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Ploidy Manipulation Using Diploid Sperm of a Wild Tetraploid Ginbuna (Japanese Silver Crucian Carp, *Carassius auratus langsdorfii*)

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Abstract

Tetraploid strains are important sources of diploid gametes available for further expansion of ploidy manipulation. Although artificial induction of tetraploid strains has been attempted by inhibition of a mitotic cell division of zygotes using hydrostatic pressure or temperature treatments at the early developmental stage, successful examples of viable and fertile tetraploid fish were very rare. Because most resultant tetraploid progeny exhibited extremely high mortality. Natural tetraploid variants are considered another source of diploid gametes. In the present study, we tried to induce new strains of tetraploid, triploid and androgenetic diploid using diploid sperm of a tetraploid ginbuna (Japanese silver crucian carp *Carassius auratus langsdorfii*), which caught at the Jounuma lake, Gunma Prefecture, Japan. New tetraploids, i.e., neo-tetraploids were induced by fertilizing eggs of a diploid goldfish *Carassius auratus auratus* with diploid sperm of the tetraploid ginbuna, followed by heat-shock (40°C for 45, 60 and 75 s) to inhibit the second polar body release at 5 min after fertilization. Although a small number of neo-tetraploid fish survived, we could not obtain any fertile gametes from them in the present study. Triploids were successfully induced by fertilizing eggs of a diploid goldfish with diploid sperm of the tetraploid ginbuna. Some resultant triploid males produced aneuploid sperm at the age of maturation. Androgenetic diploids were induced by fertilizing UV-irradiated eggs of diploid goldfish with diploid sperm of the tetraploid ginbuna. A mature androgenetic diploid produced fertile haploid sperm.

Key words : Androgenesis, Aneuploid, Chromosome manipulation, Cyprinidae, Diploid gamete, Gametogenesis, Mosaic, Polyploid, Sterility

Introduction

Tetraploid individuals are considered useful tool for chromosome manipulation to induce various kinds of polyploids, because they are expected to produce diploid gametes. Thus, diploid gametes of tetraploid individuals can be used to produce triploid progeny without any chromosome manipulation. Tetraploid individuals are induced theoretically by the inhibition of mitotic cell division using hydrostatic pressure or temperature treatment after fertilization. However, the induction of tetraploid is very difficult when compared with completely homozygous gynogenetic diploids, i.e. doubled haploids (DHs), although the same treatment is used for chromosome doubling as reviewed by Pandian and Koteeswaran (1998), Arai (2001), Piferrer et al. (2009), and Arai and Fujimoto (2019). In masu salmon, when tetraploids and gynogenetic DHs were induced by the same treating conditions using single-pair mating with the same parental fish, both tetraploids and gynogenetic DHs survived until the hatching stage. However, tetraploids could not develop beyond the feeding stage, while homozygous gynogenetic DHs could feed and grow up normally (Sakao et al., 2006). Induction of viable tetraploids is practically very difficult, but once tetraploid strains have been established, they can be maintained by cross-breeding between tetraploid females and males. For the mass production and subsequent aquaculture of sterile triploids by cross-breeding between diploid and tetraploid fish, tetraploid parents have been utilized in oyster as well as

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in rainbow trout as reviewed by Piferrer et al. (2009) and Arai and Fujimoto (2019). Unfortunately, such successful examples were so far reported in a limited number of aquatic species.

On the other hand, some fish species include polyploid variants within the species in nature. In cypriniforms, natural tetraploid variants can be seen in genera *Misgurnus* (Arai et al., 1991a, b, 1993 ; Li et al., 2008), *Cobitis* (Kusunoki et al., 1994 ; Janko et al., 2007 ; Bartoš et al., 2019), *Carassius* (Kobayasi et al., 1970 ; Liu et al., 1980 ; Onozato et al., 1983 ; Yamaha et al., 2002a). Especially in tetraploid dojo loach *M. anguillicaudatus* which were found in Japanese market specimens (Arai et al., 1991a, b, 1993) as well as in Chinese wild populations (Li et al., 2008), diploid gametes derived from the tetraploid fish were not only used for the maintenance of the tetraploid line, but also for the production of higher polyploids such as pentaploidy and hexaploidy by combining with chromosome manipulation including the inhibition of second polar body release (Zhang and Arai, 1996 ; Arai et al., 1999 ; Arai and Fujimoto, 2013). Thus, the use of diploid gametes from natural tetraploid variants is considered another option to induce polyploid fish.

