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Author(s)	Arai, Katsutoshi; Taniura, Kou; Zhang, Quanqi
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Production of Second Generation Progeny of Hexaploid Loach

Katsutoshi Arai,[†] Kou Taniura, and Quanqi Zhang

Faculty of Applied Biological Science, Hiroshima University,
Higashi-hiroshima, 739-8528 Japan

First generation of hexaploid loach *Misgurnus anguillicaudatus*, which had been produced by inhibiting the second polar body extrusion after fertilization in a natural tetraploid pair, were raised until the adult size. The sex ratio of these hexaploids did not deviate from 1:1, as predicted from the almost equal number of females and males in tetraploids as well as the all-female gynogenetic tetraploids. These observations suggested an involvement of the male heterogametic system in sex determination. Hexaploid females laid significantly larger eggs than tetraploids and diploids. Mature eggs of four mature hexaploid females (three were eu-hexaploid, while one was a hexaploid/tetraploid mosaic) were individually fertilized with spermatozoa of hexaploid (6n), tetraploid (4n), and diploid (2n) males. The progeny were viable and the majority of surviving fish from 6n female × 6n male, 6n × 4n, and 6n × 2n crosses were hexaploid, pentaploid, and tetraploid, respectively. Fertilization of eggs of diploid and tetraploid females with spermatozoa of hexaploids (2n × 6n and 4n × 6n) gave viable tetraploid and pentaploid progeny, respectively. Thus, hexaploid loach formed triploid gametes (eggs and spermatozoa) and produced viable second generation progeny.

Key words: polyploid, hexaploid, loach, *Misgurnus*, chromosome, reproduction

Triploids have been produced in many species of fish and shellfish, and their reproductive traits have been examined. However, there have been only a few studies of those in artificially induced tetraploids because of the technical difficulties in the production of tetraploids.[1-3] In the loach *Misgurnus anguillicaudatus* (Pisces: Cobitidae) which normally show 2n=50 in Japanese populations, natural tetraploids with 100 chromosomes have been found in specimens obtained from fish dealers[4,5] and their genetic tetraploidy was confirmed by the successful production of viable progeny from gynogenetically and androgenetically activated gametes of tetraploid specimens without any treatment to induce chromosome duplication.[6-8] Then, tetraploid strains were successfully reproduced by fertilizing gametes of adult tetraploids derived from the original pair of natural tetraploids.[7,9] Using diploid gametes of these tetraploid loach, it is possible to induce hexaploid individuals by inhibition of second polar body release just after fertilization. In the loach, hexaploids with 150 chromosomes were successfully induced by this method.[10] Zhang and Arai[9] examined DNA contents of erythrocytic and testicular cells of these hexaploid loach by flow cytometry and reported the occurrence of motile triploid spermatozoa in the testes of hexaploid males. However, artificial fertilization using the triploid spermatozoa has not been reported and no progeny have been produced using gametes of hexaploids. Although we have produced tetraploid strains using gametes of tetraploid loach, their reproductive traits have not yet been studied in detail.

In the present study, we observed sex ratio of diploids, tetraploids, meiotic-gynogenetic tetraploids, and hexaploids to estimate genetic sex determination in the polyploid loach. Then, we compared number and size of eggs of diploid, tetraploid, and hexaploid loach. Next, we fertilized eggs of diploid, tetraploid, and hexaploid females by spermatozoa of diploid, tetraploid, and hexaploid males in all possible combinations. Then, we assessed hatching, survival, and ploidy status of the second generation progeny of hexaploid loach.

Materials and Methods

Polyploid Fish Specimens

Normal diploid loach, which had been collected from rice fields in Sera Town, Hiroshima Prefecture, were used to obtain haploid gametes (Table 1). Diploid loach which were produced from various adult pairs (2n female × 2n male) and pooled as a stock in the facility of Hiroshima University were also used as a source of spermatozoa for breeding and examining sex ratios.

Production of the first generation of tetraploid progeny (Female coded: 024) from the original tetraploid pair was reported by Arai et al.[6,7] Parental tetraploid females and males were selected from the second generation (3026) which was produced by cross-fertilization between tetraploid individuals (4n × 4n) in the 024 family.[9] Their tetraploidy was confirmed by flow cytometry.[9] We used them to examine reproductive traits and to produce various progeny in 1996 and 1997 (Table 1).

