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Developmental Capacity and Chromosome Number in the Offspring of Artificially Produced Autotetraploids of *Rana nigromaculata*

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ABSTRACT—A large number of autotetraploids of *Rana nigromaculata* were produced in order to assess their developmental capacity and chromosome number in their offspring. An original autotetraploid male was first produced by transplanting a nucleus from an embryo (triploid) into an unfertilized egg. Next, eggs were inseminated with sperm of the autotetraploid male, then cold-treated to obtain first-, second-, and third-generation offspring. According to an investigation of the chromosome numbers of the tadpoles by the tail-tip squash method, the three generations of offspring included many tetraploids (50–80%), as well as some diploids, triploids, hexaploids and mosaics at the early tadpole stage. In addition, several percent of the second- and third-generation offspring were found to be aneuploids. Evidently, a complete set of diploid chromosomes was not precisely transferred to all of the next-generation offspring from the sperm of the artificially produced autotetraploid males. These observations suggest that there were some abnormalities in the course of spermatogenesis in the male autotetraploid frogs.

Key words: amphibia, polyploid, chromosome number, developmental capacity, nuclear transplantation

INTRODUCTION

It has proven to be very difficult to artificially produce mature tetraploid amphibians, in spite of exhaustive efforts taken by investigators from as far back as the 1940's. While it has been possible to obtain embryos of polyploid amphibians for quite some time, difficulties in nurturing tadpoles or juvenile animals in the laboratory thwarted all attempts to rear amphibians to full adulthood until 1949.

Humphrey and Fankhauser (1949) were the first to produce mature autotetraploid amphibians in their experiments using *Ambystoma mexicanum*. Thereafter, mature autotetraploids were obtained in *Xenopus laevis* by Gurdon (1959), in *Rana japonica* by Kawamura *et al*. (1960, 1963b), and in *Pleurodeles wartl* by Beetschen (1962, 1967). In each of these cases, only a very small number of tetraploid amphibians was obtained. In the most recent experiments, Kawamura and Nishioka (1960, 1963a) and Nishioka and Okumoto (1983) obtained fairly fertile allotetraploids (amphidiploids) between *R. porosa brevipoda* and *R. nigromaculata*, and between *R. nigromaculata* and *R. plancyi chosenica*. Kawamura *et al*. (1983) produced autotetraploids of *R. porosa brevipoda* and *R. nigromaculata* by mating autotriploid females with diploid males. The former autotetraploids died after metamorphosis, but the latter were reared to three males (0.1% of treated eggs) that survived to sexual maturity. Mating with these males was not attempted.

In the present study, the author attempted to produce autotetraploids of *R. nigromaculata*, examine their fertility, and establish a procedure for producing and maintaining the autotetraploidy through several generations. Moreover, he investigated the chromosome numbers of the offspring produced from fertilized eggs with autotetraploid males.

MATERIALS AND METHODS

Males and females of the Japanese pond frog *R. nigromaculata* Hallowell (2n=26) were collected from Hiro, Kure City, Hiroshima Prefecture. The following procedures were carried out to produce autotetraploid frogs (Fig. 1).

(1) Production of an original autotetraploid male

Ovulation was stimulated by cutaneous implantation of pituitary glands. Sperm suspension was prepared by anesthetizing and sacrificing a field-caught male and macerating its testes in dechlorinated tap water. Artificially inseminated eggs were refrigerated for 2.5 hr at 2°C to suppress the extrusion of their second polar bodies and make triploid embryos (Fankhauser and Griffiths, 1939; Nishioka, 1972). When these embryos developed into the late blastula stage, a single sub-surface cell nucleus from the blastula embryo was transplanted into each unfertilized egg (Briggs and King, 1952;

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Kondo, 1978). These transplanted eggs were then reared carefully and were bred to the later stage. At the early tadpole stage, the number of chromosomes was examined by the squash method after water-pretreatment of the tail-tips (Makino and Nishimura, 1952). Only one tadpole grew up to become a mature male frog.

(2) Production of the first-, second-, and third-generation offspring of autotetraploid *R. nigromaculata*

To obtain the first-generation autotetraploids, eggs from a field-caught female were inseminated with the sperm from an anesthetized autotetraploid male, and then refrigerated to suppress the extrusion of their second polar bodies. Only tadpoles confirmed to be tetraploidy were reared until maturation.

The second-generation offspring were produced by mating seven field-caught females with seven autotetraploid males of the first-generation offspring, and the third-generation offspring were produced by mating nine females of diploid control offspring with five autotetraploid males of the second-generation offspring.