In ginbuna or Japanese crucian carp *Carassius auratus langsdorfii*, diploid, triploid and tetraploid individuals synpatrically arise in Japanese wild populations (Kobayasi et al., 1970 ; Liu et al., 1980 ; Onozato et al., 1983 ; Umino et al., 1997 ; Mada et al., 2001a ; Maeda et al., 2001 ; Yamaha et al., 2002a). Although diploid and triploid fish are frequently found in Japanese wild populations, tetraploid fish were relatively rare (Onozato et al., 1983 ; Umino et al., 1997 ; Mada et al., 2001a ; Maeda et al., 2001 ; Yamaha et al., 2002a). The triploid population is essentially all-female, because its reproductive mode is clonal by gynogenesis due to apomixis (Yamashita et al., 1993). Furthermore, tetraploid females gynogenetically produce clonal tetraploid progeny as verified by the results from scale graft transplantation (Nakanishi and Ototake, 1999) and isogenic DNA genotyping on artificially reproduced progenies (Mada et al., 2001a, Yamaha et al., 2002a ; Dong et al., 2013). Thus, tetraploid ginbuna females should generate unreduced tetraploid eggs which were genetically identical to somatic cells of the mother and reproduce without any genetic contribution of sperm donor. In contrast, tetraploid males produce diploid sperm which could induce triploid progeny by artificial fertilization between diploid female and the tetraploid male (Murakami and Fujitani, 1997). Although progeny of such tetraploid males have not been analyzed genetically, they are likely to be a source of diploid gametes for further expansion of chromosome manipulation if they proceed regular gametogenesis and normal maturation.

Here, we tried to induce new tetraploid, i.e., neo-tetraploid, lines using fertilization of eggs of a diploid goldfish with fertile diploid sperm of a tetraploid ginbuna male, followed by chromosome manipulation to inhibit the second polar body release. We also produced triploid progeny by normal cross-fertilization between diploid female (haploid eggs) and tetraploid male (diploid sperm) as well as androgenetic diploid progeny exclusively with diploid genomes derived from tetraploid ginbuna by fertilization of genetically inert UV-irradiated eggs of goldfish. Then, reproductive capacity of resultant progeny was examined. The usefulness of natural tetraploid ginbuna male was discussed from the viewpoint of genetic resources to provide diploid gametes.

Materials and Methods

Experimental fish and gamete collection

A male tetraploid ginbuna, *Carassius auratus langsdorfii*, was caught at the Jounuma lake, Tatebayashi city, Gunma Prefecture, Japan, and transported to Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Nanae town, Hokkaido. Diploid female and male goldfish, *C. auratus auratus* were reared at the laboratory. Ovulation and spermiation were induced by hormonal injection of 10 U/g body weight of hCG (Asuka Pharmaceutical Co. Ltd. Tokyo). Eggs were collected on the polyvinylidene chloride film after ovulation which occurred at 12-16 h after the injection under the incubating condition at 20°C. Sperm were collected into hematocrit tube and kept at 4°C.

Induction of diploid, triploid and tetraploid progeny using sperm derived from a natural tetraploid

In this study, induction of tetraploid (neo-tetraploid) and androgenetic diploid using diploid sperm from the tetraploid ginbuna by chromosome manipulation were conducted from July to August in 2005, and triploid induction using the diploid sperm was conducted in November 2007. Artificial fertilization was performed by dry method. Eggs and sperm were activated by a modified Woynarovich's solution (0.5% NaCl and 0.4% Urea dissolved in dechlorinated tap water ; Yamaha et al., 1986) for fertilization, and then, fertilized eggs were kept at 20°C. Diploid and triploid progeny were induced by fertilizing eggs from a diploid goldfish with haploid sperm from a diploid goldfish and diploid sperm from the tetraploid ginbuna, respectively. Triploid and neo-tetraploid progeny were induced by a heat-shock treatment at 40°C to suppress second polar body release at 5 min after fertilization in the crosses using haploid sperm of a diploid goldfish and diploid sperm of the tetraploid ginbuna, respectively. To examine the optimum duration of the heat-shock treatment in the induction of tetraploid, fertilized eggs were divided into four groups, and one was intact control and the others were treated for 45, 60 and 75 s duration. For induction of neo-tetraploid and triploid progeny using the diploid sperm, six experiments (Exp. 1-6) were carried out, but hatching rate

and normal larvae rate were not recorded in Exp. 4–6.

