



Title	Reproductive capacity of neo-tetraploid loaches produced using diploid spermatozoa from a natural tetraploid male
Author(s)	Fujimoto, Takafumi; Yasui, George Shigueki; Hayakawa, Mayumi; Sakao, Suzu; Yamaha, Etsuro; Arai, Katsutoshi
Citation	Aquaculture, 308, S133-S139 <a href="https://doi.org/10.1016/j.aquaculture.2010.04.029">https://doi.org/10.1016/j.aquaculture.2010.04.029</a>
Issue Date	2010
Doc URL	<a href="http://hdl.handle.net/2115/44224">http://hdl.handle.net/2115/44224</a>
Type	article (author version)
File Information	aquaculture.pdf



[Instructions for use](#)

Reproductive capacity of neo-tetraploid loaches produced using diploid spermatozoa from a natural tetraploid male

Takafumi Fujimoto<sup>a</sup>, George Shigueki Yasui<sup>b</sup>, Mayumi Hayakawa<sup>b</sup>, Suzu Sakao<sup>b,c</sup>, Etsuro Yamaha<sup>a</sup>, Katsutoshi Arai<sup>b,\*</sup>

<sup>a</sup> *Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Nanae, Hokkaido 041-1105, Japan*

<sup>b</sup> *Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

<sup>c</sup> *Present address: Research Center for Elderly Nutrition and Development, Mukogawa Women's University, 6-46, Ikebiraki-cho, Nishinomiya, Hyogo 663-8558, Japan*

\*Corresponding author: Phone: +81-138-40-5535; Fax: +81-138-40-5537; Email: [arai@fish.hokudai.ac.jp](mailto:arai@fish.hokudai.ac.jp)

Running title: Reproduction of neo-tetraploid loach

## ABSTRACT

Tetraploid loaches have been discovered amongst specimens recovered from fish markets in Japan. These tetraploids can be used as a source of diploid gametes to assist the further expansion of ploidy manipulation. Here, we produced a first generation neo-tetraploid strain by fertilizing eggs from a normal diploid female with diploid spermatozoa from a natural tetraploid male, followed five minutes later by heat shock (42°C, 2 min duration) to inhibit release of the second polar body. Diploid spermatozoa from the neo-tetraploid males produced were then used to create androgenetic diploid progeny by fertilizing UV-irradiated eggs from a normal diploid female. Triploid progeny were produced by crossing a normal diploid female with a neo-tetraploid male. Tetraploid progeny were produced by cold shock (1°C, 40 min duration), beginning 5 min after fertilizing normal eggs with diploid spermatozoa from first generation neo-tetraploid males. Reproductive performance of second generation progeny was also examined. Androgenetic diploid males generated fertile haploid spermatozoa. Triploid males were sterile, but a triploid female laid fertile haploid eggs. Second generation neo-tetraploid males were considered sterile.

*Keywords:* polyploid, chromosome manipulation, hybrid, sterile

## 1. Introduction

Tetraploid strains are important as a source of diploid gametes, which can be used to assist the further expansion of ploidy manipulation (Arai, 2001). Using diploid gametes, sterile triploids and second generation tetraploid strains can be easily produced by simple cross-breeding. In Japan, the Nagano prefectural hatchery maintains artificially induced tetraploid rainbow trout *Oncorhynchus mykiss* strains by cross-breeding, and uses them for the commercial production of “Shinsyu Salmon” (allotriploid hybrids formed from tetraploid rainbow trout female and diploid brown trout *Salmo trutta* male) (Kohara and Denda, 2008). However, it is difficult to induce sufficient numbers of healthy tetraploid fish by inhibiting first cleavage following fertilization because of extremely low survival during embryogenesis. Poor survival rates appear to be due to side-effects of the manipulation strategies used, which presumably induce cell division without nuclear division (anuclear division), aneuploidy, and mosaicism comprising anuclear cells, macro- and micro-meres (Sakao et al., 2003). In masu salmon, *O. masou*, artificially induced tetraploid larvae frequently exhibited high mortality, even following hatching. This was reported to be caused by poorly developed vascular systems (Sakao et al., 2006). Resultant tetraploid fish were also reported to exhibit reduced survival and growth when compared with normal diploid strains in salmonids (Chourrout et al., 1986). The unexpected production of non-diploid gametes is another significant problem. In the mud loach, *Misgurnus mizolepis*, relatively small numbers

of mature tetraploids were reported to spawn diploid gametes, whilst other tetraploids generated haploid gametes (Nam and Kim, 2004).