Other groups of the second generation of tetraploid loach (4026, 4041)[9] were used to examine sex. Tetraploid gynogens (2043, 2045) which had been produced by inhibi-

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[†] Corresponding author (Present address: Laboratory of Aquatic Breeding Science, Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan).

Table 1. Summary of polyploid loach specimens used in the present study

Ploidy	Female code#	Examination for:		Cross with:		
		No. of eggs	Egg diameters	2n[2]	4n[4]	6n[5]
Diploid (2n)	6008	—	—	A	A	A
	6009	—	—	A	A	A
	6010	—	—	A	A	A
	6022	—	+	B	B	B
	6023	—	+	(B)[3]	(B)	—
	6029	—	+	C	C	C
	7020	+	+	(D)	(D)	—
	7028	+	+	(E)	—	—
	7029	+	+	(E)	—	—
	7030	—	—	(E)	—	—
	7031	+	+	(F)	—	—
	7032	+	+	(F)	(E)	—
Tetraploid (4n)	6011	—	—	A	A	A
	6020	—	+	B	B	B
	6021	—	+	B	B	—
	6030	—	+	—	C	—
	7002	+	+	(G)	(F)	(D)
	7003	+	+	(G)	(F)	(D)
	7015	+	+	H	—	—
	7016	+	+	(H)	—	—
	7017	+	+	—	G	—
	7018	+	+	—	G	—
	7022	+	+	I	H	—
	7023	+	+	J	(H)	—
	7024	+	+	—	I	—
	7025	+	+	J	I	—
	7026	+	+	K	—	—
	7027	+	+	—	I	—
Hexaploid (6n)	7036	+	+	—	JKL	D
	7037	+	+	—	JKL	D
	7038	+	+	—	—	D
	7039[1]	+	+	L	JKL	D

[1] Hexaploid/tetraploid mosaic.
[2] Letter indicates each diploid male individual.
[3] Parenthesis indicates cross made but used only to measure egg diameters because of poor egg quality.
[4] Letter indicates each tetraploid male individual.
[5] Letter indicates each hexaploid male individual.

tion of the second polar body release with hydrostatic pressure shock after fertilization with UV-irradiated carp spermatozoa (4n × UV/PS),[9] were also used to examine sex ratio.

Production of first generation of hexaploids (3047) from tetraploids was reported by Kijima et al.[10] Hexaploidy was induced by inhibiting the second polar body release with hydrostatic pressure treatment (700 Kg/cm², 1 min duration, 5 min after fertilization, 4n × 4n/PS).[10] Samples from these hexaploids were used to examine sex ratio. Mature hexaploid females were used for artificial fertilization in 1997 (Table 1), while mature males were used in both 1996 and 1997. Their hexaploidy was confirmed by flow cytometry after breeding.

Sex Ratio

Sex ratio was examined in diploids (pooled population as a stock), tetraploids (024, 3026, 4026, 4041), tetraploid gynogens (2043, 2045), and hexaploids (3047) by observing the sexual dimorphism of pectoral fins and body shape.[11] Individual gonads were microscopically observed to identify the sex as necessary.

Spawning, Fertilization and Incubation

Human chorionic gonadotropin (HCG, Gonadtropin[R], Teikoku Zouki, Co. Ltd.) was injected to induce ovulation according to the method described by Suzuki and Yamaguchi.[12] Spermatozoa were obtained by homogenizing testes and then diluted by the procedure described by Suzuki et al.[13] No HCG treatment was performed for the males in the 1996 experiments, but all the males were injected with HCG at the same dose as used in the females in 1997.

After measuring body length (BL) and body weight (BW) of females, eggs from each female were divided into three portions and each portion was fertilized with spermatozoa of either diploid, tetraploid or hexaploid loach (Table 1). Fertilized eggs of each cross were incubated in an enamel pan half-filled with freshwater. After absorption of yolk, all the resultant progeny were first fed brineshrimp *Artemia* sp. followed by artificial goldfish food about one month later. Dried water fleas, *Daphnia* spp., and ground dried grass were also fed as supplementary foods. The progeny from each cross were reared in separated tanks.

Measurement of Number and Size of Eggs

After fertilization, the total number of eggs spawned was estimated for each female by counting eggs for crossing. Diameters of eggs of diploid, tetraploid, and hexaploid females were measured at two or three h after fertilization, when the water absorption was completed and already cleaved, using a Nikon Profile Projector Model 6C (Nihon Kogaku, Co. Ltd.).