In order to induce androgenesis, eggs from a diploid goldfish were genetically inactivated by UV irradiation at a dose of 250 mJ according to the previous study (Fujimoto et al., 2007). The UV-irradiated eggs were fertilized with diploid sperm from the tetraploid ginbuna to induce diploid androgenotes and with haploid sperm from a diploid goldfish to induce haploid androgenotes, respectively. Two sets of experiments (Exp. 7 and 8) were carried out to produce androgenetic diploid lines.

After the treatment in all experiments mentioned above, the modified Woynarovich's solution used at fertilization was changed to dechlorinated tap water and eggs were incubated at 20°C. Then, dead eggs were removed every day until hatch. Larvae after yolk absorption were fed with *Artemia* larvae.

Verification of reproductive capacity of induced tetraploid, triploid and androgenetic diploid fish

Three 2-year-old neo-tetraploid, twenty three 1-year-old triploid and two 2-year-old androgenetic diploid progenies were injected with hCG (10 U/g body weight ; Asuka Pharmaceutical Co. Ltd. Tokyo) to induce ovulation and spermiation, when they grew up to mature fish size over 450 mm in standard length. Ovulation or spermiation was examined in 12–16 h after the injection. Sperm was collected into hematocrit tube and the ploidy status was analyzed (see below).

Ploidy analysis

To determine the ploidy, the relative DNA content of somatic cells and sperm of parental fish used in this study, larvae obtained from Exp. 1, 2, 4–7, semen containing sperm or sperm-like cells and testes of mature-sized progeny were measured by flow cytometry. In order to compare the relative DNA content, fluorescent peak was calibrated by somatic cells of a diploid goldfish in each experiment. Sample preparations and measurement procedures were according to the previous study (Fujimoto et al., 2007).

Results

Induction of neo-tetraploid lines

Goldfish and the tetraploid ginbuna males used in this study were diploid and tetraploid, respectively (Fig. 1A, B). The ploidy of their sperm was haploid and diploid, respectively (Fig. 1C, D).

Hatching and normal larvae rates in the induction of new tetraploid (neo-tetraploid) lines by the retention of the second polar body are shown in Table 1. The diploid sperm of the tetraploid ginbuna gave lower hatching rates (0.791–0.835) than the cases of haploid sperm of a goldfish (0.875–0.953). The hatching rates in heat shock groups using diploid sperm of the tetraploid ginbuna were lower (0.338–0.573) than those using haploid sperm of a goldfish (0.573–0.705). The normal larvae rates in heat shock groups using diploid sperm of the tetraploid ginbuna were lower (0.105–0.448) than those using haploid sperm of a goldfish (0.564–0.662). In heat-shock groups using the diploid sperm, all the treated groups showed lower hatching rates (0.338–0.573) than those of control groups (0.791–0.835). They also showed lower normal larvae rates (0.105–0.448) than those of control groups (0.981–0.990). Similar decrease was also observed in crosses using the haploid sperm of a goldfish.

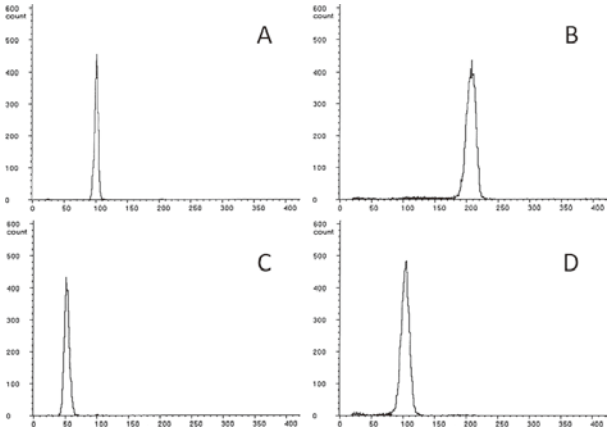

Fig. 1. Flow-cytometrical histograms for relative DNA content of nuclei of somatic cells and sperm from a diploid goldfish and the tetraploid ginbuna male, respectively. A : Somatic cell of a diploid goldfish, B : Somatic cells of the tetraploid ginbuna male, C : Haploid sperm of a diploid goldfish, D : Diploid sperm of the tetraploid ginbuna male.

