n	4	61.5	640	2.43±0.01
(B)BBNN 64–III	11	4n	4	62.5	619	2.48±0.01

50 eggs of which were 1.3～3.2 mm in diameter. These eggs were characteristic in that they distinctly varied in size. The jelly envelope was similar to that of the hybrids in hardness.

Eight 2-year-old female amphidiploids, (B)BBNN 64-I♀, Nos. 1～8, were 45.0～57.0 mm, 51.8 mm on the average in body length, and laid 439～810 eggs, 647.5 eggs on the average, 50 eggs of which were 2.05～2.63 mm in mean diameter, 2.08 mm on the whole. Three 4-year-old female amphidiploids, (B)BBNN 64-I～III♀, Nos. 9～11, were 61.5～63.0 mm in body length and laid 619～916 eggs, 725.0 eggs on the average, 50 eggs of which were 2.35～2.48 mm in mean diameter,

2.42 mm on the whole. It was evident that the female amphidiploids usually laid eggs which were distinctly fewer but remarkably larger than those laid by the females of the two species. The eggs of the amphidiploids were almost uniform in size in contrast to those of the diploid hybrids and allotriploids (Table 4). The jelly envelope became sticky more quickly than those of *Rana nigromaculata* and *Rana brevipoda* eggs. Owing to this nature of the jelly envelope, the eggs of the amphidiploids were more rapidly reduced in fertilization capacity than those of the two species.

2. Male amphidiploids

Male *Rana brevipoda* were somewhat smaller than male *Rana nigromaculata*. Two 2-year-old male *Rana brevipoda* were 37.0 mm and 40.5 mm and one 4-year-old male *Rana brevipoda* was 45.5 mm in body length, while two 2-year-old male *Rana nigromaculata* were 46.5 mm and 52.0 mm and one 4-year-old male *Rana nigromaculata* was 55.0 mm (Table 5). The testes of *Rana brevipoda* were somewhat smaller and more roundish than those of *Rana nigromaculata* which were rather long and ellipsoidal in shape.

Two 2-year-old male hybrids, (B)BN, were 45.5 mm and 47.0 mm and one 4-year-old male hybrid, (B)BN, was 52.0 mm in body length. The testes of these male hybrids were distinctly smaller than those of the two species, while they seemed to be intermediate between the two species in shape.

Two 2-year-old male allotriploids, (B)BNN, were 47.5 mm and 48.0 mm and one 4-year-old male allotriploid, (B)BNN, was 53.5 mm in body length. The

TABLE 5

Size of testes in males of diploid *Rana nigromaculata* or *Rana brevipoda*, diploid hybrids, allotriploids and amphidiploids

Kind	Individual no.	Ploidy	Age (years)	Body length (mm)	Size of testes	
					Left (mm)	Right (mm)
(N)NN 64–I	1	2n	2	46.5	5.0×3.5	5.0×3.5
	2	2n	2	52.0	6.0×3.5	5.5×3.5
	3	2n	4	55.0	6.0×4.0	6.0×4.0
(B)BB 64–I	1	2n	2	37.0	3.5×3.0	4.0×3.5
	2	2n	2	40.5	4.5×3.5	4.0×3.5
	3	2n	4	45.5	5.0×4.5	4.5×4.5
(B)BN 64–I	1	2n	2	45.5	3.0×2.5	—
	2	2n	2	47.0	3.5×3.0	—
	3	2n	4	52.0	4.0×2.5	—
(B)BNN 64–I	1	3n	2	47.5	4.0×3.0	—
	2	3n	2	48.0	4.5×3.5	—
	3	3n	4	53.5	5.5×4.0	—
(B)BBNN 64–I	1	4n	2	48.5	5.0×4.0	5.0×4.0
	2	4n	4	53.5	5.5×4.5	5.5×4.5
(B)BBNN 64–II	3	4n	4	53.5	5.5×5.0	5.0×4.5
	4	4n	4	54.5	6.0×5.0	—
(B)BBNN 64–III	5	4n	4	55.0	6.0×5.0	—
	6	4n	4	53.0	5.5×4.5	—

testes of these male allotriploids seemed to be small for their body length but larger than those of the hybrids. They were intermediate between the two species in shape.

One 2-year-old male amphidiploid, (B)BBNN, was 48.5 mm and five 4-year-old male amphidiploids, (B)BBNN, were 53.0~55.0 mm, 53.9 mm on the average, in body length. Although they were similar to the allotriploids in body length, their testes were evidently larger than those of the latter. The testes of the male amphidiploids were rather similar to those of *Rana brevipoda* in shape (Table 5).

The inner structure of the testes of male amphidiploids is almost completely normal. Many bundles of spermatozoa which were normal in shape but remarkably larger than those of diploid male *Rana nigromaculata* and *Rana brevipoda* were found in the seminiferous tubules. The first meiotic figures contained mostly 26 bivalent chromosomes. These findings remarkably differed from those in diploid male hybrids, (B)BN, and allotriploids, (B)BNN. The testes of the diploid hybrids and allotriploids contained no normal spermatozoa, but pycnotic nuclei and large deformed spermatozoa were distributed sparsely in the seminiferous tubules.

IV. Reproductive capacity of amphidiploids and ploidy of the offspring

The reproductive capacity of male and female amphidiploids produced in 1964 was examined in 1966 and 1968 by mating experiments within themselves as well as with male and female diploid *Rana brevipoda* and *Rana nigromaculata,* and by gynogenesis. All the frogs used in these mating experiments are presented in Tables 4 and 5.

1. Mating experiments in 1966

a. Mating with female *Rana nigromaculata*

In the breeding season of 1966, a male amphidiploid, (B)BBNN 64-I♂, No. 1, which had been produced in 1964 from a diploid female *Rana brevipoda,* (B)BB, and an autotetraploid male *Rana nigromaculata,* (N)NNNN 64-I♂, No. 1, was mated with two diploid female *Rana nigromaculata,* (N)NN 64-I♀, Nos. 1 and 2 produced in 1964. The results showed that 33 (24.6%) of 134 eggs cleaved normally, while nine others cleaved abnormally. The normally cleaved eggs developed almost normally and 32 (23.9%) became normally feeding tadpoles which were more than 30 mm in total length. By examining the chromosomes in the tail-tips of these tadpoles, it was found that all of them were triploids (Table 6).

b. Mating with female *Rana brevipoda*

The above male amphidiploid, (B)BBNN 64-I♂, No. 1, was mated with two diploid female *Rana brevipoda,* (B)BB 64-I♀, Nos. 1 and 2, produced in 1964. Of 114 eggs, 41 (36.0%) cleaved normally, while 19 others cleaved abnormally. Although six and four of the normally cleaved eggs died of edema, microcephaly or some other abnormalities at the tail-bud and the hatching stage, 31 (27.2%) hatched normally and became normally feeding tadpoles which were more than

TABLE 6

Developmental capacity and ploidy of the offspring of male and female amphidiploids. Experiments in 1966

Parents		No. of eggs	No. of normal cleavages	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of analyzed tadpoles	Kind of ploidy		
Female	Male							3n	4n	Mosaics, etc.
(N)NN 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	134	33 (24.6%)	33 (24.6%)	32 (23.9%)	32 (23.9%)	32	32	0	0
(B)BB 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	114	41 (36.0%)	35 (30.7%)	31 (27.2%)	31 (27.2%)	31	31	0	0
(B)BBNN 64–I, No. 1	(N)NN 64–I, No. 1	40	29 (72.5%)	25 (62.5%)	22 (55.0%)	22 (55.0%)	22	22	0	0
	(B)BB 64–I, No. 1	55	44 (80.0%)	32 (58.2%)	23 (41.8%)	23 (41.8%)	23	23	0	0
	(B)BBNN 64–I, No. 1	231	132 (57.1%)	89 (38.5%)	61 (26.4%)	59 (25.5%)	59	0	57	2
(B)BBNN 64–I, No. 2	(N)NN 64–I, No. 1	97	41 (42.3%)	35 (36.1%)	29 (29.9%)	25 (25.8%)	25	25	0	0
	(B)BB 64–I, No. 1	101	47 (46.5%)	43 (42.6%)	38 (37.6%)	38 (37.6%)	38	38	0	0
	(B)BBNN 64–I, No. 1	243	75 (30.9%)	67 (27.6%)	62 (25.5%)	62 (25.5%)	62	0	62	0
(B)BBNN 64–I, No. 3	(N)NN 64–I, No. 2	76	60 (78.9%)	40 (52.6%)	31 (40.8%)	27 (35.5%)	27	27	0	0
	(B)BB 64–I, No. 2	77	62 (80.5%)	35 (45.5%)	29 (37.7%)	25 (32.5%)	25	25	0	0
	GD	283	75 (26.5%)	41 (14.5%)	35 (12.4%)	24 (8.5%)	24	0	17	7
(B)BBNN 64–I, No. 4	(N)NN 64–I, No. 2	80	41 (51.3%)	29 (36.3%)	21 (26.3%)	17 (21.3%)	17	17	0	0
	(B)BB 64–I, No. 2	59	43 (72.9%)	24 (40.7%)	20 (33.9%)	16 (27.1%)	16	16	0	0
	GD	381	67 (17.6%)	40 (10.5%)	21 (5.5%)	18 (4.7%)	18	0	16	2
(B)BBNN 64–I, No. 5	(N)NN 64–I, No. 2	56	24 (42.9%)	17 (30.4%)	15 (26.8%)	15 (26.8%)	15	15	0	0
	(B)BB 64–I, No. 2	36	5 (13.9%)	5 (13.9%)	5 (13.9%)	5 (13.9%)	5	5	0	0
	GD	390	86 (22.1%)	63 (16.2%)	47 (12.1%)	35 (9.0%)	35	0	29	6
(B)BBNN 62–I, No. 6	(N)NN 64–I, No. 2	81	60 (74.1%)	54 (66.7%)	54 (66.7%)	54 (66.7%)	54	54	0	0
	(B)BB 64–I, No. 2	103	90 (87.4%)	53 (51.5%)	53 (51.5%)	53 (51.5%)	53	53	0	0
	GD	410	81 (19.8%)	67 (16.3%)	47 (11.5%)	32 (7.8%)	32	0	27	5
(B)BBNN 64–I, No. 7	(N)NN 64–I, No. 2	50	41 (82.0%)	25 (50.0%)	25 (50.0%)	25 (50.0%)	25	25	0	0
	(B)BB 64–I, No. 2	62	53 (85.5%)	21 (33.9%)	21 (33.9%)	21 (33.9%)	21	21	0	0
	GD	361	50 (13.9%)	35 (9.7%)	29 (8.0%)	25 (6.9%)	25	0	25	0
(B)BBNN 64–I, No. 8	(N)NN 64–I, No. 2	69	38 (55.1%)	23 (33.3%)	23 (33.3%)	23 (33.3%)	23	23	0	0
	(B)BB 64–I, No. 2	90	37 (41.1%)	20 (22.2%)	20 (22.2%)	20 (22.2%)	20	20	0	0
	GD	279	46 (16.5%)	27 (9.7%)	25 (9.0%)	20 (7.2%)	20	0	15	5

GD, Gynogenesis and refrigeration of eggs

30 mm in total length. By examining the chromosomes of these tadpoles in the tail-tips, it was found that all of them were triploids (Table 6).

c. Mating with male *Rana nigromaculata*

Eight female amphidiploids, (B)BBNN 64-I♀, Nos. 1～8, which had been produced in 1964 from *Rana brevipoda* eggs by subjecting to cold after insemination with sperm of an autotetraploid male *Rana nigromaculata,* (N)NNNN 62-I♂, No. 1, were mated with two diploid male *Rana nigromaculata,* (N)NN 64-I♂, Nos. 1 and 2, produced in 1964. The results showed that 334 (60.8%) of 549 eggs obtained from the eight female amphidiploids cleaved normally, while 54 others cleaved abnormally. Of the normally cleaved eggs, 8, 54, 24 and 28 died of edema or some other abnormalities at the blastula, the gastrula, the tail-bud and the hatching stage, respectively. After 12 others died of edema or underdevelopment shortly after hatching, 208 (37.9%) became normally feeding tadpoles which were more than 30 mm in total length. It was found that all these tadpoles were triploids, when chromosomes were examined in the tail-tips (Table 6).

d. Mating with male *Rana brevipoda*

The above eight female amphidiploids, (B)BBNN 64-I♀, Nos. 1～8, were mated with two diploid male *Rana brevipoda,* (B)BB 64-I♂, Nos. 1 and 2, produced in 1964. Of 583 eggs, 381 (65.4%) cleaved normally, while 46 others cleaved abnormally. Thereafter, 117, 31 and 24 of the normally cleaved eggs died of edema, microcephaly, underdevelopment or some other abnormalities at the gastrula, the tail-bud and the hatching stage, respectively. After eight others died of edema or underdevelopment shortly after hatching, 201 (34.5%) became normally feeding tadpoles which were more than 30 mm in total length. All these feeding tadpoles were triploids when chromosomes were examined in the tail-tips (Table 6).

e. Mating of male and female amphidiploids.