Hatching and Survival Rates

Numbers of hatched fry at three days after fertilization were recorded in each cross to calculate hatching rate. Numbers of surviving fry at one week after fertilization, just before or after the beginning of feeding were counted in each cross to calculate survival rate of feeding fry relative to the number of hatched fry.

Determination of Ploidy Status

Ploidy status of the parental fish used and the resultant progeny was assayed by measuring relative DNA contents of erythrocytes or other somatic cells isolated from various parts such as gills by flow cytometry. Preparation of samples for DNA flow cytometry was previously described by Zhang and Arai.[9] The DNA contents of samples were analyzed by excitation at 488 nm with a laser (Coulter EPICS Profile II or Becton Dickinson FACS Calibur flow cytometer).

Statistical Analysis

Fit to a 1:1 sex ratio in the progeny of each cross was examined by χ^2 test. Differences in egg numbers, egg numbers per g body weight, egg diameters, hatching rates, and survival rates between ploidies were assessed using one-way analysis of variance (ANOVA) followed by the Scheffe test. A value of $P < 0.05$ was used as the criterion for significant difference.

Results

Table 2 shows sex ratios of diploid ($2n \times 2n$), tetraploid ($4n \times 4n$), gynogenetic tetraploid ($4n \times UV/PS$), and hexaploid ($4n \times 4n/PS$) loach. Sex ratio did not deviate significantly from 1:1 (χ^2 test, $P > 0.05$) in all the crosses of diploid, tetraploid, and hexaploid loach. No males were observed among gynogenetic tetraploids ($4n \times UV/PS$).

Numbers of eggs ovulated were estimated by counting the eggs used for artificial fertilization. As shown in Table 3, both tetraploids and hexaploids laid fewer eggs than diploids. However, differences in the number of eggs per g body weight were not statistically significant among the three groups.

Average egg diameters were compared among diploid, tetraploid, and hexaploid parent groups (Fig. 1). The results demonstrated that hexaploids laid larger eggs than tetraploids ($P < 0.05$), and tetraploids laid larger eggs than diploids ($P < 0.05$).

Developmental capacity of the progeny of hexaploids was examined by observing hatching rates at three days after fertilization and survival rates at one week after fertilization (Table 4). In the progeny of diploid females, both $2n \times 2n$ and $2n \times 4n$ crosses showed similar hatching rates, but $2n \times 6n$ crosses showed significantly lower rates ($P < 0.05$). Among the progeny of tetraploid females ($4n \times 2n$, $4n \times 4n$, $4n \times 6n$), significant differences were not

Table 2. Sex ratios in diploid, tetraploid, gynogenetic tetraploid, and hexaploid loach

Ploidy	Cross	Female code#	Origin of female	No. of fish	Sex		
					Female	Male	Unknown
Diploid	$2n \times 2n$	Mixed	Wild[*4]	31	16	14	1
Tetraploid	$4n \times 4n$	024	Dealer[*5]	6	2	4	0
		3026a[*3]	024	58	30	28	0
		3026b[*3]	024	42	20	22	0
		4026	024	16	9	7	0
		4041	024	20	12	8	0
		Sum		142	73	69	0
Gynogenetic tetraploid	$4n \times UV/PS$[*1]	2043	024	17	15	0	2
		2045	024	10	10	0	0
		Sum		27	25	0	2
Hexaploid	$4n \times 4n/PS$[*2]	3047	024	23	11	10	2

[*1] Fertilized with UV-irradiated sperm, then second polar body release was inhibited by pressure shock (PS).
[*2] Second polar body release was inhibited by pressure shock (PS).
[*3] Two different crosses using the same female.
[*4] Collected from Sera Town, Hiroshima Prefecture.
[*5] Obtained from the dealer, but the exact origin is unknown.

Table 3. Number of eggs ovulated in diploid, tetraploid and hexaploid loach

| Ploidy	No. of females	BW (g)	BL (cm)	No. of eggs	No. of eggs/g BW
Diploid	5	17.58 ± 2.30	15.9 ± 0.8	$4106.6 \pm 1601.8^{a*}$	$238.4 \pm 95.5^{a*}$
Tetraploid	6	14.52 ± 4.24	14.3 ± 1.8	1833.5 ± 652.2^{b}	140.0 ± 68.6^{a}
Hexaploid	4	12.10 ± 0.69	13.7 ± 0.5	1544.0 ± 632.3^{b}	127.0 ± 48.6^{a}

* Same superscript letters indicate no significant differences ($P > 0.05$) by Scheffe test.