Ploidy of hatching larvae with normal external appearance were measured by flow-cytometry in five experiments Exp. 1, 2, 4–6, and the results were shown in Table 2. In crosses using haploid sperm from a diploid goldfish, all larvae without the heat-shock treatment were diploid. But in the heat-shock treatment, rates of triploid induction were less than 30% (3 out of 11 individuals in total) and unexpected tetraploid, mosaic and aneuploid larvae appeared. On the other hand, triploid larvae with normal external appearance were successfully induced by fertilizing eggs of a diploid goldfish with diploid sperm of the tetraploid male. Neo-tetraploid larvae with normal external appearance were successfully induced in three out of five experiments. Neo-tetraploid larvae were observed in all kinds of duration for heat-shock examined, especially in 60 and 75 s. Only exp. 1 included high rates (4 / 5) of normal neo-tetraploid larvae. Most resultant larvae in other experiments were triploid, mosaic, aneuploid and/or diploid.

Induction of androgenetic diploid

Androgenetic diploid progeny possessing diploid genome derived from the tetraploid ginbuna were induced by the fertilizing UV-irradiated eggs from a diploid goldfish with dip-

Table 1. Developmental potential of the embryos from the experimental crosses with heat-shock treatment using sperm of the tetraploid ginbuna.

	Female	Male	Treatment[1]	Number of eggs used	Hatching rate[2]	Normal larvae rate[3]
Exp. 1	2n goldfish	2n goldfish	Control	317	0.953	0.983
			60s	315	0.705	0.662
		4n ginbuna	Control	364	0.835	0.990
			45s	234	0.573	0.448
			60s	301	0.551	0.265
			75s	394	0.500	0.142
Exp. 2	2n goldfish	2n goldfish	Control	399	0.875	0.980
			60s	356	0.573	0.564
		4n ginbuna	Control	380	0.813	0.981
			45s	257	0.374	0.417
			60s	278	0.428	0.185
			75s	315	0.422	0.105
Exp. 3	2n goldfish	4n ginbuna	Control	301	0.791	No data
			45s	352	0.491	No data
			60s	461	0.447	No data
			75s	334	0.338	No data

1 : Heat shock treatments (40°C at 5 min after fertilization) were performed at three kinds of durations (45, 60 and 75s) to determine an optimum condition for suppressing second polar body release in experimental crosses using the tetraploid ginbuna male.
2 : Hatching rates were calculated from the number of hatching larvae relative to the number of total eggs used.
3 : Normal larvae rates were calculated from the number of hatching larvae with normal external appearance relative to the total number of hatching larvae.

loid sperm of the tetraploid ginbuna. Hatching and normal larvae are shown in Table 3. Ploidy of resultant larvae in Exp. 7 is shown in Table 4. Hatching rates of crosses between a diploid goldfish female and a diploid goldfish male and between a diploid goldfish female and the tetraploid gunbuna male were from 0.608 to 0.784. In contrast, crosses using UV-irradiated eggs showed decreased hatching rates from 0.019 to 0.145. Normal larvae were appeared from the crosses using eggs without UV-irradiation and the cross between UV-irradiated eggs and diploid sperm of the tetraploid ginbuna, though normal larvae never appeared in the crosses between UV-irradiated eggs and haploid sperm of a diploid goldfish. Ploidy of larvae derived from eggs fertilized with haploid sperm of a goldfish and diploid sperm of the tetraploid ginbuna were diploid and triploid, respectively. Abnormal larvae arisen from UV-irradiated eggs fertilized with haploid sperm of a diploid goldfish were haploid. Both normal and abnormal larvae in the cross between UV-irradiated eggs and diploid sperm of the tetraploid ginbuna were diploid.