In nature, spontaneous tetraploid variants are considered to be another source of diploid gametes for manipulation, but such intra-specific variation is generally rare among teleosts. In the loach, *Misgurnus anguillicaudatus*, however, tetraploid individuals possessing 100 chromosomes have been found in specimens obtained from the commercial fish market, although most loach individuals are bisexually reproducing  $2n$  diploids (i.e., 50 chromosomes) in wild populations in Japan (Arai et al., 1991a; Ojima and Takai, 1979). Although the original locality of such natural tetraploids has yet to be identified, these fish are thought to be Chinese loaches that might have been imported as food material and fishing bait, especially since tetraploid loaches can be found in central China (Li et al., 2008). Furthermore, no natural tetraploid individual has been discovered following careful and intensive screening of samples from Japanese populations (Arai, 2003; Zhang and Arai, 1999a). These natural tetraploid loaches are regarded as representing genetically true tetraploids with four sets of homologous chromosomes because gynogenetic and androgenetic progeny were viable when created from the gametes of tetraploid individuals without any manipulation to duplicate chromosomes (Arai et al., 1991b, 1993, 1995). Higher polyploid loaches, such as pentaploids and hexaploids, have been produced by fertilization and manipulation strategies using diploid gametes from the natural tetraploid loach (Arai, 2001, 2003;

Arai et al., 1999; Matsubara et al., 1995; Zhang and Arai, 1996).

In a series of studies concerning ploidy manipulation with the loach as a model animal, a neo-tetraploid strain was produced by fertilizing eggs from a normally reproducing diploid female with diploid spermatozoa from a natural tetraploid male and subsequent pressure or temperature shock to inhibit release of the second polar body (Arai et al., 1991b; Zhang and Arai, 1996). Such a neo-tetraploid strain may be different from the original natural tetraploid, and artificially induced tetraploids, because it includes two chromosome sets from a Japanese diploid female and two sets from an exotic tetraploid male. Mitochondrial DNA is transmitted from the bisexual diploid population in Japan. Thus, neo-tetraploidy is regarded not only as new lineage of tetraploids, but also a hybrid between diploid and tetraploid populations. In order to assess whether this new combination of nuclear genotypes and mitochondrial haplotypes causes alteration in reproductive performance in the neo-tetraploid, the present study aimed to examine fertility and ploidy status of gametes taken from the first generation of neo-tetraploid males. We then produced androgenetic diploid, triploid and tetraploid progeny using spermatozoa from neo-tetraploids to elucidate how this may assist in the further expansion of ploidy manipulation. Reproductive capacity of these second generation progenies was also examined.

## 2. Materials and methods

### *2.1. First generation of neo-tetraploid loach*

In June 2003, first generation neo-tetraploid fish were artificially induced by heat shock (42°C, 2 min duration) to inhibit second polar body extrusion 5 min after fertilizing eggs from a normal diploid female with sperm from a natural tetraploid male. For this manipulation, a mature female was caught during the spawning season in Kita, Iwamizawa city, central Hokkaido, Japan. The natural tetraploid male used was a survivor of the tetraploid strain, which had been created by cross-breeding between tetraploid loaches, and was selected from specimens collected in the fish market (Arai, 2001, 2003; Arai et al., 1991a, b, 1993; Zhang and Arai, 1996). Loaches from the wild population at Iwamizawa-city were reported to be genetically different from natural tetraploids based on control region sequences of the mitochondrial DNA (Morishima et al., 2008a). Resultant putative neo-tetraploid progeny was reared until maturation in the aquarium center, Faculty of Fisheries Sciences, Hokkaido University, Hakodate city, Hokkaido, Japan. In the present study, we did not collect genomic samples from the parental natural tetraploid and wild type diploid individuals used to produce the first generation neo-tetraploid. Consequently, it was not possible to undertake further genetic studies using microsatellite and other DNA markers.

### *2.2. Production of second and third generation progenies*

In order to produce second generation androgenetic diploid and triploid progeny using

gametes from the first generation neo-tetraploid fish, mature bisexual diploid loaches were caught at a paddy-field of Ohno, Hokuto-city, Hokkaido, in June 2006. Most loaches in Hokuto-city were categorized to the same genetic group as those in Iwamizawa-city (Morishima et al., 2008a). Ovulation and spermiation of mature brood stock was induced by injection of hCG (human Chorionic Gonadotropin, Aska Pharmaceuticals, Tokyo) and normal fertilization was performed, as described previously (Fujimoto et al., 2004). To induce androgenetic diploid progeny, mature eggs from the wild type female from Ohno, Hokuto-city were genetically inactivated by UV irradiation ( $150 \text{ mJ/cm}^2$ ) according to conditions previously optimized by Fujimoto et al. (2007). Irradiated eggs were then fertilized with spermatozoa taken from first generation neo-tetraploid males (#8 and #14). At the same time, triploid progeny were produced by fertilizing eggs from the same female with spermatozoa from neo-tetraploid males.

Second generation neo-tetraploid fish were produced in April 2008, by fertilizing eggs from wild type females from Kita, Iwamizawa-city with sperm from the first generation neo-tetraploid male (#16), followed by cold shock ( $1^\circ\text{C}$ , 40 min duration) 5 min after fertilization. Putative triploid progeny created by fertilizing eggs from the same wild type female with spermatozoa from the neo-tetraploid male were used as controls for each experiment.

In June 2007, normal eggs from wild type females were fertilized with sperm collected from the second generation androgenetic diploid males, which were produced in 2006. In August 2008,

we tried to collect gametes from second generation triploids by hCG stimulation, and eggs obtained were fertilized with normal sperm from a wild type male from Kita, Iwamizawa-city.

In these mating experiments, embryos were incubated at 20°C and then reared post-hatching at 20 to 25°C.