The tadpoles were fed on boiled spinach outdoors until metamorphosis, then the metamorphosed frogs were fed on crickets in a room.

In this paper, the acronyms NN, NNN, and NNNN are used to denote autodiploids, autotriploids, and autotetraploids of *R. nigromaculata,* respectively.

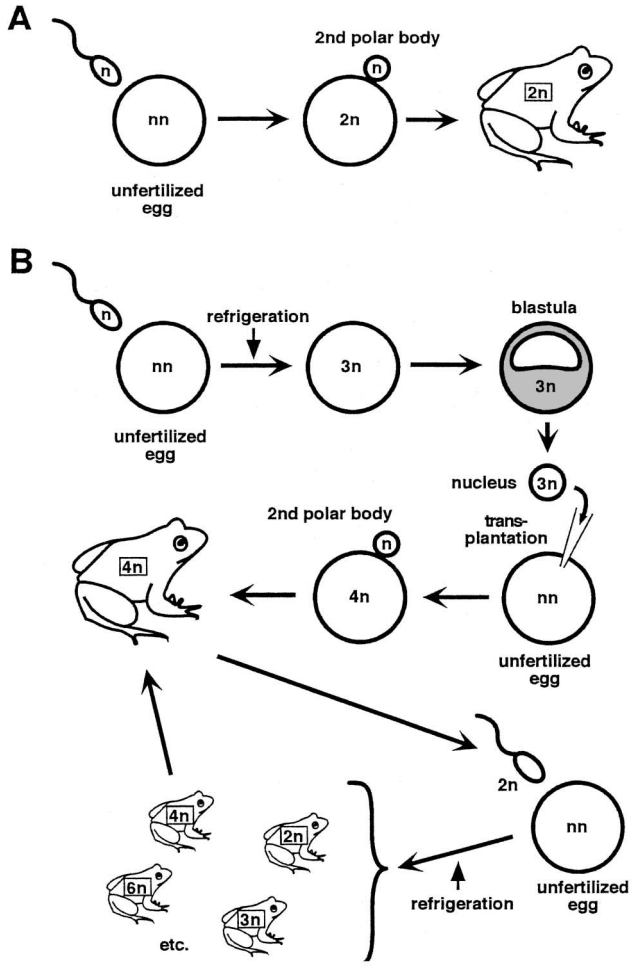

Fig. 1. Diagrams illustrating methods for producing offspring of normal control diploids (A) and the first-, second- and third-generation offspring of autotetraploids (B) in *R. nigromaculata*. See the text in details.

RESULTS

(1) An autotetraploid male parent

Several brothers from blastulae whose nuclei had been transplanted into unfertilized eggs were bred until the early tadpole stage, then their tail-tips were squashed to examine the ploidy and number of chromosomes. As expected, they were triploids possessing three genome sets consisting of five large and eight small chromosomes (Nishioka, 1972). This strongly suggested that the transplanted nucleus was also triploidy. Moreover, according to the chromosome count, a tadpole produced by nuclear transplantation was exactly tetraploid, having four genome sets consisting of five large and eight small chromosomes. This tadpole attained sexual maturity and grow to become a healthy male with a body length of 65mm.

(2) The first-generation offspring

A total of 552 eggs from one field-caught female were fertilized with the sperm of testes taken from the above-mentioned autotetraploid male in an anesthetized condition, and then refrigerated shortly after insemination. The developmental capacity of the first-generation offspring is presented in Table 1. In an analysis of the ploidy of 29 normally feeding tadpoles, 24 (82.8%) tadpoles were tetraploids, one was diploid, two were triploids, and the remaining two were diplo-tetraploid and triplo-pentaploid mosaics (Table 2). Eighteen of 24 tetraploid tadpoles completed metamorphosis and attained sexual maturity. There were nine females and nine males. Among the control diploid, autotriploid, and autotetraploid frogs of each sex, there were no differences in body sizes (64–67mm) or color pattern (Fig. 2).

Table 1. Developmental capacity of the 1st-generation offspring produced from the eggs refrigerated shortly after insemination with sperm of an autotetraploid male and the controls

Parents		Treatment of eggs	No. of eggs	No. of cleavages	No. of hatched embryos	No. of feeding tadpoles	No. of metamorphosed frogs*	No. of matured frogs*
Female	Male							
NN	NNNN	Refrig.	522	170(30.8)	46(8.3)	31(5.6)	18(3.5)	18(3.5)
NN	NNNN	None	151	55(36.4)	49(34.4)	45(32.5)	26(17.2)	26(17.2)
NN	NN	None	142	138(97.2)	116(81.7)	98(69.0)	71(50.0)	71(50.0)

*: Numerals show the number of tetraploid individuals in experimental series.
Parentheses indicate the percentages for total eggs.