Table 4. Hatching (%) and survival (%) of the progeny of diploid, tetraploid, and hexaploid loach

Cross	Hatching (%)			Survival (%)[*2]		
	Mean ± SD	(Range)	No. of cross	Mean ± SD	(Range)	No. of cross
2n × 2n	25.4 ± 5.3[a][*1]	(18.9–31.6)	5	64.2 ± 27.2[ab][*1]	(18.4–88.1)	5
2n × 4n	23.4 ± 11.1[a]	(12.4–39.8)	5	82.2 ± 13.7[b]	(60.9–95.0)	5
2n × 6n	3.0 ± 3.7[b]	(0–8.2)	5	40.4 ± 9.43[a]	(30.3–51.1)	3
4n × 2n	29.1 ± 25.8[a][*1]	(3.4–74.7)	8	54.0 ± 25.5[a][*1]	(10.4–73.3)	8
4n × 4n	30.8 ± 20.8[a]	(1.5–69.5)	10	66.1 ± 19.0[a]	(25.3–88.8)	10
4n × 6n	17.3 ± 10.3[a]	(10.0–24.5)	2	53.8 ± 39.0[a]	(26.2–81.4)	2
6n × 2n	12.5	—	1	86.1	—	1
6n × 4n	56.9 ± 13.3[a][*1]	(42.4–68.4)	3	82.6 ± 10.2[a][*1]	(73.4–93.6)	3
6n × 6n	54.8 ± 12.4[a]	(41.3–70.7)	4	82.9 ± 6.7[a]	(78.0–92.3)	4

[*1] Different letters indicate significant differences ($P < 0.05$ by Scheffe test) among the progeny of diploid, tetraploid, or hexaploid females.
[*2] At one week after fertilization, relative to the number of hatched fry.
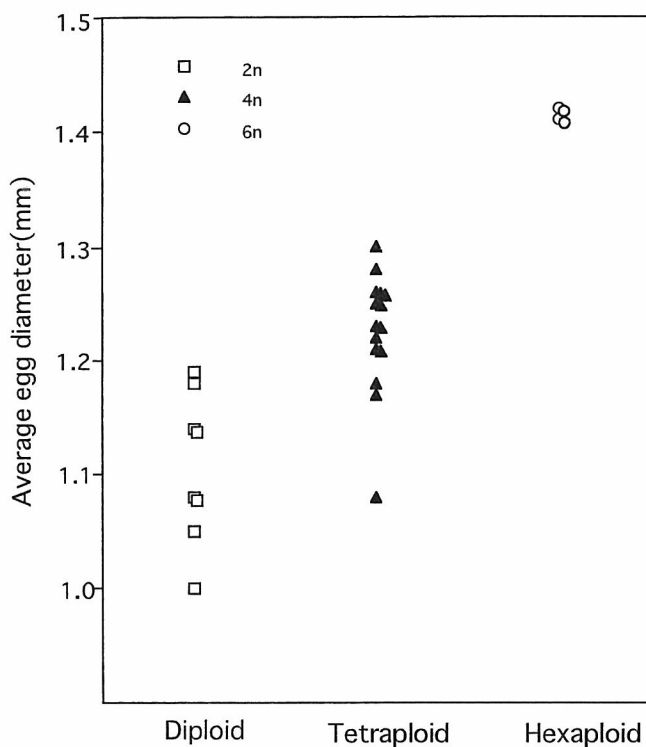
Fig. 1. Average egg diameters (mm) of diploid (open squares, number of females=8), tetraploid (solid triangles, $n=15$) and hexaploid loach (open circles, $n=4$).

Differences among the three ploidy groups were significant (Scheffe test, $P < 0.05$).