### *2.3. Ploidy determination*

DNA content was measured by flow cytometer (Ploidy Analyzer, Partec GmbH, Münster, Germany) to estimate the ploidy status of larvae, somatic cells from the caudal fin, and testis, as described previously (Fujimoto et al., 2007). Ploidy status was determined by comparing relative DNA content with the standard DNA content of somatic cells from a normal diploid loach (2.53 pg/N: Zhang and Arai 1996).

### *2.4. Histological analysis*

Adult fish, with the abdominal cavity opened, were fixed in Bouin's fixative for 16 hours. Fixed gonads were then removed from the fish and stored in 80% ethanol. Gonads were subsequently dehydrated with a graded butyl alcohol series and finally embedded in paraffin. Serial sections were cut at 6- $\mu$ m thickness and stained with hematoxylin-eosin according to standard procedures.

### 3. Results

#### *3.1. Ploidy status of somatic cells and spermatozoa from the first generation neo-tetraploid loach*

In May 2006, we used flow cytometry to examine the ploidy status of 17 three-year-old fish surviving as the first generation neo-tetraploid candidates. Fifteen survivors were tetraploid with twice the DNA content of somatic cells from the reference standard diploid loach sample (2.53 pg/N: Zhang and Arai, 1996). However, the remaining two fish exhibited mosaicism. In the mosaic individuals, one fish exhibited a large amount of tetraploid and a small amount of hypo-tetraploid cell populations, whilst the other fish exhibited a large amount of hypo-tetraploid and a small amount of hyper-tetraploid cell populations. Thus, maternally derived chromosomes were successfully duplicated by inhibiting the second polar body release from normal eggs fertilized with diploid spermatozoa from an original natural tetraploid male.

Eight of 15 neo-tetraploid survivors were identified as being male as indicated by the presence of sperm, but sex was not determined in the other seven fish. On the other hand, one of the two mosaic fish was identified as male due to the recovery of sperm following hCG injection. Flow cytometry showed that the spermatozoa taken from these eight neo-tetraploid males and one mosaic male were diploid. In the remaining fish, sex could not be determined, owing to the lack of gamete production.

### *3.2. Production of second generation progeny from neo-tetraploid loach*

In June 2006, diploid spermatozoa were obtained from two three-year-old neo-tetraploid males (#8 and #14). These fish exhibited vigorous sperm motility, as normally observed in normal haploid spermatozoa of diploid males following exposure to ambient water. Developmental capacity shown by fertilization rates, hatching rate, frequencies of normal larvae and ploidy status of progeny in each cross are shown in Table 1.

When the eggs of a normal diploid were fertilized with spermatozoa from a wild type diploid, 98.2% eggs were fertilized and 78.1% hatched. Resultant progeny were normal diploids. However, when normal eggs were fertilized with spermatozoa from neo-tetraploid males (#8 and #14), similar fertilization rates (94.0 - 96.6%) were observed, but hatching rates (59.5 - 68.1%) were notably reduced. In diploid female x neo-tetraploid male crosses, most resultant progeny were triploid with normal appearance. However, one unexpected tetraploid fish, and a mosaic fish possessing hyper- and hypo-triploid cell populations, appeared in the progeny of neo-tetraploid male #8.

When UV-irradiated eggs were fertilized with spermatozoa from a normal diploid, abnormal haploid embryos were evident upon hatched. When UV-irradiated eggs were fertilized with spermatozoa from neo-tetraploid males (#8 and #14), viable larvae hatched but the rates

were much lower than those recorded in other crosses (33.3% and 5.1%). A large majority of these hatched larvae exhibited externally normal appearance (88.6 - 90.0%) and diploidy was detected in the larvae by flow cytometry, indicating the successful induction of androgenetic diploid progeny without any treatment for chromosome duplication.

To produce second generation neo-tetraploid progenies, three experiments were conducted using cold shock to inhibit second polar body release and incorporating spermatozoa from the same neo-tetraploid male #16 and three diploid wild type females (Table 2). At the same time, diploids (control groups) and second generation neo-triploids were produced. In all experiments, control-2n groups (2n female x 2n male) produced 44.9 - 62.4% hatching rates and 86.8 - 98.8% normal larvae, 13 of which were tested and identified to be diploid. In fertilization experiments with spermatozoa from the neo-tetraploid, larvae in Exp. 1 and Exp. 3 experiments were produced at hatching with rates of 78.9% and 30.1%, respectively. Furthermore, only triploid progenies were detected by flow cytometry. In Exp. 2, most eggs did not hatch. In Exp. 1, the percentage of hatching and normal larvae created in the experimental group was reduced when compared with the control groups. Of the survivors, four unexpected diploid fish and one tetraploid fish were identified. In Exp. 3, the experimental group exhibited a relatively high percentage of hatching with normal larvae and the ploidy of resultant progeny was verified to be tetraploid by flow cytometry.