Ploidy status of six-month-old progeny

Ploidy status of 6-month-old progeny is shown in Table 5. In control groups without the heat-shock treatment, diploid and triploid progeny were confirmed in crosses using sperm of a diploid goldfish and the tetraploid ginbuna males, respectively. Triploids were also observed in fertilization between eggs of a diploid goldfish female and sperm of a diploid goldfish male followed by the heat-shock treatment. Only two neo-tetraploids out of 48 individuals were detected in fertilization between eggs of a diploid goldfish female and sperm of the tetraploid ginbuna male followed by the heat-shock treatment. In the androgenetic progeny induced by fertilization between UV-irradiated eggs and diploid sperm of the tetraploid ginbuna, two out of three progeny were diploid.

Reproductive capacity of neo-tetraploid, triploid and androgenetic diploid progeny

When three 2-year-old neo-tetraploid progeny were injected with hCG, ovulation or spermiation did not occur. In observation of the gonad of the neo-tetraploid, it was revealed that the gonad was string-like structure. Thus, the sex of the neo-tetraploid progeny could not be determined in this study.

In 1-year-old triploid progeny, which were induced by fertilization between eggs of a diploid goldfish and diploid sperm of the tetraploid ginbuna, and 2-year-old androgenetic diploid progeny, ploidy of their sperm and testes were analyzed. Ploidy of somatic cells of the progeny was also measured. Diploid goldfish were used as a standard for dip-

Table 2. Ploidy of larvae with normal external appearance obtained from the experimental crosses with heat-shock treatment using sperm from the tetraploid ginbuna.

	Female	Male	Treatment[1]	N	2n	3n	4n	Mosaic[2]	Aneuploid[3]
Exp. 1	2n goldfish	2n goldfish	Control	5	5	0	0	0	0
			60s	5	1	2	0	1	1
		4n ginbuna	Control	5	0	5	0	0	0
			45s	5	0	3	2	0	0
			60s	5	0	0	4	1	0
			75s	5	1	0	4	0	0
Exp. 2	2n goldfish	2n goldfish	Control	5	5	0	0	0	0
			60s	6	2	1	1	1	1
		4n ginbuna	Control	5	0	4	1	0	0
			45s	5	0	4	0	1	0
			60s	5	2	0	2	1	0
			75s	5	5	0	0	0	0
Exp. 4	2n goldfish	4n ginbuna	Control	6	0	5	0	0	1
			45s	11	0	8	0	0	3
			60s	12	0	11	0	0	1
			75s	10	0	6	0	0	4
Exp. 5	2n goldfish	4n ginbuna	Control	7	0	7	0	0	0
			45s	8	0	5	1	0	2
			60s	9	0	6	2	0	1
			75s	2	1	0	1	0	0
Exp. 6	2n goldfish	4n ginbuna	Control	6	0	6	0	0	0
			45s	5	0	3	0	1	1
			60s	10	0	6	0	1	3
			75s	5	0	2	0	1	2

1 : Heat shock treatments (40°C at 5 min after fertilization) were performed at three kinds of durations (45, 60 and 75s) to determine an optimum condition for suppressing second polar body release in experimental crosses using the tetraploid ginbuna male.
2 : Most mosaic individuals consisted of two different kinds of aneuploid cell populations.
3 : Most aneuploid individuals indicated DNA content between triploid and tetraploid.

Table 3. Developmental potential of androgenetic embryos induced by fertilization between UV-irradiated eggs of a diploid goldfish and sperm from the tetraploid ginbuna.

	Female	Male	Treatment[1]	Number of eggs used	Hatching rate[2]	Normal larvae rate[3]
Exp. 7	2n goldfish	2n goldfish	Control	376	0.681	0.527
			UV	485	0.144	0.000
		4n ginbuna	Control	456	0.618	0.362
			UV	1,452	0.145	0.176
Exp. 8	2n goldfish	2n goldfish	Control	232	0.608	0.567
			UV	153	0.066	0.000
		4n ginbuna	Control	278	0.784	0.569
			UV	1,223	0.019	0.087

1 : In this column, UV means UV irradiation to eggs for induction of androgenesis.
2 : Hatching rates were calculated from the number of hatching larvae relative to the number of total eggs used.
3 : Normal larvae rates were calculated from the number of hatching larvae with normal external appearance relative to the total number of hatching larvae.