Table 5. Ploidy status of the progeny obtained from crosses having hexaploid loach as female and/or male parent

Female code#	Cross	Total	Ploidy					
			3n	4n	5n	6n	5.5n	6/5n[*2]
6011	4n × 6n	4	—	—	4	—	—	—
6022	2n × 6n	5	—	5	—	—	—	—
7036	6n × 4n	10	—	—	10	—	—	—
	6n × 6n	10	—	—	—	9	1	—
7037	6n × 4n	6	—	—	6	—	—	—
	6n × 6n	6	—	—	—	6	—	—
7038	6n × 6n	11	—	—	—	11	—	—
7039[*1]	6n × 2n	4	1	3	—	—	—	—
	6n × 4n	6	—	—	6	—	—	—
	6n × 6n	4	—	—	—	3	—	1

[*1] 6n/4n mosaic.
[*2] 6n/5n mosaic.

observed in survival rate between 2n × 2n and 2n × 4n or between 2n × 2n and 2n × 6n. Surviving fry from each cross had normal external appearance. These results showed that both eggs and spermatozoa of hexaploids produced viable progeny by artificial fertilization. Thus, the gametes of hexaploids were fertile.

Flow cytometric assays after breeding revealed that all the parental loach from 2n × 2n, 4n × 4n, and 4n × 4n/PS crosses were diploid, tetraploid, and hexaploid, respectively, as predicted, except for female 7039 which was confirmed to be a hexaploid/tetraploid mosaic. Ploidy status of the progeny of hexaploid loach based on DNA content of somatic cells was shown in Fig. 2 and Table 5. The progeny obtained from 4n × 6n and 2n × 6n crosses were pentaploid and tetraploid, respectively. The majority of the progeny obtained from 6n × 6n and 6n × 4n crosses were hexaploid and pentaploid, respectively. These results clearly indicated that hexaploid loach produced triploid gametes (eggs or spermatozoa). However, one out of 10 progeny obtained from the 6n × 6n cross of female 7036 demonstrated 5.5C DNA content. This fish seemed to be a 5.5n aneuploid, but no external abnormalities were observed.

Female 7039 which was confirmed by flow cytometry to be a hexaploid/tetraploid mosaic gave one triploid and three tetraploid progeny from the cross with diploid male (Table 5). In the 6n × 4n cross, no progeny which devel-
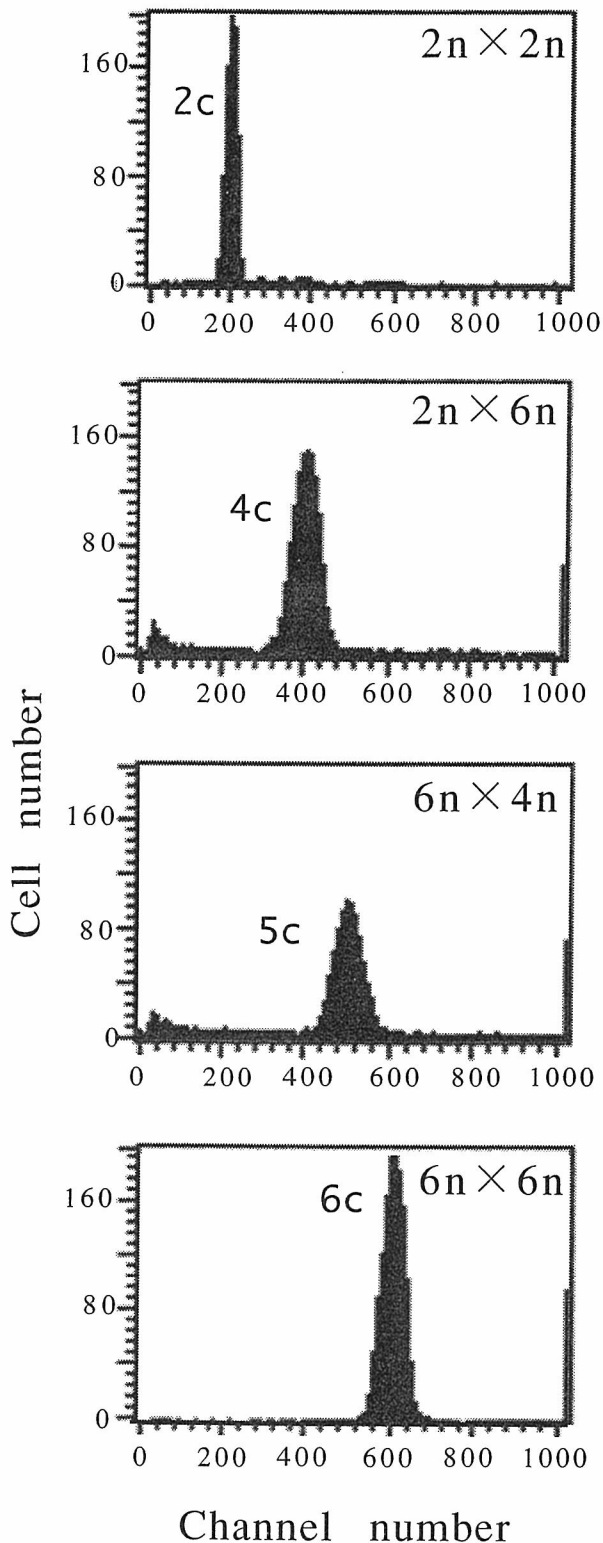
observed in hatching rate. No significant differences were observed in hatching rate between 6n × 4n and 6n × 6n crosses. Cross between hexaploid female and diploid male (6n × 2n) gave a lower rate of hatching than 6n × 4n and 6n × 6n.