### *3.3. Reproductive capacity of second generation androgenetic diploid progeny from neo-tetraploid loach*

Sixteen second-generation androgenetic diploids developed from diploid spermatozoa from neo-tetraploid (#8) and approximately one-year-old survivors showed secondary sex characteristics that were specific to males. No female androgenotes were identified in this experiment. Five androgenetic diploid males (Andro-2n #1-5) were used to examine reproductive capacity (Table 3). In fertilization experiments using spermatozoa from androgenetic diploids, four (Andro-2n #2-5) generated a few larvae due to very low fertilization rates (0.5-19.4%), but andro-2n #1 yielded no fertilized eggs. Most third-generation progeny were diploid, but a few unexpected triploids were identified (Table 3).

In April 2009, histological analysis revealed that a small number of spermatozoa were observed in the testes of the four males exhibiting fertility (Fig. 1B), when compared with a normal diploid whose testes were occupied by a large number of spermatozoa (Fig. 1A). On the other hand, an infertile second generation androgenetic male #1 possessed a tube-like gonad but without evidence of any germ cells.

### *3.4. Reproductive capacity of second generation triploid progeny from neo-tetraploid loach*

In August 2008, 23 triploids (22 males and one female) derived from #8 neo-tetraploid male and 12 further triploids (11 males and one female) from #14, were examined for reproductive capacity by gentle squeezing the abdomen following the injection of hCG. Somatic ploidy status was judged as being triploid by flow cytometry analysis. All males produced a small quantity of sperm, including cells without motility. Ploidy status of these sperm cells could be detected in 13 males, but the other 20 males did not provide sufficient sperm cells for ploidy analysis. Triploid cells were detected in four of 13 males, hexaploid cells were detected in seven males and both triploid and hexaploid cells were detected in two males.

In April 2009, we analysed the histological structure and ploidy status of testicular cells from three second-generation triploid fish derived from #16 neo-tetraploid male. Spermatogenesis had progressed in these testes as shown by the presence of type A and type B spermatogonia, and primary spermatocytes undergoing meiosis (Fig.1C). However, spermatozoa and spermatids were not observed.

In normal wild type males with diploid somatic cells (Fig. 2A), only haploid cell populations corresponding to spermatozoa were detected in the testis (Fig. 2D). In triploid males, ploidy status of somatic cells was verified as being triploid (Fig. 2B), while testicular cells were comprised of both triploid and hexaploid cell populations (Fig. 2E).

In contrast, eggs laid by one of the two triploid females were able to develop normally after

fertilization with spermatozoa from a wild type male. One hundred and eighty seven (23.3%) of the total number of eggs used (801 eggs) hatched normally. Flow cytometry revealed that 47 normal larvae out of 49 samples were diploid, but the other two larvae were haploid-triploid mosaic and aneuploid, revealing cells of intermediate value between haploid and diploid. Although another female laid eggs, no fertilized eggs were obtained because her eggs were apparently over-ripe.

### *3.5. Reproductive capacity of second generation tetraploid progeny from neo-tetraploid loach*

Seven one-year-old males from the second-generation tetraploid loach derived from #16 neo-tetraploid male were used to examine reproductive capacity. No mature females were present among the survivors. When stimulated by hCG, the males produced a small quantity of sperm, but no motile spermatozoa were evident.

In the testes of these second-generation neo-tetraploid males, we identified type A and type B spermatogonia, and primary spermatocytes, but spermatozoa and spermatids were not found (Fig.1D).

The somatic cells of these neo-tetraploids were tetraploid (Fig. 2C). Tetraploid and octaploid cell populations were detected by flow cytometry in sperm samples from five males and only octaploid cell populations were found in sperm of the two males. Flow cytometry of testicular

cells from the other two neo-tetraploid males, collected two months later, also exhibited tetraploid and octaploid cell populations (Fig. 2F).

#### 4. Discussion

When eggs of wild type females were fertilized with sperm of neo-tetraploid males, triploid progeny were produced. Fertilization of UV-irradiated eggs from wild type female with sperm of neo-tetraploid males yielded viable androgenetic diploid progeny. These results indicate that first-generation neo-tetraploid males generate viable diploid spermatozoa and their reproductive capacity is similar to that seen in the original natural tetraploid loach that reproduces bisexually (Arai et al., 1991b, 1993, 1995). Unfortunately, reproductive capacity of neo-tetraploid females remains unknown at present.

Only males were found in the androgenetic diploid progeny. A much higher frequency of males (female:male = 1:22 or 1:11) was observed in the triploid progeny of neo-tetraploid males. These results suggest that spermatozoa from neo-tetraploid males might carry male-determinant gene(s). Since artificially induced gynogenetic diploids were almost all-female, the sex determination system was elucidated as being male heterogametic (XX female, XY male) in the loach (Suzuki et al., 1985). Furthermore, Arai et al. (1999) reported normal sex ratios (female:male = 1:1) in a natural tetraploid strain and suggested that the genotype of the natural

tetraploid male was XXXY and likely to generate a balanced (normal; 1 female : 1 male) sex ratio by fertilizing eggs with a XXXX genotype. However, if our first-generation natural tetraploid has an XXYY genotype and produces much more XY- and YY-spermatozoa than XX-spermatozoa, then an unbalanced sex ratio skewed towards a male population can be achieved in triploid progeny. However, it is difficult to explain the occurrence of all-male androgenotes by this hypothesis. Thus, at present, we cannot propose any conclusive mechanism responsible for the observed deviation from a balanced 1:1 sex ratio in triploid and androgenetic progeny of neo-tetraploid males. One other possible explanation for the skewed sex ratio may be female to male sex reversal by environmental factors. In the loach, higher temperature during the period of sexual differentiation is reported to induce many more males (Nomura et al., 1998).