Table 2. Ploidy of feeding tadpoles of the 1st-generation offspring and the controls

Parents		Treatment	No. of	No. of chromosomes				
Female	Male	of eggs	analyzed tadpoles	26 (2n)	39 (3n)	52 (4n)	Mosaics 2n+4n	Mosaics 3n+5n
NN	NNNN	Refrig.	29	1	2	24	1	1
				3.4%	6.9%	82.8%	3.4%	3.4%
NN	NNNN	None	30	0	30	0	0	0
NN	NN	None	30	30	0	0	0	0

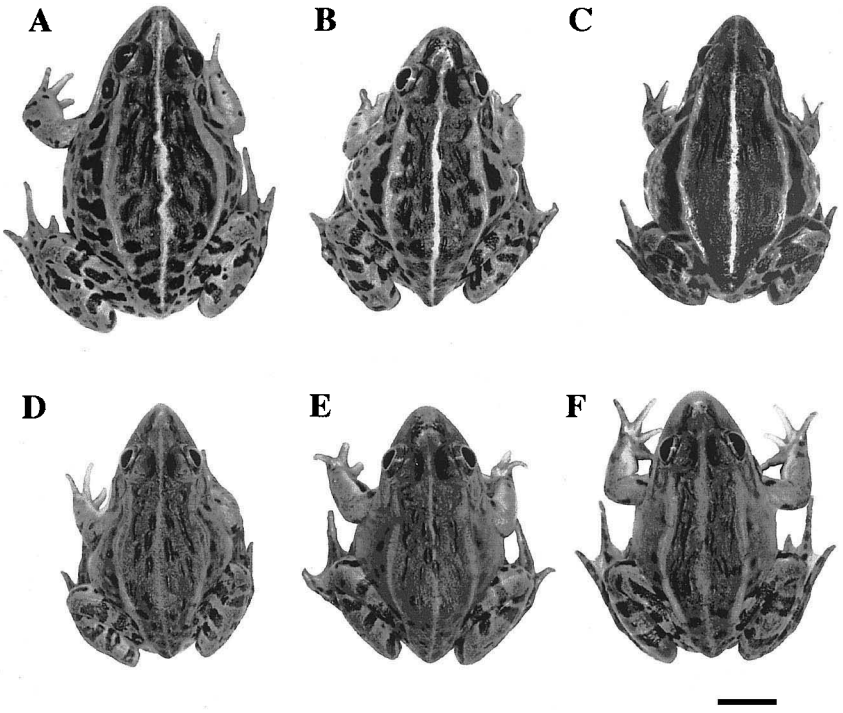

Fig. 2. Mature control diploid (A, D), control autotriploid (B, E) and the first-generation autotetraploid *R. nigromaculata* (C, F). A, B and C are females and D, E and F are males. There was no difference among diploid, autotriploid and autotetraploid frogs in appearance. Scale bar represents 1cm.

Table 3. Developmental capacity of the 2nd-generation offspring produced from the eggs refrigerated shortly after insemination with sperm of autotetraploid males and the controls

Parents		Treatment of eggs	No. of eggs	No. of cleavages	No. of hatched embryos	No. of feeding tadpoles	No. of metamorphosed frogs*	No. of matured frogs*
Female	Male							
NN1	NNNN1	Refrig.	697	119(17.7)	67(9.6)	54(7.7)	37(5.3)	29(4.2)
NN2	NNNN2	Refrig.	750	229(30.5)	162(21.6)	83(11.1)	40(5.3)	14(1.9)
NN2,3,4	NNNN3	Refrig.	1815	230(12.7)	144(7.9)	101(5.6)	74(4.1)	38(2.1)
NN5	NNNN4	Refrig.	290	6(2.1)	2(0.7)	1(0.3)	1(0.3)	0
NN6	NNNN5	Refrig.	1069	221(20.7)	174(16.3)	125(11.7)	83(7.8)	36(3.4)
NN7	NNNN6	Refrig.	945	41(4.3)	17(1.8)	13(1.4)	4(4.2)	3(3.2)
NN7	NNNN7	Refrig.	498	4(0.8)	2(0.4)	2(0.4)	2(0.4)	2(4.0)
	Total		6064	850(14.0)	568(9.4)	379(6.3)	241(4.0)	122(2.0)
NN1-7	NNNN1–7	None	524	174(33.2)	164(31.3)	164(31.3)	28	24
NN1-7	NN1	None	320	304(95.0)	212(66.3)	212(66.3)	28	27

*: In experimental series, numerals show the number of tetraploid individuals. In control series, 30 feeding tadpoles were bred and numerals show the number of metamorphosed and matured frogs derived from these 30 tadpoles. Parentheses indicate the percentages for total eggs.