Survival rates at one week after fertilization showed no significant differences among the progeny of tetraploid females (4n × 2n, 4n × 4n, 4n × 6n). No significant differences were observed in survival rate between 6n × 4n and 6n × 6n crosses. The 6n × 2n cross showed almost the same survival rate as those observed for 6n × 4n and 6n × 6n crosses. Progeny of hexaploid males (2n × 6n) showed significantly lower survival rates than those of tetraploid males (2n × 4n) ($P < 0.05$). No significant differences were

Fig. 2. Flow cytometric histograms of somatic cell suspension of 2n (female) × 2n (male), 2n × 6n, 6n × 4n and 6n × 6n crosses, representing 2C, 4C, 5C and 6C DNA content, respectively.

oped from diploid eggs derived from tetraploid cells of the mosaic parent were observed. Three hexaploids and a hexaploid/pentaploid mosaic were recorded in the 6n × 6n cross.

Discussion

Genetic Sex Determination

Genetic sex determination in the diploid loach has been estimated to be due to male heterogamety (XX female, XY male), because of the exclusive occurrence of females (95%) in diploid gynogens generated by inhibiting the second polar body release after fertilization with UV-irradiated spermatozoa and predicted to be all genetic females (XX).[13] The sex ratio of normally fertilized diploid loach (2n × 2n) is 1:1, as observed in the present crosses. The present results regarding the sex ratios of tetraploids (4n × 4n) also showed the occurrence of almost equal numbers of females and males. The all-female offspring observed from gynogenetic tetraploids (4n × UV/PS) produced by inhibiting the second polar body release after fertilization with UV-irradiated spermatozoa strongly suggest that sex is determined by male heterogamety in tetraploid loach. The predominance of males was reported in the second generation of artificially induced tetraploid rainbow trout produced by suppression of the first cleavage.[1] This was elucidated by the XXYY male genotype of the first generation of tetraploids, which were likely to produce more males than females due to more XY and YY than XX spermatozoa.[1] However, this is not the case in natural tetraploid loach, because they showed a 1:1 sex ratio in the progeny from 4n × 4n crosses. These results suggest that the genotype of the first generation of natural tetraploid males is XXXY and likely to generate the balanced 1:1 sex ratio by fertilization with eggs probably with the XXXX genotype. Thus, the first generation hexaploid males (4n × 4n/PS), which were produced by inhibiting the second polar body release after 4n × 4n cross-fertilization,[10] are probably XXXXXY and likely to form equal numbers of XXX and XXY spermatozoa which will allow the 1:1 sex ratio in the second generation. However, this assumption should be confirmed in future by examining the sex ratio of the second generation of hexaploids (6n × 6n) produced in the present study.

Number and Size of Eggs

The present observations regarding the fecundity of polyploid loach revealed that eggs of hexaploids were larger than those of diploids and tetraploids. The results obtained here were in accordance with those observed in tissue structures of triploid stickleback[14] and ayu,[15] and in erythrocytes of triploid loach,[5] grass carp × silver carp hybrid,[16] carp,[17] and Atlantic salmon.[18] In the triploid loach produced by hybridization beween normal diploids and natural tetraploids, unreduced triploid eggs are larger than normal haploid eggs.[19] Thus, it is generally accepted that cellular or egg sizes increase in proportion with ploidy status. In somatic tissues such as erythrocytes,[16-18] retinal cells,[14,15] kidney,[14,15] and intestine,[14,15] polyploid fish showed fewer numbers of cells when compared with diploids. In the present study, fewer numbers of eggs were observed in hexaploid females than in tetraploids and diploids. However, statistically significant decreases in the number of eggs per *g* body weight were not seen in the three groups of loach.