In the present study, triploid males were sterile, but one of two females generated fertile haploid eggs. Identification of fertile females but sterile males arising from triploid progeny of neo-tetraploid loach represent a differing scenario from the situation in artificially induced triploid fish, which normally exhibit retarded oogenesis in the female and formation of a small quantity of aneuploid spermatozoa in the male (Arai, 2001; Arai and Inamori, 1999; Zhang and Arai, 1999b). However, reproductive characteristics of triploid progeny arising from neo-tetraploids were similar to those reported in other types of triploid loaches, such as triploid individuals created by cross-fertilization between diploid and natural tetraploids (Arai and

Mukaino, 1997, 1998; Matsubara et al., 1995; Zhang et al., 1998), those occurring via the fertilization of unreduced eggs from diploid females in nature (Zhang and Arai, 1999a), and those derived from clonal diploid eggs by the accidental incorporation of a sperm nucleus (Morishima et al., 2008b; Oshima et al., 2005; Yoshikawa et al., 2007). All of the above-mentioned triploid females usually produce a large number of haploid eggs together with a small number of triploid eggs (Arai and Mukaino, 1997, 1998; Matsubara et al., 1995; Morishima et al., 2008b; Oshima et al., 2005; Yoshikawa et al., 2007; Zhang and Arai, 1999a; Zhang et al., 1998). Haploid eggs are likely to be produced by meiotic hybridogenesis, i.e., synapsis between two chromosomes that are more homologous and the elimination of the exotic haploid set (Arai and Mukaino, 1998; Morishima et al., 2008b). Thus, formation of haploid eggs in triploid progeny arising from neo-tetraploid loach may be, in part, similar to the reproductive strategy of the above mentioned triploids.

Triploid progeny arising from the neo-tetraploid males did not produce fertile spermatozoa. Sterility in these triploid males is similar to the reproductive capacity of the above mentioned natural triploid loaches. In the testes of normal diploid males, we used flow cytometry to detect a haploid cell population, corresponding to spermatids and/or spermatozoa, which were confirmed histologically. On the other hand, both triploid and/or hexaploid cell populations were found in testes from the neo-triploid. Neither spermatids nor spermatozoa were histologically detected in

the testis, but spermatogonia and spermatocytes occupied a large part of the testis. Flow-cytometry and histological data from the neo-triploid progeny suggest that an aberrant and unusual meiotic process occurred in the testes, such as proliferation and replication of triploid germ cells and subsequent arrest of spermatogenesis. Thus, most spermatocytes would not have completed meiotic division and could not develop to the spermatid or spermatozoa stages. Similar observations, such as the presence of triploid and hexaploid cell populations in testes, and arrested spermatogenesis, have been reported in natural triploids (Oshima et al., 2005; Zhang and Arai 1999a).

Second-generation progeny from neo-tetraploids were successfully produced by inhibition of second polar body release after cross-fertilization between a diploid wild type female and sperm from a first generation neo-tetraploid male, but unexpected diploid progeny were detected for unknown reasons. Although fertility is expected in second-generation neo-tetraploid progeny, seven second-generation males examined did not generate fertile spermatozoa and thus a neo-tetraploid strain remains difficult to establish at present. Flow cytometric and histological observation did not detect spermatids and spermatozoa, as already reported for triploid progeny.

On the other hand, four out of five second-generation androgenetic diploid males generated a small amount of fertile haploid sperm and third-generation diploid progeny were produced by fertilization of normal eggs with sperm from androgenotes. The results obtained using

androgenetic diploids suggest that some androgenotes might have two normal sets of homologous chromosomes that are able to pair and then undergo regular meiotic divisions for spermatogenesis. In some fertile androgenetic diploids, two sets of chromosomes transmitted from paternal neo-tetraploids were able to pair to form bivalents and segregate in a regular meiotic manner in order to form haploid spermatozoa. The other sterile androgenotes are likely to have homoeologous chromosomes from neo-tetraploid males, which are difficult to pair and make bivalents and then segregate gametes. Reproductive capacity is presumably dependent on the pairing of homologous chromosomes transmitted from neo-tetraploids.

In the second-generation neo-tetraploids, infertility can be explained not only by pairing capacity between chromosomes from the neo-tetraploid, but also that between maternally derived chromosomes from wild type and paternally derived chromosomes transmitted from neo-tetraploid males. In most second-generation tetraploid progeny, four chromosomes (two homologous chromosomes from the diploid and a further two homoeologous chromosomes from the tetraploid), may experience difficulty to pair and then segregate in the meiotic process and subsequent gametogenesis. Failure of the normal chromosome pairing process may give rise to sterility in the second-generation neo-tetraploid progeny.