Table 4. Ploidy of larvae derived from the induction of androgenesis using sperm from a diploid goldfish and the tetraploid ginbuna.

Female	Male	Treatment[1]	Morphology	N	1n	2n	3n	Mosaic[2]
2n goldfish	2n goldfish	Control	Normal	20	0	19	1	0
		UV	Abnormal	10	10	0	0	0
	4n ginbuna	Control	Normal	22	0	0	22	0
		UV	Normal	5	0	5	0	0
			Abnormal	10	0	10	0	0

1 : In this column, UV means UV irradiation to eggs for induction of androgenesis.
2 : The individual including this column had two cell populations consisted of hypo-triploid and hyper-triploid cells.

Table 5. Ploidy in 6 month-old fish derived from tetraploidization and androgenesis using sperm form the tetraploid ginbuna.

Female	Male	Treatment[1]	N	2n	3n	4n
2n goldfish	2n goldfish	Control	4	4	0	0
		HS	4	1	3	0
	4n ginbuna	Control	4	0	4	0
		HS	48	1	45	2
		UV	3	2	1	0

1 : In this column, HS means heat shock treatment to suppress a second polar body release and UV means UV irradiation to eggs for induction of androgenesis.

loid DNA content (Fig. 2A). The results of ploidy analyses were shown in Table 6. Sperm samples of six diploid goldfish were haploid (Fig. 2C). Two out of the six goldfish were sacrificed to measure the ploidy of their testicular cells. The testes of the goldfish were composed of haploid, diploid and tetraploid cell populations (Fig. 2B). In the triploid progeny, 23 individuals were sacrificed to confirm their sex and measure their ploidy of somatic cells and gonads. Twenty individuals were males, in which 19 males were triploid and one male was diploid, and three individuals were triploid female (Fig. 2D). We tried to obtain semen from nine triploid males by injection of hCG. Three out of the eight triploid males produced small amount of semen containing aneuploid (1.5n) sperm-like cells (Fig. 2G). Semen obtained from one triploid male contained only triploid cells. Any distinct cell populations in the semen were not detected by flow cytometry in the rest individuals. In the testes of triploid males, two types of ploidy compositions were observed. Thirteen out of 19 triploids showed two cell populations (3n

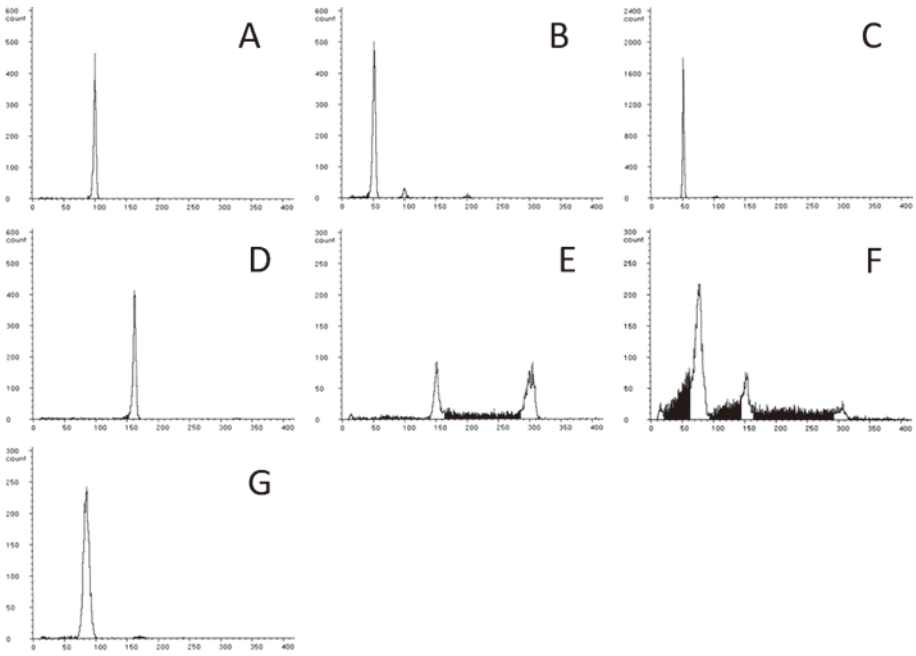

Fig. 2. Flow-cytometrical histograms for relative DNA content of somatic cells, testes and sperm of a diploid goldfish and triploid progeny. A : Somatic cells of a diploid goldfish, B : Testis of a diploid goldfish consisting of haploid, diploid and tetraploid cell population, C : Haploid sperm from a diploid goldfish, D : Somatic cells of a triploid progeny, E : Testis consisting of triploid and hexaploid cell population in a triploid progeny, F : Testis consisting of 1.5n, triploid and hexaploid cell population in a triploid progeny, G : Semen from a triploid progeny containing 1.5n cell population.