Two of the above eight female amphidiploids, (B)BBNN 64-I♀, Nos. 1 and 2, were mated with the above male amphidiploid, (B)BBNN 64-I♂, No. 1. The results showed that 207 (43.7%) of 474 eggs cleaved normally, while 16 others cleaved abnormally. After 10, 41 and 33 of the normally cleaved eggs died of various abnormalities at the gastrula, the tail-bud and the hatching stage, respectively, 123 hatched normally. While two of them died shortly after hatching, the other 121 (25.5%) became normally feeding tadpoles which were more than 30 mm in total length. By examining the chromosomes of these tadpoles in the tail-tips, it was found that 119 (98.3%) were amphidiploids, another was a 2n-4n mosaic and the remainder was a 2n-6n mosaic (Table 6).

f. Gynogenesis from female amphidiploids

In the breeding season of 1966, 2104 eggs obtained from six of the above eight female amphidiploids, (B)BBNN 64-I♀, Nos. 3～8, were subjected to 1～2°C for 2.5 hours, 20～25 minutes after the eggs were inseminated with UV-irradiated sperm of *Rana nigromaculata.* The results showed that 405 (19.2%) cleaved normally, while 774 others cleaved abnormally. Of the normally cleaved eggs,

38, 94 and 69 died of edema, underdevelopment or some other abnormalities at the gastrula, the tail-bud and the hatching stage, respectively. Shortly after hatching, 50 tadpoles died of edema or underdevelopment. Eventually, 154 (7.3%) became normally feeding tadpoles which were more than 30 mm in total length.

By examining chromosomes of these tadpoles in the tail-tips, it was found that 129 (83.8%) were amphidiploids, (B)BBNN, one was a hexaploid, 15 were mosaics and the remaining nine were aneuploids. Of the mosaics, 14 were 2n-4n and the remainder was a 2n-6n. While one of the aneuploids was a hypertetraploid having 53 chromosomes, the other eight were hypertriploids; two were 40, two were 41, two were 42, one was 43 and the remainder was 44 in chromosome number (Table 6).

2. Mating experiments in 1968

a. Mating with a female *Rana nigromaculata*

In the breeding season of 1968, eggs of a diploid female *Rana nigromaculata,* (N)NN 64-I♀, No. 3, produced in 1964 were inseminated with sperm of a 4-year-old male amphidiploid, (B)BBNN 64-II♂, No. 3, produced in 1964. The results showed that only 24 (16.4%) of 146 eggs cleaved normally. Thereafter, eight, two and two of the normally cleaved eggs became abnormal and died at the tail-bud, the hatching and the earliest tadpole stage, respectively, while 12 (8.2%) became normally feeding tadpoles which were more than 30 mm in total length. These tadpoles were all triploids, (N)NNB, when their chromosomes were examined in the tail-tips (Table 7).

TABLE 7

Developmental capacity and ploidy of the offspring of male and female amphidiploids. Experiments in 1968

Parents		No. of eggs	No. of normal cleavages	No. of normal tail-bud embryos	No. of normally hatched tadpoles	No. of normally feeding tadpoles	No. of analyzed tadpoles	Kind of ploidy		
Female	Male							3n	4n	Mosaics, etc.
(N)NN 64–I, No. 3	(B)BBNN 64–II, No. 3	146	24 (16.4%)	16 (11.0%)	14 (9.6%)	12 (8.2%)	12	12	0	0
(B)BB 64–I, No. 3	(B)BBNN 64–II, No. 3	127	56 (44.1%)	53 (41.7%)	50 (39.4%)	47 (37.0%)	47	46	0	1
(B)BBNN 64–I, No. 9	(N)NN 64–I, No. 3	87	63 (72.4%)	59 (67.8%)	53 (60.9%)	47 (54.0%)	46	46	0	0
	(B)BB 64–I, No. 3	121	102 (84.3%)	98 (81.0%)	83 (68.6%)	76 (62.8%)	74	72	0	2
	(B)BBNN 64–II, No. 3	604	465 (77.0%)	414 (68.5%)	316 (52.3%)	292 (48.3%)	288	0	285	3
(B)BBNN 64–II, No. 10	(N)NN 64–I, No. 3	25	14 (56.0%)	12 (48.0%)	7 (28.0%)	7 (28.0%)	7	7	0	0
	(B)BB 64–I, No. 3	39	28 (71.8%)	25 (64.1%)	15 (38.5%)	15 (38.5%)	15	15	0	0
	(B)BBNN 64–II, No. 3	377	326 (86.5%)	313 (83.0%)	300 (79.6%)	298 (79.0%)	291	0	286	5
(B)BBNN 64–III, No. 11	(N)NN 64–I, No. 3	54	30 (55.6%)	22 (40.7%)	19 (35.2%)	19 (35.2%)	19	19	0	0
	(B)BB 64–I, No. 3	59	36 (61.0%)	29 (49.2%)	24 (40.7%)	24 (40.7%)	24	24	0	0
	(B)BBNN 64–II, No. 3	401	292 (72.8%)	286 (71.3%)	281 (70.1%)	273 (68.1%)	268	0	266	2

b. Mating with a female *Rana brevipoda*

A total of 127 eggs of a diploid female *Rana brevipoda,* (B)BB 64-I♀, No. 3, produced in 1964 were inseminated with sperm of the above male amphidiploid, (B)BBNN 64-II♂, No. 3. The results showed that 56 (44.1%) cleaved normally and 47 (37.0%) became normally feeding tadpoles which were more than 30 mm in total length, while three became edematous and one each died at the tail-bud, the hatching and the earliest tadpole stage. When the chromosomes of the feeding tadpoles were examined in the tail-tips, it was found that 46 of them were triploids, (B)BBN, and the remainder was a diploid (Table 7).

c. Mating with a male *Rana nigromaculata*

Three 4-year-old female amphidiploids, (B)BBNN 64-I~III♀, Nos. 9~11, were matcd with a diploid male *Rana nigromaculata* obtained in 1964. Of 166 eggs obtained from the three females, 107 (64.5%) cleaved normally, while 24 others cleaved abnormally. Of the normally cleaved eggs, 14, 14 and 6 became abnormal and died of edema or underdevelopment at the embryonic, the hatching and the earliest tadpole stage, respecitvely. Eventually, 73 (44.0%) became normally feeding tadpoles which were more than 30 mm in total length. When the chromosomes of these tadpoles were examined in the tail-tips, it was found that 72 of them were all triploids, (B)BNN, while the ploidy of the remainder was not determined, owing to paucity of analyzable mitotic figures (Table 7).

d. Mating with a male *Rana brevipoda*

A total of 219 eggs obtained from the above three female amphidiploids, (B)BBNN 64-I~III♀, Nos. 9~11, were inseminated with sperm of a male diploid *Rana brevipoda,* (B)BB 64-I♂, No. 3, produced in 1964. The results showed that 166 (75.8%) eggs cleaved normally, while 26 others cleaved abnormally. Of the normally cleaved eggs, 115 (52.5%) became normally feeding tadpoles which were more than 30 mm in total length, while 14, 30 and 7 became abnormal and died of edema or underdevelopment at the embryonic, the hatching and the earliest tadpole stage, respectively.

When the chromosomes of the 115 feeding tadpoles were examined in the tail-tips, it was found that 111 (96.5%) were triploids, (B)BBN, and two were pentaploids, while the remaining two were unknown, owing to paucity of analyzable mitotic figures (Table 7).

e. Mating of male and female amphidiploids

The above three female amphidiploids, (B)BBNN 64-I~III♀, Nos. 9~11, were mated with the above male amphidiploids, (B)BBNN 64-II♂, No. 3. Of a total of 1382 eggs, 1083 (78.4%) cleaved normally, while 20 others cleaved abnormally. Some of the normally cleaved eggs showed incomplete invagination at the gastrula stage and became abnormal neurulae. While 70, 116 and 34 died of edema, underdevelopment or some other abnormalities at the tail-bud, the hatching and

the earliest tadpole stage, respectively, 863 (62.4%) became normally feeding tadpoles.

When the chromosomes of these tadpoles were examined in the tail-tips, it was found that 837 (97.0%) of them were tetraploids, (B)BBNN, two were hexaploids,

TABLE 8

Growth of allotriploid and amphidiploid offspring produced from amphidiploid parents. Experiments in 1966