Consequently, reproductive capacity of the resultant progeny from neo-tetraploid males may be determined by pairing capacity between two chromosomes transmitted from the ancestral

tetraploid and those from wild type diploid individuals. In *Misgurnus* loaches, disruption of normal meiosis and gametogenesis has been reported in inter-specific hybrids (Fujimoto et al., 2008) and sometimes in inter-populational hybrids between genetically distinct groups (Arias-Rodriguez et al., 2009). Genetic difference between ancestral natural tetraploid loach with unknown origin and wild type diploid loach collected from central or southern Hokkaido, Japan (Morishima et al., 2008a) may involve the expression of fertility or sterility in neo-tetraploid loach and its progeny, because these are presumably regarded as being hybrids that lie between different genotypes.

In the present study, we produced neo-tetraploid fish as a source of diploid gametes which are necessary for the expansion of chromosome manipulation research. First-generation neo-tetraploid loaches were fertile and produced functional gametes. However, reproductive capacity decreased in second-generation neo-tetraploids, and fertile neo-tetraploid strains have not yet been established. Thus, neo-tetraploid loaches are not useful for practical breeding programs at present.

#### Acknowledgments

This study was supported in part by Grants-in-Aid for the 21<sup>st</sup> Century COE (Center of Excellence) Program (K-02, 2004-2008) from the Ministry of Education, Culture, Sports, Science,

and Technology (MEXT) of Japan and for Scientific Research (B) (No.18380108) from Japan

Society for the Promotion of Science (JSPS) to KA.

## References

- Arai, K., 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. *Aquaculture* 197, 205-228.
- Arai, K., 2003. Genetics of the loach, *Misgurnus anguillicaudatus*: recent progress and perspective. *Folia Biologica (Krakow)* 51 supplement, 107-117.
- Arai, K., Ikeno, M., Suzuki, R., 1995. Production of androgenetic diploid loach *Misgurnus anguillicaudatus* using spermatozoa of natural tetraploids. *Aquaculture* 137, 131-138.
- Arai, K., Inamori Y., 1999. Viable hyperdiploid progeny between diploid female and induced triploid male in the loach, *Misgurnus anguillicaudatus*. *Suisanzoshoku* 47, 489-495.
- Arai, K., Matsubara, K., Suzuki, R., 1991a. Karyotype and erythrocyte size of spontaneous tetraploidy and triploidy in the loach *Misgurnus anguillicaudatus*. *Nippon Suisan Gakkaishi* 57, 2167-2172.
- Arai, K., Matsubara, K., Suzuki, R., 1991b. Chromosomes and developmental potential of progeny of spontaneous tetraploid loach *Misgurnus anguillicaudatus*. *Nippon Suisan Gakkaishi* 57, 2173-2178.
- Arai, K., Matsubara, K., Suzuki, R., 1993. Production of polyploids and viable gynogens using spontaneously occurring tetraploid loach, *Misgurnus anguillicaudatus*. *Aquaculture* 117, 227-235.

- Arai, K, Mukaino, M., 1997. Clonal nature of gynogenetically induced progeny of triploid (diploid x tetraploid) loach *Misgurnus anguillicaudatus* (Pisces:Cobitidae). Journal of Experimental Zoology 278, 412-420.
- Arai, K, Mukaino, M., 1998. Electrophoretic analysis of the diploid progenies from triploid x diploid crosses in the loach *Misgurnus anguillicaudatus* (Pisces: Cobitidae). Journal of Experimental Zoology 280: 368-374.
- Arai, K., Taniura, K., Zhang, Q., 1999. Production of second generation progeny of hexaploid loach. Fisheries Science 65, 186-192.
- Arias-Rodriguez, L., Yasui, G. S., Arai, K., 2009. Disruption of normal meiosis in artificial inter-populational hybrid female *Misgurnus* loach. Genetica 136, 49-56.
- Chourrout, D., Chevassus, B., Krieg, F., Happe, A., Burger, G., Renard, P., 1986. Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females—Potential of tetraploid fish. Theoretical and Applied Genetics, 72, 193-206.
- Fujimoto, T., Kataoka, T., Otani, S., Saito, T., Aita, T., Yamaha, E., Arai, K., 2004. Embryonic stages from cleavage to gastrula in the loach *Misgurnus anguillicaudatus*. Zoological Science 21, 747-751.
- Fujimoto, T., Sakao, S., Yamaha, E., Arai, K., 2007. Evaluation of different doses of UV