Viable Progeny of Hexaploid Loach

The present study demonstrated that hexaploid females produced viable progeny by fertilization of their eggs with spermatozoa from diploid, tetraploid, and hexaploid males. Similarly, tetraploid females showed no significant differences in hatching rate when crossed with diploid, tetraploid, and hexaploid males. In contrast, spermatozoa from hexaploid males gave significantly low hatching rates and survival of feeding fry when used to fertilize eggs of diploid females. In this study, although we did not record fertilizability by counting cleaved eggs in the early developmental stages, the low hatching rates might be related to the low fertilizability of triploid spermatozoa formed in hexaploid loach. In crosses between diploid females and tetraploid males rainbow trout, low fertilizability was reported and was explained as a result of the difficult penetration of diploid spermatozoa with larger head sizes than haploid spermatozoa into the micropyle canal of normal eggs.[1] To verify this idea in the loach, further studies are required not only for fertilizability of triploid spermatozoa but also for electron microscopy for sperm head and micropyle sizes.

Ploidy Status of the Progeny of Hexaploid Loach

The majority of the progeny of hexaploid females (7036, 7937, and 7038) assayed by flow cytometry in the crosses 6n × 4n and 6n × 6n were pentaploid and hexaploid, respectively. The 2n × 6n and 4n × 6n crosses of hexaploid males gave tetraploid and pentaploid progeny, respectively. These results indicated that hexaploid loach normally produced triploid gametes (eggs and spermatozoa) by normal meiotic division and reproduced by normal bisexual fertilization.

However, a 5.5n loach was detected in the 6n × 6n progeny of the 7036 hexaploid female. This was very likely to be viable aneuploid with chromosome numbers between hexaploid and pentaploid. Therefore, we cannot exclude the possibility of the formation of hypo- and hypertriploid gametes in hexaploids in addition to the major population of triploid gametes. In addition to the occurrence of such a clear aneuploid, we cannot exclude the possibility of aneuploids with minor surplus and/or loss of chromosome(s), because we did not determine ploidy by chromosome counting but by determining DNA content by flow cytometry. It is generally difficult to identify aneuploids with minor changes in chromosome number by flow cytometry. The same problem may occur in the gametes of natural tetraploids. The products of unbalanced gametes and resultant aneuploid individuals have been reported in tetraploid rainbow trout[2] and polyploid anurans.[20-22] Thereby, further cytogenetic studies of the progeny of hexaploid loach are required.

Mosaicisim

A hexaploid-tetraploid mosaic female (7039) produced two types of eggs, triploid and diploid, probably derived from hexaploid and tetraploid cells, respectively. However, the occurrence of the progeny of diploid eggs, i.e. a triploid in the 6n × 2n and a hexaploid/pentaploid mosaic in the 6n × 6n, was relatively low (14.3%, 2/14). Such a ratio between the two egg types (14% diploid eggs and 86% triploid eggs) in this mosaic individual coincided with the previous result probably in the same fish which showed 84% hexaploid cells and 16% tetraploid cells in erythrocytes.[9] Thus, somatic mosaicism might be transmitted to the germline in almost the same ratio. Consequently, the majority (86%) of eggs laid by female 7039 were considered to be triploid. One hexaploid/pentaploid progeny was detected in the 6n × 6n progeny of this female, but it was quite difficult to elucidate the mechanism responsible for the occurrence of such mosaic progeny.

Additional Evidence for Genetic Tetraploidy

Successful reproduction of the hexaploid loach provides additional evidence to support the hypothesis that natural tetraploid loach are not rediploidized tetraploids (2n=100) which are now diploid and have evolved back from a tetraploid ancestor, but evolutionarily young tetraploids (4n=100) with four sets of homologous chromosomes.[6-8] If the tetraploids were rediploidized tetraploid (2n=100), hexaploid loach with 150 chromosomes would be "triploid (3n=150)" and then "expression of sterility" would be predicted as reported in artificial triploids induced from normal diploid loach by chromosome manipulation.[23] However, the hexaploid loach produced by the manipulation of fertilized eggs of tetraploid loach were apparently fertile as shown in the present study. Thus, the natural tetraploid progenitor in the loach was very likely to be a genetically true tetraploid (4n=100) with four sets of homologues.

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