Parents		No. of mitoses	Ploidy	Constitution	No. of tadpoles	Mean total length of 50-day-old tadpoles (mm)	Period of tadpole stage (days)	No. of metamorphosed frogs	Mean body length soon after metamorphosis (mm)
Female	Male								
(N)NN 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	3~5	3n	(N)NNB	32	69.4 ± 0.2	65~82 (73.6)	31	18.2 ± 0.3
(B)BB 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	3~5	3n	(B)BBN	31	66.0 ± 0.2	70~84 (74.0)	30	19.6 ± 0.4
(B)BBNN 64–I, No. 1	(N)NN 64–I, No. 1	3~6	3n	(B)BNN	22	69.5 ± 0.3	65~80 (73.4)	21	18.1 ± 0.2
	(B)BB 64–I, No. 1	4~6	3n	(B)BBN	23	68.7 ± 0.3	69~82 (74.2)	21	18.6 ± 0.2
	(B)BBNN 64–I, No. 1	4~6	4n	(B)BBNN	57	69.0 ± 0.3	65~90 (76.1)	52	19.3 ± 0.2
(B)BBNN 64–I, No. 2	(N)NN 64–I, No. 1	3~5	3n	(B)BNN	25	65.3 ± 0.3	68~81 (74.3)	23	17.7 ± 0.2
	(B)BB 64–I, No. 1	3~5	3n	(B)BBN	38	66.0 ± 0.3	66~80 (76.8)	37	18.3 ± 0.3
	(B)BBNN 64–I, No. 1	3~6	4n	(B)BBNN	62	67.3 ± 0.2	66~87 (76.8)	59	19.0 ± 0.3
(B)BBNN 64–I, No. 3	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	27	67.4 ± 0.5	60~76 (70.2)	25	17.3 ± 0.4
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	25	66.4 ± 0.4	63~83 (68.5)	25	17.6 ± 0.4
	GD	5~7	4n	(B)BBNN	17	65.3 ± 0.7	60~77 (73.3)	15	17.1 ± 0.4
(B)BBNN 64–I, No. 4	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	17	65.1 ± 0.4	65~75 (71.2)	17	17.4 ± 0.4
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	16	64.3 ± 0.5	61~72 (69.6)	14	18.0 ± 0.5
	GD	4~6	4n	(B)BBNN	16	64.1 ± 0.9	60~74 (67.2)	12	17.6 ± 1.2
(B)BBNN 64–I, No. 5	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	15	63.7 ± 0.4	65~70 (67.5)	13	17.5 ± 0.3
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	5	63.5~66.0 (64.7)	60~73 (68.0)	4	17.0~19.5 (17.9)
	GD	3~6	4n	(B)BBNN	29	65.3 ± 0.6	61~74 (71.4)	22	18.4 ± 0.3
(B)BBNN 64–I, No. 6	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	54	65.8 ± 0.7	62~76 (73.1)	54	17.4 ± 0.4
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	53	63.6 ± 0.7	60~79 (72.5)	52	17.3 ± 0.4
	GD	3~6	4n	(B)BBNN	27	69.2 ± 0.4	63~74 (71.2)	27	18.1 ± 0.2
(B)BBNN 64–I, No. 7	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	25	66.0 ± 0.5	60~71 (68.6)	25	17.7 ± 0.2
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	21	63.4 ± 0.4	61~75 (70.0)	21	17.5 ± 0.2
	GD	3~5	4n	(B)BBNN	25	67.1 ± 0.4	67~73 (69.5)	23	18.4 ± 0.2
(B)BBNN 64–I, No. 8	(N)NN 64–I, No. 2	3~5	3n	(B)BNN	23	67.3 ± 0.5	62~70 (67.3)	20	17.0 ± 0.3
	(B)BB 64–I, No. 2	3~5	3n	(B)BBN	20	64.7 ± 0.8	60~72 (67.1)	20	17.0 ± 0.4
	GD	3~7	4n	(B)BBNN	15	65.2 ± 0.3	65~76 (72.3)	15	17.8 ± 0.2

three were 2n-4n mosaics, four were 2n-6n mosaics and the remainder was a hypertriploid having 42 chromosomes. The chromosomes of each mosaic tadpole were observed in more than six mitotic figures. The resting nuclei of each mosaic tadpole were evidently divided into two groups in size. The ploidy of the remaining 16 tadpoles was not determined, owing to paucity of analyzable mitotic figures (Table 7).

V. Growth and sex of the offspring of amphidiploids

1. Offspring produced in 1966

a. Offspring of female *Rana nigromaculata* and a male amphidiploid

Thirty-two triploids, (N)NNB, produced from two diploid female *Rana nigromaculata*, (N)NN 64-I♀, Nos. 1 and 2, by mating with a male amphidiploid, (B)BBNN 64-I♂, No. 1, were continuously reared. They were 69.4 mm in mean total length at the age of 50 days (Table 8). Thereafter, 31 of them com-

TABLE 9

Sex of allotriploid and amphidiploid offspring produced from

Parents		Ploidy	Constitution	No. of metamorphosed frogs
Female	Male			
(N)NN 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	3n	(N)NNB	31
(B)BB 64–I, Nos. 1, 2	(B)BBNN 64–I, No. 1	3n	(B)BBN	30
(B)BBNN 64–I, No. 1	(N)NN 64–I, No. 1	3n	(B)BNN	21
	(B)BB 64–I, No. 1	3n	(B)BBN	21
	(B)BBNN 64–I, No. 1	4n	(B)BBNN	52
(B)BBNN 64–I, No. 2	(N)NN 64–I, No. 1	3n	(B)BNN	23
	(B)BB 64–I, No. 1	3n	(B)BBN	37
	(B)BBNN 64–I, No. 1	4n	(B)BBNN	59
(B)BBNN 64–I, No. 3	(N)NN 64–I, No. 2	3n	(B)BNN	25
	(B)BB 64–I, No. 2	3n	(B)BBN	25
	GD	4n	(B)BBNN	15
(B)BBNN 64–I, No. 4	(N)NN 64–I, No. 2	3n	(B)BNN	17
	(B)BB 64–I, No. 2	3n	(B)BBN	14
	GD	4n	(B)BBNN	12
(B)BBNN 64–I, No. 5	(N)NN 64–I, No. 2	3n	(B)BNN	13
	(B)BB 64–I, No. 2	3n	(B)BBN	4
	GD	4n	(B)BBNN	22
(B)BBNN 64–I, No. 6	(N)NN 64–I, No. 2	3n	(B)BNN	54
	(B)BB 64–I, No. 2	3n	(B)BBN	52
	GD	4n	(B)BBNN	27
(B)BBNN 64–I, No. 7	(N)NN 64–I, No. 2	3n	(B)BNN	25
	(B)BB 64–I, No. 2	3n	(B)BBN	21
	GD	4n	(B)BBNN	23
(B)BBNN 64–I, No. 8	(N)NN 64–I, No. 2	3n	(B)BNN	20
	(B)BB 64–I, No. 2	3n	(B)BBN	20
	GD	4n	(B)BBNN	15

♀$_{N1}$, Females with normal ovaries ♀$_{N2}$, Females with somewhat underdeveloped ovaries

pleted metamorphosis at the age of 65～82 days, 73.6 days on the average. When measured immediately after metamorphosis, they were 18.2 mm in body length. Within 3 weeks after metamorphosis, 21 of them were killed to examine the sex. It was found that two were females with somewhat underdeveloped ovaries, nine were females with distinctly underdeveloped ovaries, six were hermaphrodites and the remaining four were males. The other 10 living triploids attained sexual maturity. Four of them were females and the other six were males. When the hermaphrodites were counted as males, 15 of the 31 frogs in total were females and the other 16 (51.6%) were males (Table 9).

b. Offspring of female *Rana brevipoda* and a male amphidiploid

Thirty-one triploids, (B)BBN, produced from two diploid female *Rana brevipoda,* (B)BB 64-I♀, Nos. 1 and 2, by mating with the above male amphidiploid, (B)BBNN 64-I♂, No. 1, were 66.0 mm in mean total length at the age of 50 days (Table 8). Of these triploids, 30 completed metamorphosis at the age of 70～84 days, 74.0 days on the average. They were 19.6 mm in mean body length

amphidiploid parents. Experiments in 1966

Sex of frogs dead or killed within three months after metamorphosis						Sex of frogs, one or two years old			Sex of all frogs examined			
Total	♀$_{N1}$	♀$_{N2}$	♀$_{U}$	♀	♂	Total	♀	♂	Total	♀	♂	(%)
21	0	2	9	6	4	10	4	6	31	15	16	(51.6)
20	0	3	9	7	1	10	5	5	30	17	13	(43.3)
11	0	3	2	0	6	10	6	4	21	11	10	(47.6)
12	2	4	3	1	2	9	4	5	21	13	8	(38.1)
37	17	4	5	3	8	15	6	9	52	32	20	(38.5)
12	0	1	4	0	7	10	4	6	22	9	13	(59.1)
26	0	4	15	3	4	9	5	4	35	24	11	(31.4)
33	19	5	2	2	5	22	9	13	55	35	20	(36.4)
25	0	3	10	0	12	—	—	—	25	13	12	(48.0)
25	0	2	8	0	15	—	—	—	25	10	15	(60.0)
5	0	4	1	0	0	10	7	3	15	12	3	(20.0)
17	0	2	6	0	9	—	—	—	17	8	9	(52.9)
14	0	2	4	0	8	—	—	—	14	6	8	(57.1)
4	0	4	0	0	0	8	7	1	12	11	1	(8.3)
13	0	1	6	0	6	—	—	—	13	7	6	(46.2)
4	0	0	2	0	2	—	—	—	4	2	2	(50.0)
11	8	1	2	0	0	10	10	0	21	21	0	
27	0	7	8	3	9	27	14	13	54	29	25	(46.3)
26	0	6	8	2	10	26	13	13	52	27	25	(48.1)
12	7	2	2	1	0	15	14	1	27	25	2	(7.4)
25	0	5	6	0	14	—	—	—	25	11	14	(56.0)
21	0	2	8	0	11	—	—	—	21	10	11	(52.4)
15	7	3	3	1	1	8	6	2	23	19	4	(17.4)
5	0	0	2	0	3	15	6	9	20	8	12	(60.0)
5	0	0	3	0	2	15	7	8	20	10	10	(50.0)
13	10	1	2	0	0	2	1	1	15	14	1	(6.7)

♀$_{U}$, Females with remarkably underdeveloped ovaries

immediately after metamorphosis. When 20 of them were killed to examine the sex within 3 weeks after metamorphosis, it was found that three were females with somewhat underdeveloped ovaries, nine were females with distinctly underdeveloped ovaries, seven were hermaphrodites, and the remainder was a male. The other 10 living triploids consisted of five females and five males when they sexually matured. When the hermaphrodites were counted as males, 17 of the 30 triploid frogs in total were females and the other 13 (43.3%) were males (Table 9).

c. Offspring of female amphidiploids and male *Rana nigromaculata*

Two hundred and eight triploids, (B)BNN, produced from eight female amphidiploids, (B)BBNN 64-I♀, Nos. 1~8, by mating with two diploid male *Rana nigromaculata*, (N)NN 64-I♂, Nos. 1 and 2, were continuously reared. At the age of 50 days, they were 63.7~69.5 mm in mean body length in eight experimental series (Table 8). Of these tadpoles, 198 completed metamorphosis at the age of 67.3~74.3 days on the average in the eight series, 60~81 days on the whole. They were 17.0~18.1 mm in mean body length when measured immediately after metamorphosis.

Of the 198 metamorphosed frogs, 135 were killed or died within 3 months after metamorphosis. When the gonads of these frogs were observed, it was found that 22 were females with somewhat underdeveloped ovaries, 44 were females with remarkably underdeveloped ovaries, three were hermaphrodites and the remaining 66 were males. It was evident that the gonads of the triploids were considerably retarded in differentiation as compared with those of the control diploid *Rana nigromaculata* and *Rana brevipoda*. Of the other 63 metamorphosed frogs reared continuously, 62 attained sexual maturity. They consisted of 30 females and 32 males. When the hermaphrodites were counted as males, 96 of the 197 triploids in total were females and the other 101 (51.3%) were males (Table 9).

d. Offspring of female amphidiploids and male *Rana brevipoda*

Two hundred and one triploids, (B)BBN, produced from eight female amphidiploids, (B)BBNN 64-I♀, Nos. 1~8, by mating with two diploid male *Rana brevipoda*, (B)BB 64-I♂, Nos. 1 and 2, were continuously reared. At the age of 50 days, they were 63.4~68.7 mm in mean total length in eight experimental series (Table 8). Thereafter, 194 tadpoles completed metamorphosis at the age of 67.1~76.8 days on the average in the eight series, 60~83 days on the whole. When measured immediately after metamorphosis, they were 17.0~18.6 mm in mean body length. Of these metamorphosed triploids, 133 were killed or died within 3 months after metamorphosis. When their sex was examined, it was found that two were females with normal ovaries, 20 were females with somewhat underdeveloped ovaries, 51 were females with remarkably underdeveloped ovaries, six were hermaphroidtes and the remaining 54 were males. Of the other 61 metamorphosed triploids which were reared continuously, 59 attained sexual maturity. Of these mature frogs, 29 were females and the other 30 were males. When the hermaphrodites were counted as males, 102 of the 192 triploids in total

were females and the other 90 (46.9%) were males (Table 9).

e. Offspring of male and female amphidiploids

One hundred and nineteen amphidiploids, (B)BBNN, produced from matings between two female amphidiploids, (B)BBNN 64-I♀, Nos. 1 and 2, and a male amphidiploid, (B)BBNN 64-I♂, No. 1, were continuously reared. They were 69.0 mm and 67.3 mm in mean total length at the age of 50 days in two experimental series (Table 8). Thereafter, 111 of these tadpoles completed metamorphosis at the age of 76.1 and 76.8 days on the average in the two series, 65～90 days on the whole. When measured immediately after metamorphosis, they were 19.3 mm and 19.0 mm in mean body length.