(3) The second-generation offspring

A total of 6,064 eggs from seven field-caught females were fertilized with the sperm of seven autotetraploid males of the first-generation offspring and then refrigerated shortly after insemination. Table 3 shows the developmental capacity of the second generation. In total, 379 (6.3%) embryos developed into normally feeding tadpoles.

According to a chromosomal analysis on 364 of 379 normal tadpoles, 272 (74.7%) tadpoles were tetraploids, 30 (8.2%) were diploids, two (0.5%) were triploids, three (0.8%) were hexaploids, nine (2.5%) were aneuploids, and the remaining 48 (13.2%) were mosaics. The nine aneuploids consisted of five hypotetraploids [including two (4n-3), one (4n-2), and two (4n-1)] and four hypertetraploids [including two (4n+1), one (4n+2) and one (4n+3)]. The other 48 tadpoles consisted of one (2n+4n), 41 (2n+6n), and six (3n+5n) mosaics (Table 4, Fig. 3).

Of the 272 tetraploid tadpoles, 241 (88.6%) completed metamorphosis and 122 (44.9%) attained sexual maturity. Of a total 202 juvenile and mature frogs analyzed, 152 (75.2%) were females and 50 (24.8%) were males.

(4) The third-generation offspring

The nine females used for the production of the third-generation offspring were derived from the diploid control offspring of the first generation. Of these nine females, five (Nos. 1–5) were mated with three autotetraploid males (Nos.

Table 4. Number of chromosomes of feeding tadpoles of the 2nd-generation offspring and the controls

Parents		Treatment	No. of	No. of chromosomes								
Female	Male	of eggs	analyzed	26	39	49–51	52	53–55	78	Mosaics		
			tadpoles	(2n)	(3n)	(4n–)	(4n)	(4n+)	(6n)	2n+4n	2n+6n	3n+5n
NN1	NNNN1	Refrig.	54	4	0	0	40	0	0	0	10	0
NN2	NNNN2	Refrig.	81	13	1	0	56	0	1	0	9	1
NN2,3,4	NNNN3	Refrig.	96	5	1	2	82	2	0	1	2	2
NN5	NNNN4	Refrig.	1	0	0	0	1	0	0	0	0	0
NN6	NNNN5	Refrig.	118	4	0	3	87	2	2	0	16	3
NN7	NNNN6	Refrig.	12	4	0	0	4	0	0	0	4	0
NN7	NNNN7	Refrig.	2	0	0	0	2	0	0	0	0	0
	Total		364	30	2	5	272	4	3	1	41	6
				8.2%	0.5%	1.4%	74.7%	1.0%	0.8%	48(13.2%)		
NN1-7	NNNN1-7	None	30	0	30	0	0	0	0	0	0	0
NN1-7	NN1	None	30	30	0	0	0	0	0	0	0	0