- irradiation to loach eggs for genetic inactivation of the maternal genome. *Journal of Experimental Zoology* 307A, 449-462.
- Fujimoto, T., Yasui, G. S., Yoshikawa, H., Yamaha, E., Arai, K., 2008. Genetic and reproductive potential of spermatozoa of diploid and triploid males obtained from interspecific hybridization of *Misgurnus anguillicaudatus* female with *M.mizolepis* male. *Journal of Applied Ichthyology* 24, 430-437.
- Kohara, M., Denda, I., 2008. Production of allotriploid "Shinsyu Salmon" by chromosome manipulation. *Fish Genetics and Breeding Science (Suisanikussyu)* 37, 61-65. (In Japanese with English abstract)
- Li, Y-J., Yin, J., Wang J-B., Yuan, X., Wei, J., Sun, X-W., Arai, K. 2008. A study on the distribution of polyploidy loaches in China. *Nippon Suisan Gakkaishi* 74, 177-182.(in Japanese with English abstract)
- Matsubara, K., Arai, K., Suzuki, R., 1995. Survival potential and chromosomes of progeny of triploid and pentaploid females in the loach *Misgurnus anguillicaudatus*. *Aquaculture* 131, 37-48.
- Morishima, K., Nakamura-Shiokawa, Y., Bando, E., Li, Y-J., Boron, A., Khan, M.M.R., Arai, K. 2008a. Cryptic clonal lineages and genetic diversity in the loach *Misgurnus anguillicaudatus* (Teleostei:Cobitidae) inferred from mitochondrial DNA analyses. *Genetica*

132, 159-171.

Ojima, Y., Takai, A., 1979. The occurrence of spontaneous polyploidy in the Japanese common loach, *Misgurnus anguillicaudatus*. Proceedings of Japan Academy 55B, 487-491.

Oshima, K., Morishima, K., Yamaha, E., Arai, K., 2005. Reproductive capacity of triploid loaches obtained from Hokkaido island, Japan. Ichthyological Research 52, 1-8.

Sakao, S., Fujimoto, T., Tanaka, M., Yamaha, E., Arai, K., 2003. Aberrant and arrested embryos from masu salmon eggs treated for tetraploidization by inhibition of the first cleavage. Nippon Suisan Gakkaishi 69, 738-748. (In Japanese with English abstract)

Sakao, S., Fujimoto, T., Kimura, S., Yamaha, E., Arai, K., 2006. Drastic mortality in tetraploid induction results from the elevation of ploidy in masu salmon *Oncorhynchus masou*. Aquaculture 252, 147-160.

Suzuki, R., Oshiro, T., Nakanishi, T., 1985. Survival, growth and fertility of gynogenetic diploids induced in the cyprinid loach, *Misgurnus anguillicaudatus*. Aquaculture 48, 45-55.

Yoshikawa, H., Morishima, K., Fujimoto, T., Yamaha, E., Arai K. 2007. Simultaneous formation of haploid, diploid and triploid eggs in diploid-triploid mosaic loaches. Journal of Fish Biology 71(Suppl. B), 250-263.

Zhang, Q., Arai, K., 1996. Flow cytometry for DNA contents of somatic cells and spermatozoa in the progeny of natural tetraploid loach. Fisheries Science 62, 870-877.

Zhang, Q., Arai, K., 1999a. Distribution and reproductive capacity of natural triploid individuals and occurrence of unreduced eggs as a cause of polyploidization in the loach, *Misgurnus anguillicaudatus*. Ichthyological Research 46, 153-161.

Zhang, Q., Arai, K., 1999b. Aberrant meioses and viable aneuploid progeny of induced triploid loach (*Misgurnus anguillicaudatus*) when crossed to natural tetraploids. Aquaculture 175, 63-76.

Zhang, Q., Arai, K., Yamashita, M., 1998. Cytogenetic mechanisms for triploid and haploid egg formation in the triploid loach *Misgurnus anguillicaudatus*. Journal of Experimental Zoology 281, 608-619

Figure legends.

Figure 1. Photomicrographs of testis cross-sections from a wild type diploid male (A), androgenetic diploid progeny of the first generation neo-tetraploid male (B), triploid progeny of the first generation neo-tetraploid male (C), tetraploid progeny of first generation neo-tetraploid male (D). Note relatively fewer numbers of spermatozoa or spermatids in the testis of the androgenetic diploid male and absence of spermatozoa or spermatids in the testis of triploid and tetraploid progeny. Scale indicates 100  $\mu\text{m}$ .

Figure 2. Flow cytometry histograms of somatic tissue from caudal fin (A-C) and testis (D-F) of wild type diploid (A, D), triploid progeny of neo-tetraploid (B, E) and tetraploid progeny from a neo-tetraploid (C, F).

Table 1. Developmental capacity shown by the percentage of fertilized eggs, hatching larvae and normal larvae, and ploidy status examined by flow cytometry in second generation progeny produced by fertilization and induction of androgenesis using spermatozoa from first generation neo-tetraploid males. Only diploid progeny appeared in Control-2n, while all or most progeny were triploid in 2n x neo-4n crosses. Haploid and diploid androgenotes occurred in 2n (UV irradiated eggs) x 2n cross and 2n (UV irradiated) x neo-4n crosses, respectively.