Of the metamorphosed amphidiploids, 74 were killed or died within 3 months after metamorphosis. The sex was not determined in four of them, owing to postmortem changes of the gonads. Of the other 70, 36 were females with normal ovaries, nine were females with somewhat underdeveloped ovaries, seven were females with remarkably underdeveloped ovaries, five were hermaphrodites and the remaining 13 were males. The other 37 living amphidiploids attained sexual maturity. They consisted of 15 females and 22 males. When the hermaphrodites were counted as males, 67 of the 107 amphidiploids in total were females, while the other 40 (37.4%) were males (Table 9).

f. Offspring produced from female amphidiploids by gynogenesis

One hundred and twenty-nine amphidiploids raised from eggs of six female amphidiploids, (B)BBNN 64-I♀, Nos. 3～8, by subjecting to 1～2°C for 2.5 hours after insemination with UV-irradiated sperm of *Rana nigromaculata* were continuously reared. They were 64.1～69.2 mm in mean total length at the age of 50 days in six experimental series (Table 8). Of these tadpoles, 114 completed metamorphosis at the age of 67.2～73.3 days on the average in the six series, 60～77 days on the whole. When measured immediately after metamorphosis, they were 17.1～18.4 mm in mean body length.

Of these metamorphosed amphidiploids, 60 were killed or died within three months after metamorphosis. When their gonads were examined, it was found that 32 were females with normal ovaries, 15 were females with somewhat underdeveloped ovaries, 10 were females with remarkably underdeveloped ovaries, two were hermaphrodites and the remainder was a male. Of the other 54 living amphidiploids, 53 attained sexual maturity. They consisted of 45 females and 8 males. When the hermaphrodites were counted as males, 102 of the 113 metamorphosed amphidiploids in total were females and the other 11 (9.7%) were males (Table 9).

2. Offspring produced in 1968

a. Offspring of a female *Rana nigromaculata* and a male amphidiploid

Twelve triploids, (N)NNB, produced from a diploid female *Rana nigromaculata*, (N)NN 64-I♀, No. 3, by mating with a male amphidiploid, (B)BBNN 64-II♂,

TABLE 10

Growth of allotriploid and amphidiploid offspring produced from amphidiploid parents. Experiments in 1968

Parents		No. of mitoses	Ploidy	Constitution	No. of tadpoles	Mean total length of 50-day-old tadpoles (mm)	Period of tadpole stage (days)	No. of metamorphosed frogs	Mean body length soon after metamorphosis (mm)
Female	Male								
(N)NN 64–I, No.3	(B)BBNN 64–II, No. 3	3~6	3n	(N)NNB	12	67.4±0.2	55~74 (65.5)	10	17.5±0.1
(B)BB 64–I, No.3	(B)BBNN 64–II, No. 3	4~6	3n	(B)BBN	46	66.1±0.2	56~73 (64.9)	46	17.8±0.2
(B)BBNN 64–I, No. 9	(N)NN 64–I, No. 3	4~6	3n	(B)BNN	46	69.9±0.5	55~77 (63.4)	46	18.3±0.2
	(B)BB 64–I, No. 3	4~7	3n	(B)BBN	72	65.3±0.4	55~88 (65.5)	67	17.1±0.2
	(B)BBNN 64–II, No. 3	5~7	4n	(B)BBNN	285	67.7±0.2	57~98 (66.0)	252	17.9±0.2
(B)BBNN 64–II, No. 10	(N)NN 64–I, No. 3	3~5	3n	(B)BNN	7	67.2±0.5	55~64 (62.0)	7	18.0±0.3
	(B)BB 64–I, No. 3	3~6	3n	(B)BBN	15	65.9±0.4	56~77 (63.7)	15	17.3±0.2
	(B)BBNN 64–II, No. 3	3~8	4n	(B)BBNN	286	68.2±0.3	58~101 (72.4)	280	18.2±0.2
(B)BBNN 64–III, No. 11	(N)NN 64–I, No. 3	3~5	3n	(B)BNN	19	69.1±0.3	55~69 (64.6)	17	18.0±0.2
	(B)BB 64–I, No. 3	4~5	3n	(B)BBN	24	65.5±0.4	55~68 (63.2)	22	17.2±0.2
	(B)BBNN 64–II, No. 3	3~7	4n	(B)BBNN	266	67.6±0.3	59~100 (73.2)	265	17.8±0.2

No. 3, were continuously reared. They were 67.4 mm in mean total length at the age of 50 days. Thereafter, 10 of them completed metamorphosis at the age of 55~74 days, 65.5 days on the average. When measured immediately after metamorphosis, they were 17.5 mm in mean body length (Table 10).

All the 10 metamorphosed triploids were killed about one month after metamorphosis to examine their sex. The results showed that four were females with somewhat underdeveloped ovaries, two were females with remarkably underdeveloped ovaries, one was a hermaphrodite and the remaining three were males.

TABLE 11

Sex of allotriploid and amphidiploid offspring produced from

Parents		Ploidy	Constitution	No. of metamorphosed frogs
Female	Male			
(N)NN 64–I, No. 3	(B)BBNN 64–II, No. 3	3n	(N)NNB	10
(B)BB 64–I, No. 3	(B)BBNN 64–II, No. 3	3n	(B)BBN	46
(B)BBNN 64–I, No. 9	(N)NN 64–I, No. 3	3n	(B)BNN	46
	(B)BB 64–I, No. 3	3n	(B)BBN	67
	(B)BBNN 64–II, No. 3	4n	(B)BBNN	252
(B)BBNN 64–II, No. 10	(N)NN 64–I, No. 3	3n	(B)BNN	7
	(B)BB 64–I, No. 3	3n	(B)BBN	15
	(B)BBNN 64–II, No. 3	4n	(B)BBNN	280
(B)BBNN 64–III, No. 11	(N)NN 64–I, No. 3	3n	(B)BNN	17
	(B)BB 64–I, No. 3	3n	(B)BBN	22
	(B)BBNN 64–II, No. 3	4n	(B)BBNN	265

♀$_{N1}$, Females with normal ovaries ♀$_{N2}$, Females with somewhat underdeveloped ovaries

When the hermaphrodite was counted as a male, six were females and four (40%) were males (Table 11).

b. Offspring of a female *Rana brevipoda* and a male amphidiploid

Forty-six triploids, (B)BBN, produced from a diploid female *Rana brevipoda*, (B)BB 64-I♀, No. 3, by mating with a male amphidiploid, (B)BBNN 64-II♂, No. 3, were 66.1 mm in mean total length at the age of 50 days. These tadpoles all completed metamorphosis at the age of 56～73 days, 64.9 days on the average. When measured immediately after metamorphosis, they were 17.8 mm in mean body length (Table 10).

All the 46 metamorphosed frogs were killed about one month after metamorphosis to examine their sex. It was found that three were females with normal ovaries, 17 were females with somewhat underdeveloped ovaries, four were females with remarkably underdeveloped ovaries, two were hermaphrodites and the remaining 20 were males. When the hermaphrodites were counted as males, 24 were females and 22 (47.8%) were males (Table 11).

c. Offspring of female amphidiploids and a male *Rana nigromaculata*

Seventy-two triploids, (B)BNN, produced from three female amphidiploids, (B) BBNN 64-I～III♀, Nos. 9～11, by mating with a diploid male *Rana nigromaculata*, (N)NN 64-I♂, Nos. 3, were continuously reared in three experimental series. They were 69.9 mm, 67.2 mm and 69.1 mm in mean body length at the age of 50 days in the three series. Thereafter, 70 of them completed metamorphosis at the age of 63.4 days, 62.0 days and 64.6 days on the average, 55～77 days on the whole. When measured immediately after metamorphosis, they were 18.3 mm, 18.0 mm and 18.0 mm in mean body length in the three series (Table 10).

Of the 70 metamorphosed triploids, 40 were killed or died within one month after metamorphosis and thereafter eight others died within three months after

amphidiploid parents. Experiments in 1968

Sex of frogs dead or killed within three months after metamorphosis						Sex of frogs, one or two years old			Sex of all frogs examined		
Total	♀N1	♀N2	♀U	♀	♂	Total	♀	♂	Total	♀	♂ (%)
10	0	4	2	1	3	—	—	—	10	6	4(40.0)
46	3	17	4	2	20	—	—	—	46	24	22(47.8)
31	0	2	12	0	17	10	5	5	41	19	22(53.7)
47	2	4	17	0	24	12	7	5	59	30	29(49.2)
202	34	47	23	3	95	47	24	23	249	128	121(48.6)
5	0	0	4	0	1	2	1	1	7	5	2(28.6)
9	0	0	4	0	5	6	3	3	15	7	8(53.3)
230	55	32	25	7	111	44	20	24	274	132	142(51.8)
12	0	0	6	0	6	5	2	3	17	8	9(52.9)
17	0	0	11	0	6	5	2	3	22	13	9(40.9)
232	62	19	27	4	120	30	17	13	262	125	137(52.3)

♀U, Females with remarkably underdeveloped ovaries

metamorphosis. When the gonads of these 48 triploids were examined, it was found that two were females with somewhat underdeveloped ovaries, 22 were females with remarkably underdeveloped ovaries and the remaining 24 were males. Of the other 22 living triploids, 17 attained sexual maturity. These consisted of eight females and nine males. Thus, of the 65 triploids in total, 32 were females and the other 33 (50.8%) were males (Table 11).

d. Offspring of female amphidiploids and a male *Rana brevipoda*

One hundred and eleven triploids, (B)BBN, produced from three female amphidiploids, (B)BBNN 64-I～III♀, Nos. 9～11, by mating with a diploid male *Rana brevipoda*, (B)BB 64-I♂, No. 3, were continuously reared in three experimental series. They were 65.3 mm, 65.9 mm and 65.5 mm in mean total length at the age of 50 days in the three series. Of these tadpoles, 104 completed metamorphosis at the age of 65.5 days, 63.7 days and 63.2 days on the average, 55～88 days on the whole. When measured immediately after metamorphosis, they were 17.1 mm, 17.3 mm and 17.2 mm in mean body length in the three experimental series (Table 10).

Of the 104 metamorphosed triploids, 74 were killed or died within one month after metamorphosis and thereafter, seven others died within 3 months after metamorphosis. The sex was not determined in eight of these 81 triploids in total, owing to postmortem changes of the gonads. When the sex was examined in the other 73 triploids, it was found that two were females with normal ovaries, four were females with somewhat underdeveloped ovaries, 32 were females with remarkably underdeveloped ovaries and the remaining 35 were males. The other 23 living triploids attained sexual maturity. They consisted of 12 females and 11 males. Thus, 50 of the 96 metamorphosed triploids in total were females and the other 46 (47.9%) were males (Table 11).

e. Offspring of male and female amphidiploids

Eight hundred and thirty-seven amphidiploids, (B)BBNN, produced from matings between three female amphidiploids, (B)BBNN 64-I～III♀, Nos. 9～11, and a male amphidiploid, (B)BBNN 64-II♂, No. 3, were continuously reared in three experimental series. They were 67.7 mm, 68.2 mm and 67.6 mm in mean total length at the age of 50 days in the three series. Thereafter, 797 of the 837 tadpoles completed metamorphosis at the age of 66.0 days, 72.4 days and 73.2 days on the average, 57～101 days on the whole. When measured immediately after metamorphosis, they were 17.9 mm 18.2 mm and 17.8 mm in mean body length in the three series (Table 10).