Table 6. Ploidy of testis and semen containing sperm or sperm-like cells in triploid and androgenetic progeny derived from the tetraploid ginbuna.

Female	Male	Treatment	Ploidy of somatic cells	N	Ploidy of testis			N	Ploidy of cells in semen			
					1n/2n/4n	1.5n/3n/6n	3n/6n		1n	1.5n	3n	ND[1]
2n goldfish	2n goldfish	Control	2n	2	2	0	0	6	6	0	0	0
	4n ginbuna	Control	3n	19	0	6	13	8	0	3	1	4
	4n ginbuna	UV[2]	2n	–	–	–	–	2	1	0	0	1

1 : ND means ploidy of cells included in the semen was not detected by flow cytometry because of low number of the cells.
2 : UV means UV irradiation to eggs for induction of androgenesis.

Table 7. Developmental potential and ploidy of progeny from an experimental cross using sperm of the androgenetic diploid.

Female	Male	Developmental potential			Ploidy of larvae		
		Number of eggs used	Hatching rate[2]	Normal larvae rate[3]	N	2n	3n
Goldfish	Goldfish	117	0.376	0.523	15	14	1
	Androgenetic diploid[1]	162	0.333	0.648	15	14	1

1 : This male was arisen from the cross between UV irradiated eggs and sperm from the tetraploid ginbuna.
2 : Hatching rates were calculated from the number of hatching larvae relative to the number of total eggs used.
3 : Normal larvae rates were calculated from the number of hatching larvae with normal external appearance relative to the total number of hatching larvae.

and 6n) in their testes (Fig. 2E). The others showed three cell populations (1.5n, 3n and 6n) in their testes (Fig. 2F).

In androgenetic diploid progeny, haploid sperm were obtained from one out of the two individuals (Table 6). When the sperm of the androgenetic diploid were fertilized with eggs of a diploid goldfish, viable diploid larvae were obtained as well as a control cross (Table 7).

Discussion

Tetraploids are theoretically induced by heat-shock or hydrostatic pressure shock before entering into the first cleavage after fertilization, but induction rates of viable tetraploids are always very low (Pandian and Koteeswaran, 1998 ; Arai, 2001 ; Piferrer et al., 2009 ; Arai and Fujimoto, 2019). On the other hand, natural tetraploid variant is an alternative resource to establish tetraploid lines. Production of neo-tetraploid lines by fertilizing eggs from diploid fish with diploid sperm from natural tetraploid variant was previously reported in the dojo loach, *Misgurnus anguillicaudatus* (Arai et al., 1991b, Zhang and Arai, 1996 ; Fujimoto et al., 2010). In the present study, neo-tetraploid line was produced by fertilizing eggs from diploid goldfish female with diploid sperm from the tetraploid ginbuna male, followed by the suppression of the second polar body release. The neo-tetraploid progeny were successfully obtained from some heat shocked experiments after fertilization with diploid sperm, but resultant neo-tetraploid progeny gave relatively low normal rates when compared with triploids produced by cross-fertilization in control experiments. In goldfish, Yamaha et al. (2002b) reported that heat and hydrostatic pressure shocks caused developmental abnormality in body axis formation. The decrease of normal larvae rate with the increase of heat-shock duration (strength) is explained by the side effect due to the disruption of body axis formation in the early development. Artificially induced tetraploid masu salmon could not survive beyond feeding stage presumably due to the same mechanism (Sakao et al., 2006). Furthermore, higher frequencies of abnormality observed in the tetraploid inductions are presumably caused by the elevation of ploidy level. In other species, neo-tetraploid dojo loach induced by polar-body release inhibition after cross-fertilization between diploid female and tetraploid male were reported to show lower rates of normal larvae (Fujimoto et al., 2010). In the same dojo loach, deleterious effect of the ploidy elevation on body growth was also shown by lower body weight of tetraploid clone than diploid clone, both of which are genetically identical except for the number of chromosome sets (Morishima et al., 2012). Retarded growth was also reported in hexaploid dojo loach, when compared with tetraploid dojo loach (Horie et al., 2004). In the present study, diploid, triploid, aneuploid and mosaic progeny often arose and frequencies of successful induction of neo-tetraploid with normal appearance varied among the experiments. The variation among the results of neo-tetraploid induction is likely explained by difference of egg quality of the parental fish used.