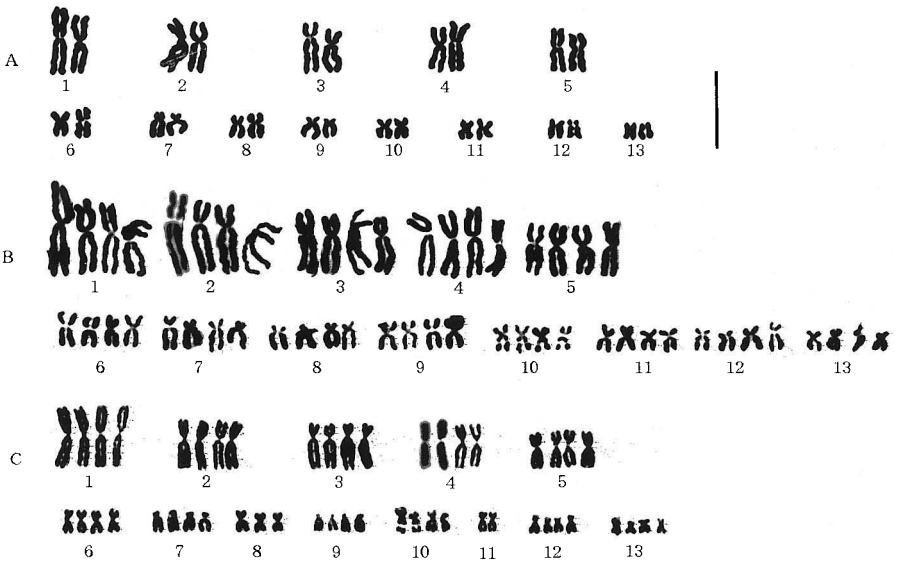

Fig. 3. Karyotypes of a control diploid (A), an autotetraploid (B), and an aneuploid (C) of the second-generation offspring observed by the tail-tip squash method. Karyotype of the autotetraploid (B) possessed four genome sets consisting of five large (Nos. 1–5) and eight small chromosomes (Nos. 6–13). One of chromosome No. 8 and two of chromosome No. 11 were lost in the karyotype of the hypotetraploid (4n-3) (C). Scale bar represents 10 μm.

Table 5. Developmental capacity of the 3rd generation offspring produced from the eggs refrigerated shortly after insemination with sperm of autotetraploid males and the controls

Parents		Treatment of eggs	No. of eggs	No. of cleavages	No. of hatched embryos	No. of feeding tadpoles	No. of metamorphosed frogs*	No. of matured frogs*
Female	Male							
NN1	NNNN1	Refrig.	1208	37(3.1)	25(2.1)	20(1.7)	8(0.7)	4(0.0)
NN2,3	NNNN2	Refrig.	2628	1147(34.7)	543(16.4)	528(15.9)	220(8.4)	180(3.8)
NN4,5	NNNN3	Refrig.	2784	1371(49.2)	913(32.8)	890(32.0)	244(8.8)	219(7.8)
NN6,7	NNNN4	Refrig.	1112	294(26.4)	179(16.1)	155(13.9)	62(5.6)	31(2.8)
NN8,9	NNNN5	Refrig.	1400	219(15.6)	128(9.1)	115(8.2)	41(2.9)	32(2.3)
	Total		9132	3070(33.6)	1788(19.6)	1708(18.7)	575(6.3)	466(5.1)
NN1	NNNN1	None	107	6(5.6)	4(3.7)	4(3.7)	3(2.8)	1(0.9)
NN2,3	NNNN2	None	222	135(60.8)	102(45.9)	98(44.1)	89(40.1)	79(35.6)
NN4,5	NNNN3	None	241	197(81.7)	121(50.2)	121(50.2)	120(49.8)	115(47.7)
	Total		570	338(59.3)	227(39.8)	223(39.1)	212(37.2)	195(34.2)

*: Numerals show the number of tetraploid individuals in experimental series.
Parentheses indicate the percentages for total eggs.

Table 6. Number of chromosomes of feeding tadpoles of the 3rd-generation offspring and the contro

Parents		Treatment	No. of	No. of chromosomes													
Female	Male	of eggs	analyzed	26	27,28	37,38	39	40–42	49–51	52	53–55	65	78	Mosaics			
			tadpoles	(2n)	(2n+)	(3n–)	(3n)	(3n+)	(4n–)	(4n)	(4n+)	(5n)	(6n)	2n+4n	2n+6n	3n+5n	Others
NN1	NNNN1	Refrig.	20	1	0	1	1	0	0	14	0	0	0	0	2	0	1
NN2,3	NNNN2	Refrig.	337	17	0	2	34	2	0	227	2	0	2	9	31	4	7
NN4,5	NNNN3	Refrig.	568	28	3	5	197	4	8	246	10	0	1	26	38	2	0
NN6,7	NNNN4	Refrig.	111	21	0	1	7	0	1	62	0	0	0	6	12	0	1
NN8,9	NNNN5	Refrig	92	17	2	0	18	0	1	41	0	1	0	3	9	0	0
	Total		1128	84	5	9	257	6	10	590	12	1	3	44	92	6	9
				(7.4%)			(22.8%)			(52.3%)				151(13.4%)			
NN3	NNNN2	None	40	0	0	0	40	0	0	0	0	0	0	0	0	0	0

1–3) derived from the male No. 2 used to produce the second generation, and the other four females (Nos. 6–9) were mated with two autotetraploid males (Nos. 4 and 5) derived from the male No. 5 used to obtain the second generation. A total of 9,132 eggs were fertilized with the sperm of autotetraploid males and refrigerated shortly after insemination to block the extrusion of their second polar bodies. Table 5 shows the developmental capacity of the third generation. In total, 1,708 (18.7%) embryos developed into normally feeding tadpoles.