Of the 797 metamorphosed amphidiploids, 647 were killed or died within one month after metamorphosis and thereafter 29 other amphidiploids died within three months after metamorphosis. In 12 of these 676 metamorphosed frogs in total, the sex was not determined, owing to postmortem changes of the gonads. When the sex was examined in the other 664 amphidiploids, it was found that 151 were females with normal ovaries, 98 were females with somewhat underdeveloped

ovaries, 75 were females with remarkably underdeveloped ovaries, 14 were hermaphrodites and the remaining 326 were males. The other 121 living amphidiploids attained sexual maturity. They consisted of 61 females and 60 males. When the hermaphrodites were counted as males, 385 of the 785 metamorphosed amphidiploids in total were females and the other 400 (51.0%) were males (Table 11).

3. Sex of the offspring produced in 1966 and 1968

The allotriploids and amphidiploids produced in the two breeding seasons of 1966 and 1968 from matings between male or female diploid *Rana nigromaculata* or *Rana brevipoda* and male or female amphidiploids were totalled to determine their sex ratio.

Of 41 (N)NNB allotriploids produced from matings between female diploid

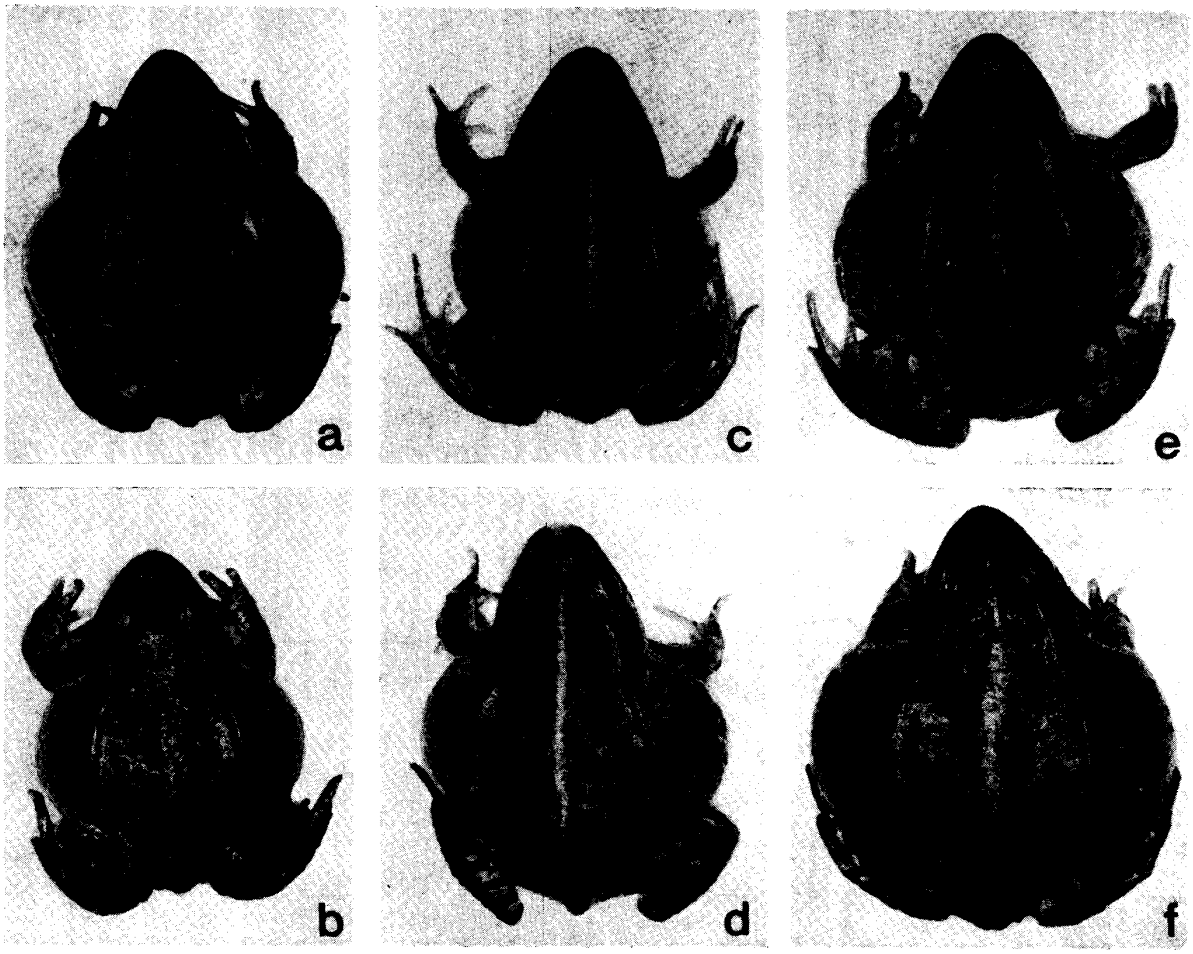

Fig. 9. Diploid *Rana brevipoda* and reciprocal diploid hybrids and reciprocal allotriploids between *Rana nigromaculata* and *Rana brevipoda*. ×0.7

a. Diploid female *Rana brevipoda*, 2 years old
b. Diploid male *Rana brevipoda*, 2 years old
c. Diploid female (N)NB hybrid, 2 years old
d. Diploid female (B)BN hybrid, 2 years old
e. Female (N)NNB allotriploid produced from (N)NN 64-I ♀, No. 1 × (B)BBNN 64-I ♂, No. 1, 3 years old
f. Female (B)BBN allotriploid produced from (B)BB 64-I ♀, No. 1 × (B)BBNN 64-I ♂, No. 1, 3 years old

Rana nigromaculata and male amphidiploids, 21 were females and 20 (48.8%) were males, while 41 were females and 35 (46.1%) were males in a total of 76 (B)BBN allotriploids produced from matings between diploid female *Rana brevipoda* and male amphidiploids.

Of 262 (B)BNN allotriploids produced from female amphidiploids by mating with diploid male *Rana nigromaculata,* 128 were females and 134 (51.1%) were males. Of 288 (B)BBN allotriploids produced from female amphidiploids by mating with diploid male *Rana brevipoda,* 152 were females and 136 (47.2%) were males.

Matings between male and female amphidiploids performed in 1966 and 1968 produced 892 amphidiploids in total. Of these amphidiploids, 452 were females and 440 (49.3%) were males.

The numbers of males and females in the allotriploids or amphidiploids produced from each of the five kinds of matings using amphidiploids as one or both parents all seem to indicate a sex ratio of 1 : 1.

VI. Some characters of allotriploids and amphidiploids produced from amphidiploids

1. External characters

The external characters of three kinds of mature allotriploids, (N)NNB, (B)BNN and (B)BBN, and mature amphidiploids, (B)BBNN, produced in 1966 and 1968 from amphidiploids, (B)BBNN, by mating with diploid *Rana nigromaculata,* diploid *Rana brevipoda* or amphidiploids were compared with those of diploid *Rana nigromaculata,* diploid *Rana brevipoda* and reciprocal diploid hybrids between these two species. As presented in Table 12, the amphidiploids, (B)BBNN, were very

TABLE 12
External characters of eight kinds of frogs

Kinds	(N)NN	(B)BB	(N)NB	(B)BN
Body shape	Slender	Dumpy	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Dorso-median stripe	Pres	Abs	Pres	Pres
Dorsal black spots in female	Fus	Is	Is	Is
Whiteness of ventral surface	Pres	Abs	Pres	Pres
Black bands on hind legs	Abs	Pres	Pres	Pres
Dermal protuberances:				
Arrangement	Par	Ir	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Height	High	Low	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Shape	Long	Short	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Dorsal black spots:				
Number	>30	6~12	12~20	10~18
Size	Small	Large	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Shape	Rod	Round	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Dorsal ground color	Pale	Brownish Gray	Med (N)NN & (B)BB	Med (N)NN & (B)BB
Length of hind limbs	Long	Short	Med (N)NN & (B)BB	Med (N)NN & (B)BB

Abs, Absent Fus, Fused Ir, Irregular Is, Isolated Med, Intermediate between Par, Parallel

similar to the reciprocal diploid hybrids, (N)NB and (B)BN, in appearance (Fig. 9).

a. Amphidiploids

The dorsomedian stripe and whiteness of the ventral surface found in *Rana nigromaculata* revealed themselves in the amphidiploids, as they are found in the reciprocal diploid hybrids as dominant characters. In contrast, the black bands on the hind limbs of *Rana brevipoda* and non-fusion of the dorsal black spots in the mature females of this species were found as dominant characters in the amphidiploids like those in the reciprocal diploid hybrids. The amphidiploids were intermediate between the two species like the reciprocal diploid hybrids in parallelism, height and shape of the dorsal protuberances, number of the dorsal black spots, body shape, dorsal ground color and hind limb length. However, the amphidiploids were more similar to the diploid *Rana brevipoda* than the reciprocal diploid hybrids in size and shape of the dorsal black spots. The dorsal body surface of the amphidiploids was remarkably rougher than that of the reciprocal hybrids. The dorsal black spots of the amphidiploids were also generally deeper in color tone than those of the reciprocal hybrids (Fig. 10).

b. Allotriploids

The three kinds of allotriploids, (N)NNB, (B)BNN and (B)BBN, were similar to the diploid *Rana nigromaculata* in existence of the dorsomedian stripe and whiteness of the ventral surface. They were the same in these respects as the reciprocal diploid hybrids as well as the amphidiploids. In contrast, these three kinds of allotriploids were similar to the diploid *Rana brevipoda* in non-fusion of the dorsal black spots of mature females like the reciprocal diploid hybrids and the amphidiploids.

in combination of the nucleus and cytoplasm

(N)NNB	(B)BNN	(B)BBN	(B)BBNN
Med (N)NN & (N)NB	Med (N)NN & (N)NB	Somewhat dumpy	Med (N)NN & (B)BB
Pres	Pres	Pres	Pres
Is	Is	Is	Is
Pres	Pres	Pres	Pres
Pres or abs	Pres or abs	Pres	Pres
Par	Par	Med (B)BB & (B)BN	Med (N)NN & (B)BB
High	High	Med (B)BB & (B)BN	Med (N)NN & (B)BB
Long	Long	Med (B)BB & (B)BN	Med (N)NN & (B)BB
20～30	18～30	10～15	10～20
Small	Small	Med (B)BB & (B)BN	Med (B)BB & (B)BN
Rod	Rod	Med (B)BB & (B)BN	Med (B)BB & (B)BN
Near (N)NN	Near (N)NN	Near (B)BB	Med (N)NN & (B)BB
Near (N)NN	Near N()NN	Near (B)BB	Med (N)NN & (B)BB