Neo-tetraploids, which were successfully induced in the present study, did not produce any gamete, when hCG was injected to induce ovulation or spermiation. One of these neo-tetraploids had string like gonad, which sex was not determined by observing external shape of the gonad. Such a sterility was also reported in the second generation of neo-

tetraploid dojo loach males newly induced by using diploid sperm of the first generation of neo-tetraploid dojo loach and was considered as a result of disruption of meiosis due to genetic difference among four chromosome sets (Fujimoto et al., 2010). As androgenic diploid progeny produced haploid sperm, neo-tetraploid in the present study is theoretically regarded as amphidiploids comprising two sets of homologous chromosomes from goldfish and two different sets of homologues from tetraploid ginbuna. Such an amphidiploid (allotetraploid or tetraploid hybrid) is expected to proceed normal meiotic process and gametogenesis, because two sets of homologous chromosomes from each origin subspecies can be regularly paired to form bivalents at meiosis. It was also reported that diploid gametes were obtained from amphidiploid fish, which spontaneously appeared in interspecific hybrids between red crucian carp female and common carp male (Liu et al., 2001).

In resultant triploid males of the present study, two cases were observed. Some fish produced 1.5n (aneuploid) sperm, while the others did not give any gamete. At present, the reason for such a reproductive difference within the same triploid fish is unclear. Triploid fish were easily generated by crossing diploid goldfish female with the tetraploid ginbuna male, but resultant triploid males produced aneuploid sperm. Such a reduced reproductive ability of triploid males has been often reported in many other fish species (Piferrer et al., 2009). In the dojo loach, natural triploid males showed infertility and triploid and hexaploid cell populations were observed in their testes (Zhang and Arai, 1999 ; Oshima et al., 2005). Similar results were also obtained in triploid progeny of neo-tetraploid produced by using diploid sperm of natural tetraploid male (Fujimoto et al., 2010). Although aneuploid sperm occasionally showed a little motility and very low fertilizing ability, most progeny died due to abnormalities (Piferrer et al., 2009). Thus, the triploid produced by using diploid sperm of natural tetraploid ginbuna male can be used as sterile fish. Reproductive ability of the triploid female induced in this study is unknown at present, because we could not induce maturation in the triploid females.

Androgenetic diploids were induced by fertilizing UV-irradiated eggs with diploid sperm from the tetraploid ginbuna male. In the dojo loach, such androgenetic diploids derived from the neo-tetraploid line generated fertile haploid sperm (Fujimoto et al., 2010). However, androgenetic diploid progeny generated from diploid sperm of amphidiploid males between red crucian carp and common carp produced unreduced diploid gametes (Sun et al., 2007). An androgenetic diploid in the present study produced fertile haploid sperm like normal diploid goldfish male. The result suggests that genomic construction of the tetraploid ginbuna used in this study should be conspecific.

In conclusion, the tetraploid ginbuna male could be used for the expansion of chromosome manipulation as a source of diploid sperm. Therefore, natural tetraploid ginbuna is an important genetic resource, though it is difficult to discover tetraploid individuals among wild triploid populations which reproduce clonally by gynogenesis in nature. Another option to obtain tetraploid is fertilization of unreduced triploid eggs from clonal triploid line with haploid sperm, followed by temperature treatments to incorporate sperm nucleus into eggs (Dong et al., 1997 ; Mada et al., 2001b). Such an approach using diploid gametes may provide possibilities to generate new tetraploid lines, such as autotetraploid, allotetraploid and amphidiploid, instead of the traditional chromosome manipulation using the inhibition of mitotic division.

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