A chromosomal examination of 1,128 of the 1,708 normal tadpoles revealed 590 (52.3%) tetraploids, 84 (7.4%) diploids, 257 (22.8%) triploids, one (0.1%) pentaploid, and three (0.3%) hexaploids. The remaining 193 tadpoles were 42 (3.7%) aneuploids and 151 (13.4%) mosaics. The 42 aneuploids consisted of five (0.4%) hyperdiploids [including three (2n+1) and two (2n+2)], nine (0.8%) hypotriploids [including five (3n-2) and four (3n-1)], six (0.5%) hypertriploids [including three (3n+1), one (3n+1), and two (3n+3)], 10 (0.9%) hypotetraploids [including two (4n-3), two (4n-2), and six (4n-1)], and 12 (1.1%) hypertetraploids [including six (4n+1), five (4n+2), and one (4n+3)]. The remaining 151 individuals consisted of 44 (2n+4n), 92 (2n+6n), six (3n+5n), and nine (3n+4n, 2n+3n+4n+5n, and others) mosaics (Table 6).

Of the 590 tetraploid tadpoles, 575 (97.5%) completed metamorphosis and 466 (79.0%) of the frogs attained sexual maturity. Of a total 511 of analyzed juvenile and mature frogs, there were 328 (64.2%) females and 183 (35.8%) males.

DISCUSSION

The present results on *R. nigromaculata* show that this species was able to produce next-generation autotetraploids from more than half of the total eggs used by consecutive procedures of nuclear transplantation of a triploid nucleus, fertilization with diploid sperm, and egg refrigeration. Numerous descendants with the same viability as the control diploids were obtained through three generations. On the other hand, a small number of mosaics and aneuploid tadpoles were also produced through the three generations. If the

aneuploids were the product of abnormal processes of meiosis in testis, it may be valuable to examine the pairing of meiotic chromosomes in the first reduction division of spermatogenesis of autotetraploid males. The autotetraploid male used for the present study developed from an egg transplanted with a triploid blastula nucleus taken from a sibling fertilized egg. That is to say, the original autotetraploid possessed three genome sets from the same mother and one genome set from a father. Fankhauser and Humphrey (1950) produced autotetraploids of *Ambystoma mexicanum* by matings between triploid females and diploid males. Kawamura and Nishioka (1960) obtained autotetraploids of *R. japonica* by two methods, i.e., nuclear transplantation of a diploid blastula nucleus into diploid fertilized eggs, or heat-shock treatment of the diploid fertilized eggs before the first cleavages. If such methods can be applied to produce autotetraploids of *R. nigromaculata*, it is interesting to analyze the chromosome numbers of the offspring of autotetraploids to compare the incidence of the aneuploids among tetraploid tadpoles produced by different methods.

Autotetraploid males from the species *A. mexicanum* (Humphrey and Fankhauser, 1956; Fankhauser and Humphrey, 1959) and *X. laevis* (Gurdon, 1959) were completely sterile. Among tetraploid silkworms obtained by Vereiskaya and Astaurov (1962), amphidiploid females derived from matings between *Bombyx mori* and *B. mandarina* were as fertile as autotetraploid females of *B. mori*, while amphidiploid males from the same parentage were partly fertile, and autotetraploid males were completely sterile. In the plant kingdom, a number of artificially synthesized tetraploid plants have been reported (Myers, 1940; Lamm, 1943). A true autopolyploid is usually sterile because of improper pairing of the chromosomes at meiosis (Bell, 1967); the gametogenesis in autotetraploid plants is generally abnormal. The above findings are remarkably different from the findings on the fertility of autotetraploids from the species *R. nigromaculata* or *R. japonica*. However, it may be possible to produce fertile autotetraploid males of the species urodeles or *Xenopus* artificially in the future.

Fertile autotetraploid males are very useful materials for producing autotetraploids, amphidiploids, androgenetic homospermic diploids, and diploid nucleo-cytoplasmic hybrids in amphibians (Kawamura and Nishioka, 1963b).

When the same methods are applied to lower vertebrates, these artificial polyploids and diploids will be useful for the improvement of breeds in many species. Moreover, the artificial polyploid and diploid animals may help clarify the process of the polyploid species formation found in some areas of the world.

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