Pres, Present

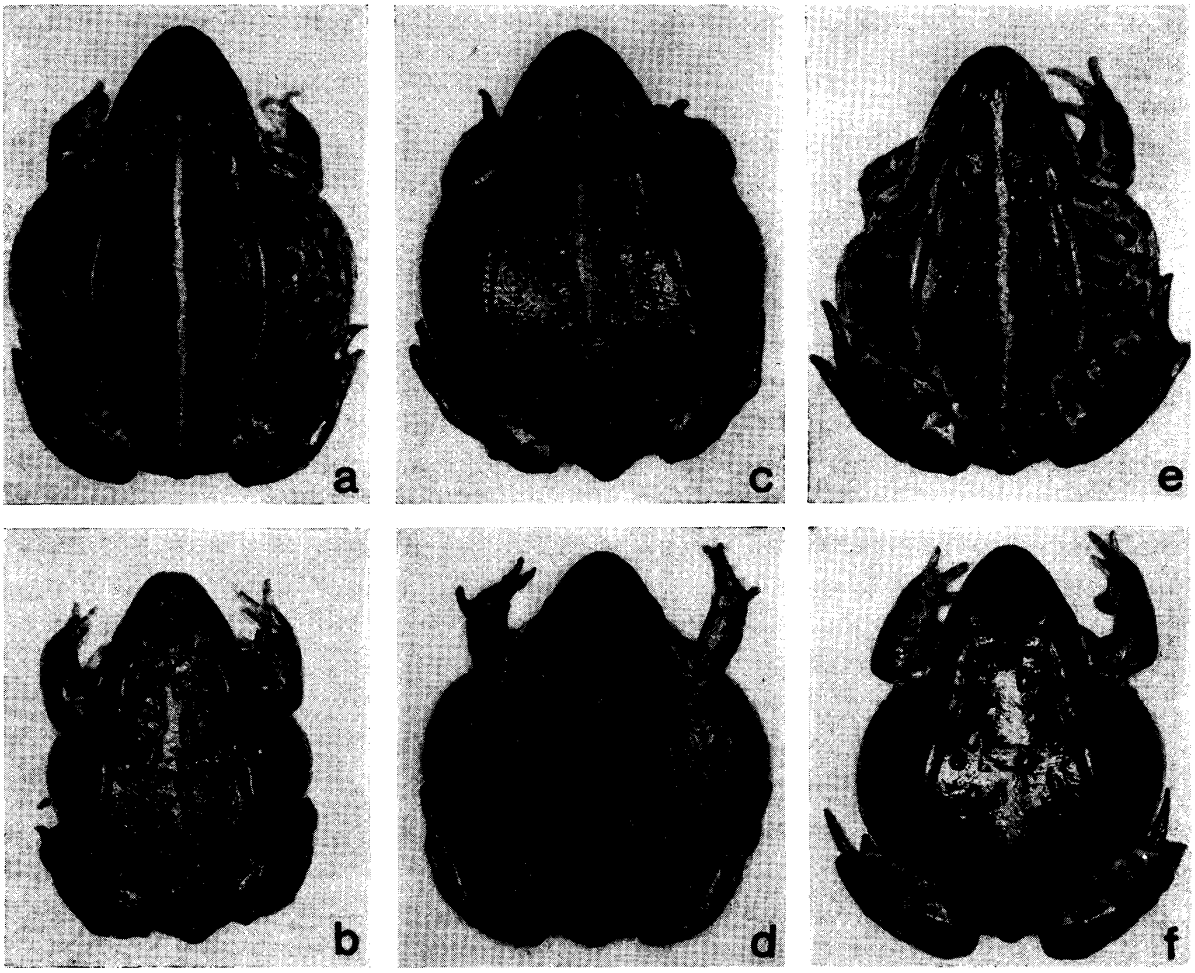

Fig. 10. Three-year-old allotriploids and amphidiploids produced from a female amphidiploid by mating with a male *Rana nigromaculata*, *Rana brevipoda* or amphidiploid. ×0.7

a, b. Female and male (B)BNN allotriploids produced from (B)BBNN 64-I♀, No. 1 × (N)NN 64-I♂, No. 1

c, d. Female and male (B)BBN allotriploids produced from (B)BBNN 64-I♀, No. 1 × (B)BB 64-I♂, No. 1

e, f. Female and male (B)BBNN amphidiploids produced from (B)BBNN 64-I♀, No. 1 × (B)BBNN 64-I♂, No. 1

The (N)NNB and (B)BNN allotriploids were very similar to the diploid *Rana nigromaculata* in parallelism, height and shape of the dermal protuberances, size and shape of the dorsal black spots, dermal ground color and length of the hind limbs, while the (B)BBN allotriploids were very similar to the diploid *Rana brevipoda* in shape of the dorsal black spots, dorsal ground color, length of the hind limbs and existence of black bands on the hind limbs. In parallelism, height and shape of the dorsal protuberances, number and size of the dorsal black spots and body shape, the (B)BBN allotriploids were intermediate between the diploid *Rana brevipoda* and the reciprocal hybrids. On the other hand, the (N)NNB and (B)BNN allotriploids were intermediate between the diploid *Rana nigromaculata* and the reciprocal hybrids in number of the dorsal black spots, existence of black bands on the hind limbs and body shape.

Thus, the allotriploids were rather intermediate in appearance between the diploid hybrids and the diploid *Rana nigromaculata* or *Rana brevipoda,* whereas the amphidiploids were very similar to the diploid hybrids which were intermediate between the two species in appearance.

2. Inner structure of testes

The testes of the three kinds of mature male allotriploids, (N)NNB, (B)BNN and (B)BBN, were very abnormal in inner structure. No normal spermatozoa were found in all the testes of these male allotriploids. The seminiferous tubules contained a few abnormal spermatozoa and many pycnotic nuclei in the central part and many spermatocytes and some spermatogonia in the peripheral part (Fig. 11).

In contrast, the testes of the male amphidiploids were very similar in inner structure to those of the diploid *Rana nigromaculata* and *Rana brevipoda.* The seminiferous tubules contained compact bundles of normally shaped and remarkably large spermatozoa (Fig. 11).

3. Electrophoresis

In order to compare biochemically the three kinds of allotriploids, (N)NNB, (B)BNN and (B)BBN, and the amphidiploids with the diploid *Rana nigromaculata, Rana brevipoda* and reciprocal hybrids of these two species, albumin (Ab), transferrin (Tf) and protein-C from blood serum, hemoglobin (Hb) from erythrocytes and 12 kinds of enzymes, lactate dehydrogenase (LDH), malate dehydrogenase (MDH), α-glycerophosphate dehydrogenase (α-GDH), isocitrate dehydrogenase (IDH), superoxide dismutase (SOD), aspartate aminotransferase (AAT), creatine kinase (CK), pyruvate kinase (PK), adenylate kinase (AK), phosphoglucomutase (PGM), glucosephosphate isomerase (GPI) and glutamate dehydrogenase (GaDH), from skeletal muscles were analyzed by the method of starch-gel electrophoresis. The three kinds of allotriploids and the amphidiploids were those obtained in 1968 from matings of male and female amphidiploids and preserved in a refrigerator in October, 1975 together with diploid *Rana nigromaculata,* diploid *Rana brevipoda* and reciprocal diploid hybrids. The diploid *Rana nigromaculata* distinctly differed from the diploid *Rana brevipoda* in the following nine proteins: Ab, Tf, protein-C, Hb, LDH, MDH, α-GDH, IDH and SOD.

The reciprocal diploid hybrids showed the sum of the characteristic bands of the two species in the electrophoretic pattern of each protein. They revealed two bands in each of Ab, Tf and protein-C and four bands in each of SOD and Hb. They also showed the sum of the bands or the characteristic hybrid bands derived from the two species in the electrophoretic patterns of LDH, MDH, α-GDH and IDH (Nishioka, Ueda and Sumida, 1981).

The three kinds of allotriploids were the same as the reciprocal diploid hybrids in the electrophoretic patterns of the above nine kinds of proteins, except that the bands derived from the species giving two genomes were darker than those derived from the species giving one genome in each kind of allotriploids. No differences

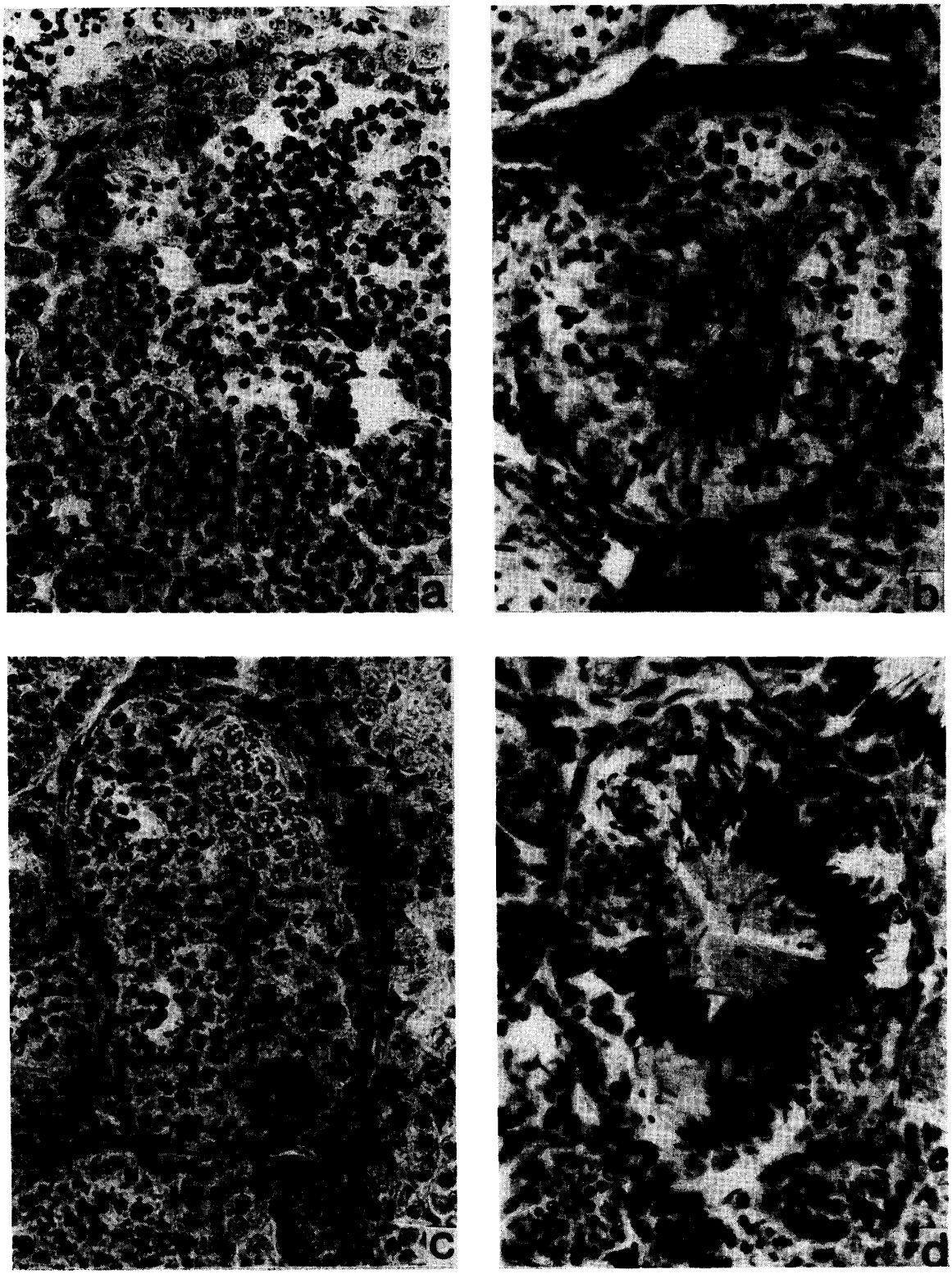

Fig. 11. Cross-sections of the testes of diploid hybrid, allotriploid and amphidiploid males. ×260

a. Diploid (B)BN hybrid produced from (B)BB 64W♀, No. 1 × (N)NN 64W♂, No. 1
b. (B)BNN allotriploid produced from (B)BBNN 64-I♀, No. 1 × (N)NN 64W♂, No. 1
c. (B)BBN allotriploid produced from (B)BBNN 64-I♀, No. 1 × (B)BB 64W♂, No. 1
d. (B)BBNN amphidiploid produced from (B)BBNN 64-I♀, No. 1 × (B)BBNN 64-I♂, No. 1