Putative biotype	Cross		Egg no.	Fertility %	Hatch %	Normal %	Sample no.	Ploidy status				
	Female(UV irradiated)	Male						1n	2n	3n	4n	Mosaic
Control-2n	2n	2n	169	98.2	78.1	96.2	10	0	10	0	0	0
Andro-1n	(2n)	2n	182	95.6	33.5	0.0	10	10	0	0	0	0
3n	2n	neo-4n #8	116	94.0	59.5	94.2	10	0	0	8	1	1*
Andro-2n	(2n)	neo-4n #8	105	93.3	33.3	88.6	10	0	10	0	0	0
3n	2n	neo-4n #14	119	96.6	68.1	98.8	10	0	0	10	0	0
Andro-2n	(2n)	neo-4n #14	198	93.9	5.1	90.0	9	0	9	0	0	0

\*including hyper- and hypo-triploid cell populations

Table 2. Developmental capacity shown by percentage of hatching larvae and normal larvae, and ploidy status determined by flow cytometry in second generation progeny produced by fertilization with spermatozoa from a first generation neo-tetraploid male and subsequent inhibition of second polar body release by cold shocking fertilized eggs. In Exp. no.1 and no.3, only diploid progeny appeared in Control 2n crosses, while triploid progeny appeared in 2n female x neo-4n male crosses. In the cold shock group after 2n female x neo-4n male crosses, tetraploid progeny appeared both in Exp. no. 1 and no. 3. But the rate of tetraploidy was low in Exp. no. 1.

Exp. no.	Putative biotype	Cross		Cold shock	Egg no.	Hatch %	Normal %	Sample no.	Ploidy status		
		Female	Male						2n	3n	4n
1	Control-2n	2n	2n	no	346	59.8	96.1	8	8	0	0
	3n	2n	neo-4n #16	no	114	78.9	97.8	5	0	5	0
	4n	2n	neo-4n #16	yes	897	6.9	43.5	5	4	0	1
2	Control-2n	2n	2n	no	178	44.9	98.8	ND*	-	-	-
	3n	2n	neo-4n #16	no	275	1.8	100.0	ND	-	-	-
	4n	2n	neo-4n #16	yes	234	1.7	25.0	ND	-	-	-
3	Control-2n	2n	2n	no	170	62.4	86.8	5	5	0	0
	3n	2n	neo-4n #16	no	326	30.1	83.7	5	0	5	0
	4n	2n	neo-4n #16	yes	196	71.9	84.4	10	0	0	10

\*ND indicates no data.

Table 3. Developmental capacity shown by percentage of fertilized eggs, hatching larvae and normal larvae, and ploidy status determined by flow cytometry, in third generation progeny produced by fertilization with spermatozoa from second generation androgenetic diploid males produced using spermatozoa from a first generation neo-tetraploid male. In controls, most larvae (10/12) were diploid, but spontaneous triploids (2/12) also appeared. In Exp. no. 2-5, 2n female x andro-2n male crosses, most survivors were diploid.

Exp. no.	Cross		Egg no.	Fertility %	Hatch %	Normal %	Sample no.	Ploidy status	
	Female	Male						2n	3n
Control	2n	2n	165	67.9	66.1	94.5	12	10	2
1	2n	Andro-2n #1	285	0.0	0.0	0.0	ND*	-	-
2	2n	Andro-2n #2	212	0.5	0.5	100	1	1	0
3	2n	Andro-2n #3	320	19.4	19.4	88.7	12	11	1
4	2n	Andro-2n #4	215	5.6	5.6	91.7	11	10	1
5	2n	Andro-2n #5	243	3.7	3.7	100	9	9	0

\*ND indicates no data.

Fig. 1

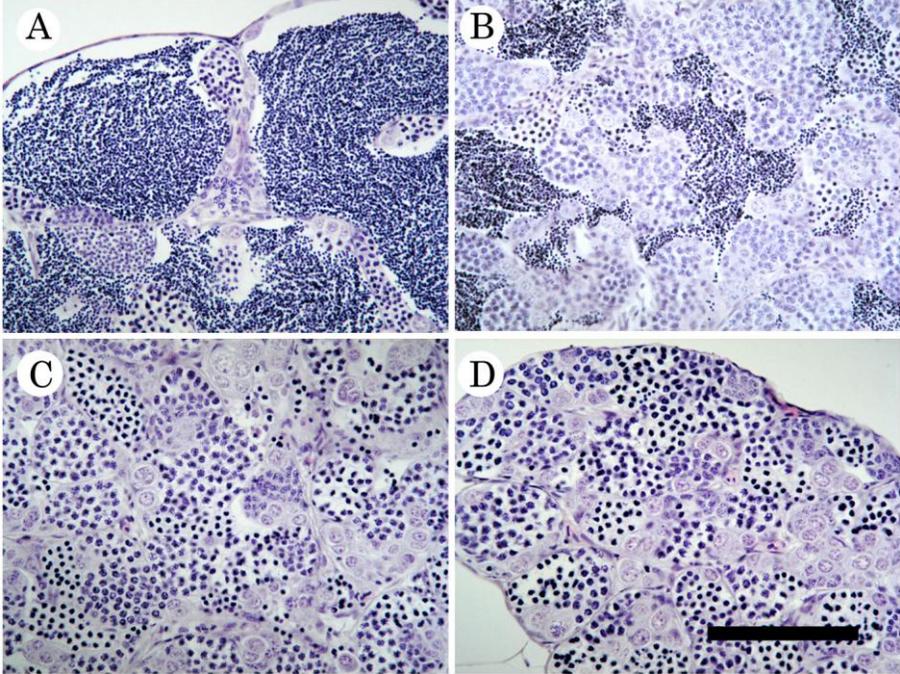


Fig. 2