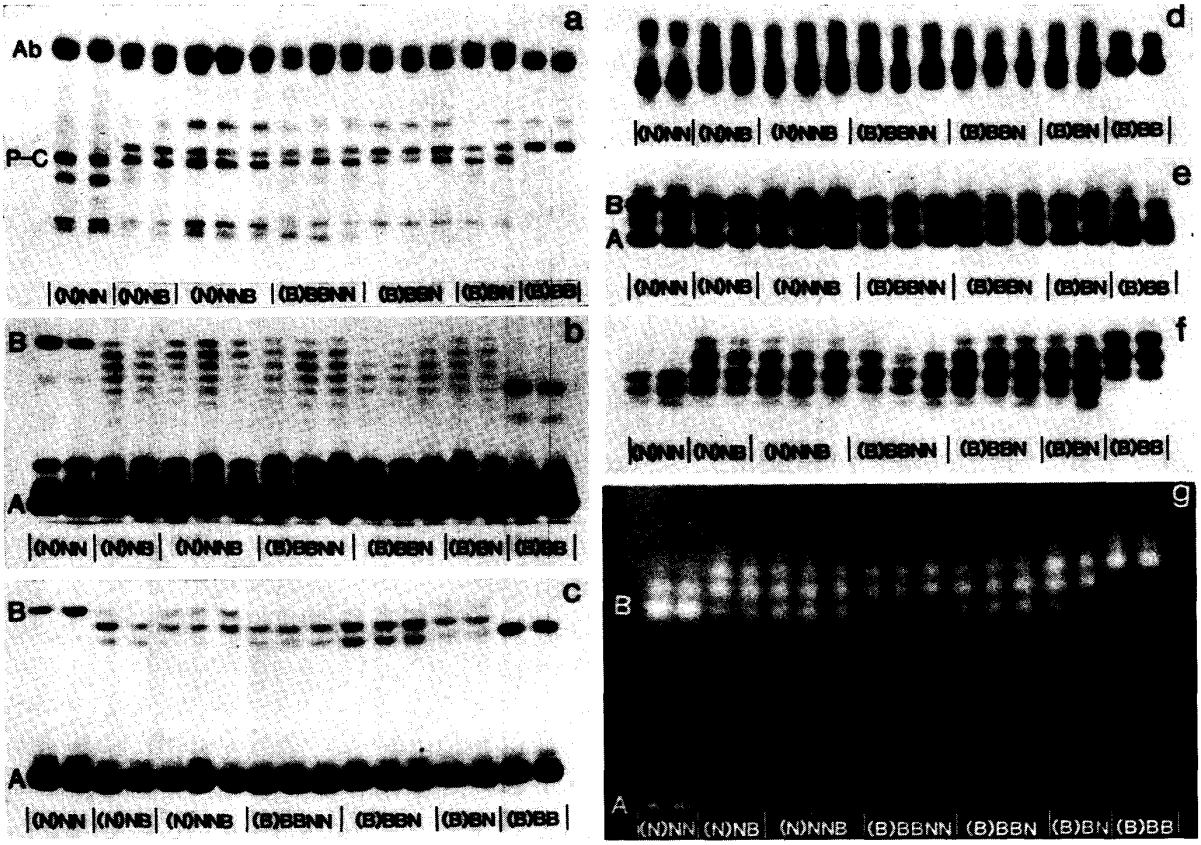

Fig. 12. Electrophoretic patterns of three blood proteins and five enzymes from reciprocal diploid hybrids, reciprocal allotriploids and amphidiploids between *Rana nigromaculata* and *Rana brevipoda*, and the control diploids.

a. Serum albumin (Ab) and protein-C (P-C) b. Lactate dehydrogenase (LDH)
c. Isocitrate dehydrogenase (IDH) d. Hemoglobin (Hb)
e. Malate dehydrogenase (MDH) f. α-Glycerophosphate dehydrogenase (α-GDH)
g. Superoxide dismutase (SOD)

were found in the electrophoretic patterns of the above nine proteins between the reciprocal hybrids and the amphidiploids (Fig. 12).

DISCUSSION

1. Production of amphidiploids

The first two tetraploid urodeles were described by FANKHAUSER (1939) in *Eurycea bislineata*. They were discovered together with 13 triploids in 134 larvae raised from eggs which had been laid by females after pituitary implantation. These tetraploids were slightly smaller and grew more slowly than the controls. FANKHAUSER (1941, 1942, 1945) thereafter found one tetraploid among 2448 larvae of *Notophthalmus viridescens* and one tetraploid among more than 3000 larvae of axolotl. KAWAMURA and SANADA (1944) found a single tetraploid among many larvae raised from cold-treated eggs of *Cynops pyrrphogaster*. This tetraploid could not attain completion of metamorphosis, although it grew

normally during the larval stage. FISCHBERG (1947) reported four tetraploids besides 184 triploids among 553 larvae raised from cold-treated fertilized eggs of *Triturus alpestris*. One of these tetraploids completed metamorphosis and died at the age of 9.5 months. HUMPHREY and FANKHAUSER (1949) and FANKHAUSER and HUMPHREY (1950) reported in axolotls that about 11% of the larvae produced from triploid females by mating with diploid males were tetraploids, and that a considerable number of tetraploid females became sexually mature and produced triploid or near-triploid offspring. Unlike the tetraploid females, tetraploid males were subnormal in size and vigor, and completely sterile.

The first four tetraploid anurans were discovered by KAWAMURA (1939) in 413 parthenogenetically developed individuals of *Rana nigromaculata,* although they died at the age of eight to 30 days. The oldest of them was approximately normal in appearance. The second tetraploid anuran was reported by KAWAMURA and MORIWAKI (1953) in *Rana limnocharis*. This tetraploid was found accompanied with many triploids raised from cold-treated eggs. Although killed at the age of 151 days because of infection, this frog began metamorphosis earlier than the controls and grew rapidly for about two months. It was a female whose ovaries were normal and contained many growing oocytes. KING and BRIGGS (1954) and BRIGGS and KING (1957) obtained some tetraploid embryos by transplantation of embryonic nuclei into enucleated eggs in *Rana pipiens*. SAMBUICHI (1959) also obtained tetraploid embryos from both fertilized and unfertilized eggs injected with blastula nuclei in *Rana japonica*.

All the tetraploids stated above were rather those which were unexpectedly obtained or incidentally detected. In contrast, SANADA (1951) refrigerated eggs of *Cynops pyrrhogaster* immediately before the first cleavage for the purpose of producing tetraploids by disturbing formation of the first cleavage furrow. By this method he obtained 10 tetraploids. While one of them died at the early larval stage, the others metamorphosed at the same rate as the controls. KAWAMURA and UTSUNOMIYA (1957) reported that auto- and allotetraploids were produced in *Cynops pyrrhogaster* and *Cynops ensicauda* by exposing eggs to supersonic waves when the first cleavage furrow began to appear by insemination with sperm of their own or foreign species. However, these allotetraploid *Cynops* were autotetraploids in reality, as the two species are considered to be two subspecies. FISCHBERG (1958) obtained high percentages of autotetraploids in *Triturus vulgaris* and *Triturus helveticus* by heat-shock treatment of eggs at the 2-cell stage in most cases. ROMANOVSKY and SPICAROVA (1961) described that 28 larvae raised from *Triturus vulgaris* eggs heat-shocked by FISCHBERG's method were all tetraploids. JAYLET (1972) and GAILLARD and JAYLET (1975) also reported that all the larvae obtained by FISCHBERG's method were tetraploids in *Pleurodeles waltl.* GURDON (1959) obtained 11 tetraploid adults in *Xenopus laevis* by nuclear transplantation. In this case, both males and females were very abnormal in gametogenesis and entirely sterile, whereas they were almost identical to normal diploids in external morphology.

The first four amphidiploid frogs were produced by KAWAMURA and NISHIOKA

(1960) between *Rana nigromaculata* and *Rana brevipoda* by application of three different methods (cf. NISHIOKA, 1971). One of the amphidiploids was found among 66 tadpoles obtained from fertilized *Rana brevipoda* eggs injected with blastula nuclei of *Rana nigromaculata*. This tetraploid grew into a mature male which was completely fertile. Another amphidiploid was found among 16 tadpoles obtained from unfertilized *Rana nigromaculata* eggs injected with blastula nuclei of an allotriploid consisting of two genomes of *Rana brevipoda* and one genome of *Rana nigromaculata*. This tetraploid became a mature male with paralyzed hind limbs. The other two amphidiploids were found among 520 tadpoles produced from *Rana brevipoda* eggs by heat-shock treatment after insemination with sperm of *Rana nigromaculata*. One of them grew into a mature male, while the other attained near sexual maturity. KAWAMURA and NISHIOKA (1963a) reported that the above mature male amphidiploid obtained from a fertilized *Rana brevipoda* egg injected with a blastula nucleus of *Rana nigromaculata* was not inferior to the control *Rana nigromaculata* and *Rana brevipoda* in reproductive capacity. KAWAMURA, NISHIOKA and MYOREI (1963) produced 14 autotetraploid tadpoles in *Rana japonica* by two different methods; seven by heat-shock treatment of fertilized eggs and the other seven by transplanting diploid blastula nuclei into fertilized eggs. Ten of these tetraploids metamorphosed normally and seven attained sexual maturity. All the metamorphosed tetraploids were males. Four tetraploid males were slightly fertile and produced a few triploid offspring by mating with diploid females. NISHIOKA (1963) obtained four and 10 metamorphosed amphidiploids between *Rana japonica* and *Rana ornativentris* by nuclear transplantation and heat-shock treatment, respectively. Of a total of 14 amphidiploids, five were females and 9 were males. The finding that female amphidiploids were produced was remarkable, as the diploid hybrids between the two species all became males. Two of three female amphidiploids reared for more than six months had entirely or approximately normal ovaries. Two of four mature male amphidiploids were partially fertile and produced a small number of offspring by mating with female *Rana japonica*. These offspring were all allotriploids which attained sexual maturity.

KAWAMURA and NISHIOKA (1963b) produced 19 autotetraploid tadpoles from fertilized eggs of *Rana nigromaculata* by heat-shock treatment. Six of them metamorphosed normally, while six others remained as tadpoles for a long time. The six frogs and four old tadpoles were all males. Three of the autotetraploid frogs attained sexual maturity and two produced auto- and allotriploids by mating with diploid female *Rana nigromaculata* and *Rana brevipoda*, respectively. From the same matings, autotetraploids and amphidiploids were also produced by refrigeration of inseminated eggs. Four of 16 male autotetraploid frogs produced from one (60 N12H7) of the two paternal autotetraploids were used in the present study as the animals for producing abundant amphidiploids (cf. KAWAMURA and NISHIOKA, 1963b: Table 9).

In the present study, by mating with the four male autotetraploids of *Rana nigromaculata*, 7.4～19.7% of the respective number of eggs obtained from two

diploid females of the same species cleaved normally, and 2.8~17.1% became feeding tadpoles which were almost totally autotriploids. This seems to indicate that the fertile spermatozoa of the autotetraploid males were mostly diploid. As a matter of fact, most of the tadpoles raised from refrigerated eggs were autotetraploids, owing to retention of the second polar body nucleus, although the percentages of normal cleavages and feeding tadpoles somewhat dropped, as compared with the non-refrigerated eggs. When the same four autotetraploid males were mated with three female *Rana brevipoda,* 19.3~36.5% became feeding tadpoles by three of the four males. These tadpoles were all allotriploids as far as their ploidy was elucidated. The high percentages of normal cleavages in this case seem to show that the large diploid spermatozoa of the autotetraploid males were apt to penetrate into the eggs of the foreign species. As in the production of autotetraploids, 140 (69.7%) of 201 tadpoles raised from refrigerated eggs were amphidiploids.

It is noteworthy that autotetraploid males of *Rana nigromaculata* are normal in growth and produced viable offspring by mating with diploid females, although they are not always complete in reproductive capacity. They distinctly differ in this respect from those of *Xenopus laevis* (GURDON, 1959), axolotls (HUMPHREY and FANKHAUSER, 1946) and *Pleurodeles waltl* (BEETSCHEN, 1962, 1967). Autotetraploid males were completely sterile in the former two species. They have not yet been produced in the latter species, while there are some autotetraploid females which are fairly fertile. Thus, autotetraploid males in *Rana nigromaculata* are a unique material in producing abundant amphidiploids from eggs of a foreign species by cold-treatment after insemination.

2. Reproductive capacity of amphidiploids

The amphidiploid offspring produced from autotetraploid males seemed to be somewhat retarded in metamorphosis, although they were slightly larger soon after metamorphosis as compared with the control autotetraploids. Mature amphidiploid females were intermediate in body length between the diploid females of the two species. The eggs laid by the former were nearly half the number of those laid by the latter. While the eggs of *Rana nigromaculata* were distinctly larger than those of *Rana brevipoda,* the eggs of amphidiploids were generally larger than those of *Rana nigromaculata.* While the testes of male *Rana nigromaculata* were longer than those of male *Rana brevipoda,* amphidiploid males had testes which were rather similar to those of *Rana brevipoda* in shape and not inferior in size to those of *Rana nigromaculata.* The testes of the amphidiploid males were almost normal in inner structure in contrast to those of male diploid hybrids which were very abnormal and almost completely sterile.

When 11 amphidiploid females were mated with three diploid male *Rana nigromaculata* and three diploid male *Rana brevipoda,* 42.3~82.0% and 13.9~87.4% of the respective number of eggs cleaved normally and 21.3~66.7% and 13.9~62.8% became feeding tadpoles, respectively. When two amphidiploid males were mated with three diploid female *Rana nigromaculata,* 24.6% and 16.4%

of the respective number of eggs cleaved normally and 23.9% and 8.2% became feeding tadpoles, while 36.0% and 44.1% cleaved normally and 27.2% and 37.0% became feeding tadpoles when they were mated with three female diploid *Rana brevipoda*. When five amphidiploid females were mated with two amphidiploid males, 30.9~86.5% of the respective number of eggs cleaved normally and 25.5~79.0% became feeding tadpoles. The tadpoles obtained from amphidiploid females or males by mating with diploid males or females of *Rana nigromaculata* or *Rana brevipoda* were all allotriploids. Of 968 tadpoles produced from matings between amphidiploid males and females, 956 (98.8%) were amphidiploids. Of 154 tadpoles obtained from six amphidiploid females by cold treatment of gynogenetic eggs, 129 (83.8%) were also amphidiploids. These findings seem to show that both male and female amphidiploids produce nearly exclusively diploid gametes by regular gametogenesis and that they already establish a new artificial species.

3. Sex chromosome constitution of amphidiploids

The sex chromosome constitutions of the four male autotetraploids (N)NNNN 62-I♂, Nos. 1~4, which were used to produce autotetraploids and amphidiploids by mating with diploid female *Rana nigromaculata* and *Rana brevipoda* are considered as follows. From the sex ratio of the offspring including all the autotriploids, allotriploids, autotetraploids and amphidiploids produced by each of the paternal autotetraploids, two male autotetraploids, (N)NNNN 62-I♂, Nos. 3 and 4, seem to be XXXY, as the sex ratio was about 1: 1. It was also very probable that another male autotetraploid, (N)NNNN 62-I♂, No. 2 is XXXY, as 17 of 48 offspring were males. In contrast with these three autotetraploids, it is very difficult to assume the sex chromosome constitution of the remaining male autotetraploid, (N)NNNN 62-I♂, No. 1. It seems to be either XXXY or XXXX, as 39 (18.9%) of 209 offspring were males. Although six of 14 autotriploids and three of six autotetraploids were males, there was a great unbalance in sex ratio of allotriploids (B)BNN obtained by mating with diploid female *Rana brevipoda*; only 9 (9.2%) of 98 offspring were males. In the offspring produced from *Rana brevipoda* eggs by refrigeration after inseminating with sperm of the male autotetraploid, four (23.5%) of 17 allotriploids and 17 (23.0%) of 72 amphidiploids were males. If this autotetraploid is assumed to be XXXX in sex chromosome constitution, the small number of male offspring should be considered to be sex-reversed genetic females. The problem whether the two male autotetraploids, (N)NNNN 62-I♂, Nos. 1 and 2, were XXXX or XXXY in sex chromosome constitution was solved by examining the sex of their grandchildren.

One (No. 1) of the 17 male amphidiploids, (B)BBNN 64-I♂, which had been obtained from autotetraploid (N)NNNN 62-I♂, No. 1 by mating with a female diploid *Rana brevipoda*, produced 31 (N)NNB and 30 (B)BBN allotriploid offspring by mating with diploid female *Rana nigromaculata* and *Rana brevipoda*, respectively. Of these allotriploid offspring, 16 (51.6%) and 13 (43.3%) were males, respectively. When this male amphidiploid (No. 1) was mated with two

female amphidiploids, 20 (38.5%) of 52 and 20 (36.4%) of 55 amphidiploid offspring were males. If the sex was examined in one- or two-year-old offspring produced from the paternal amphidiploid, 11 of 20 allotriploids and 22 of 37 amphidiploids were males. These findings seem to indicate that the paternal amphidiploid is XXXY and maternal amphidiploids are XXXX in sex chromosome constitution. The same situation was found in the offspring of one (No. 3) of three male amphidiploids, (B)BBNN 64-II♂, which had been obtained from male autotetraploid (N)NNNN 62-I♂, No. 2 by mating with a diploid female *Rana brevipoda*. When this male amphidiploid was mated with diploid female *Rana nigromaculata* and *Rana brevipoda*, 4 (40.0%) of 10 (N)NNB and 22 (47.8%) of 46 (B)BBN allotriploid offspring were males, respectively. When it was mated with three female amphidiploids, 121 (48.6%) of 249, 142 (51.8%) of 274 and 137 (52.3%) of 262 amphidiploid offspring were males. These findings evidently seem to show that the paternal amphidiploid as well as the autotetraploid grandfather was XXXY in sex chromosome constitution. The paucity of males in the triploid and tetraploid offspring of two male autotetraploid *Rana nigromaculata* (N)NNNN 62-I♂, Nos. 1 and 2, may be attributable to scantiness in number or inseminating capacity of XY chromosomes produced by these autotetraploid males as compared with those of XX chromosomes.

SUMMARY

1. Amphidiploids were produced from eggs of diploid female *Rana brevipoda* fertilized with sperm of autotetraploid male *Rana nigromaculata* by refrigeration 25 minutes after insemination. The autotetraploids used in these experiments were the progenies of a male autotetraploid which had been obtained from a fertilized diploid eggs by applying a heat shock before the appearance of the first cleavage furrow. These progenies were obtained from eggs of diploid female *Rana nigromaculata* fertilized with sperm of the single male autotetraploid by refrigeration 25 minutes after insemination.

2. Of 46 feeding tadpoles produced from matings between diploid female and tetraploid male *Rana nigromaculata*, 45 (97.8%) were triploids, (N)NNN, while 20 (64.5%) of 31 feeding tadpoles produced from similar matings by refrigerating the eggs were tetraploids, (N)NNNN. All 222 feeding tadpoles produced from matings between diploid female *Rana brevipoda* and autotetraploid male *Rana nigromaculata* were allotriploids, (B)BNN, while 140 (69.7%) of 201 feeding tadpoles produced from the same matings by refrigerating the eggs were amphidiploids, (B)BBNN.

3. Amphidiploids obtained from three of four autotetraploid male *Rana nigromaculata* seemed to be slightly larger than allotriploids obtained from the same males in mean total length of 50-day-old tadpoles and mean body length immediately after metamorphosis, while autotetraploids were not always larger than autotriploids.

4. Of 98 metamorphosed amphidiploids produced from matings between

diploid female *Rana brevipoda* and autotetraploid male *Rana nigromaculata* by refrigeration of eggs, 69 were females and 29 (29.6%) were males, while 157 of 218 allotriploids produced from the same parents were females and the other 61 (28.0%) were males. Of 29 metamorphosed autotriploids and 14 metamorphosed autotetraploids produced from the same autotetraploid males, 17 and 8 were females and 12 (41.4%) and 6 (42.9%) were males, respectively.

5. Eight 2-year-old female amphidiploids laid 439~810 eggs, 647.5 eggs on the average which were 2.05~2.63 mm in mean diameter, while three 2-year-old diploid hybrids laid 650~1139 eggs, 959.3 eggs on the average which were 1.69~1.70 mm in mean diameter and four 2-year-old female allotriploids laid 490~716 eggs, 585.3 eggs on the average, which were 1.3~3.2 mm in diameter.

6. The testes of male amphidiploids had many bundles of normally shaped large spermatozoa in the seminiferous tubules, whereas those of male diploid hybrids and allotriploids had no normally shaped spermatozoa.

7. Amphidiploid offspring were produced from matings of male and female amphidiploids and from female amphidiploids by gynogenesis accompanied with refrigeration of eggs, while allotriploid offspring were produced from male or female amphidiploids by mating with male or female diploid *Rana nigromaculata* and *Rana brevipoda*. When male or female amphidiploids were mated with male or female diploids, usually 16~53% of the respective total number of eggs became normally feeding tadpoles which were triploids with a few exceptions.

8. When male and female amphidiploids were mated in two breeding seasons, 25.5% and 62.4% of the respective total number of eggs became feeding tadpoles which were almost totally amphidiploids. By gynogenesis accompanied with refrigeration of eggs, 7.3% of the total number of eggs obtained from female amphidiploids became feeding tadpoles, of which 83.8% were amphidiploids.

9. There were no large differences between allotriploids produced from male or female amphidiploids by mating with diploids and amphidiploids produced from both amphidiploid parents or from female amphidiploids by gynogenesis in mean total length of 50-day-old tadpoles, period of tadpole stage, metamorphosis rate and mean body length of newly metamorphosed frogs. However, the amphidiploids obtained from seven of 11 female amphidiploids seemed to be slightly larger than the allotriploids obtained from the same females.

10. There was nearly an equal number of males and females in the two kinds of allotriploids produced from female amphidiploids by mating with diploid male *Rana nigromaculata* and *Rana brevipoda* as well as in the amphidiploids produced from matings between female and male amphidiploids. Of the amphidiploids obtained from female amphidiploids by gynogenesis, only 9.6% were males.

11. Male and female amphidiploids seemed to be XXXY and XXXX in sex chromosome constitution, respectively.

12. Three kinds of mature allotriploids, (N)NNB, (B)BNN and (B)BBN, and mature amphidiploids, (B)BBNN, were compared with diploid *Rana nigromaculata*, diploid *Rana brevipoda* and reciprocal hybrids in external characters as well as in the electrophoretic patterns of nine kinds of proteins. The amphidiploids were

very similar in appearance to the diploid hybrids which were intermediate between the two species, whereas the allotriploids were rather intermediate between the diploid hybrids and one of the two species. No differences were found in the electrophoretic patterns of the proteins between the diploid hybrids and the amphidiploids. The three kinds of allotriploids were the same as the diploid hybrids, except that the bands derived from the species giving two genomes were darker than those derived from the spccies giving one genome.

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