Genetic and reproductive potential of spermatozoa of diploid and triploid males obtained from interspecific hybridization of *Misgurnus anguillicaudatus* female with *M. mizolepis* male

Takafumi Fujimoto[1*], George Shigueki Yasui[1], Hiroyuki Yoshikawa[1], Etsuro Yamaha[2] and Katsutoshi Arai[1]

[1] Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

[2] Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Nanae, Kameda, Hokkaido 041-1105, Japan

*Corresponding author: Tel: +81.138.40.5614, Fax: +81.138.40.5537, Email: motchan@fish.hokudai.ac.jp

Short title: Diploid and triploid *Misgurnus* hybrid males

Summary

Diploid and triploid interspecific hybrid male progeny obtained from mating *Misgurnus anguillicaudatus* with *M. mizoleis* were reported to have histologically fertile and sterile testes, respectively. However, their reproductive capacity is still unclear because mating tests have not been examined using mature hybrids. Here, we examined physiological and genetic characteristics of spermatozoa of diploid and triploid hybrids. In diploid hybrid males, 1n, 2n and 4n spermatozoa showing low motility were detected. However, spermatozoa of three diploid hybrid males could generate 2n larvae. Therefore, only 1n spermatozoa of diploid hybrid males was fertile to produce larva. The chromosomes of diploid hybrid males were transmitted to spermatozoa by random segregation between the homologous chromosomes because most larvae had one allele derived from both *M. anguillicaudatus* and *M. mizolepis* at all loci examined. In triploid hybrid males, spermatozoa could be categorized to three different types based on their ploidy status. Type 1: In the first and second males, sperm samples mainly comprised 6n spermatozoa. Motility and fertility were not recorded. Type 2: The third male gave a large proportion of 6n spermatozoa as well as a small proportion of 1n spermatozoa. Although no motility was observed, larvae arose from eggs inseminated with such spermatozoa. Type 3: In the fourth male, only 1n spermatozoa were detected and their motility was vigorous. When eggs were fertilized with such 1n spermatozoa, normal larvae hatched. 1n spermatozoa of the triploid hybrid male only included the *M. anguillicaudatus* genome. In *Misgurnus* fishes, diploid hybrid males exhibited semi-sterility or slight fertility. On the contrary, triploid hybrid males were sometime fertile due to the production of 1n spermatozoa by a kind of transformation of meiosis like meiotic hybridogenesis.

Keywords: *Misgurnus,* hybrid male, sterility, triploid, spermatozoa

Introduction

Diploid hybrids between two different fish species exhibit gonadal, gametic, or zygotic sterility depending on the combination of species (Chevassus, 1983). However, fertile hybrid fish often show alternative atypical reproduction, such as unreduced gametogenesis, gynogenesis, or hybridogenesis, especially in females (reviewed by Dawley, 1989). On the contrary, hybrid males are usually sterile in most cases, but a reproduction with fertile unreduced sperm was reported in the Iberian minnow with a natural hybrid origin (Alves et al., 1999) and common carp × crucian carp hybrids (Cherfas et al 1994; Liu et al., 2001; Sun et al., 2007).

Interspecific hybridization has been regarded as a useful tool for aquaculture to improve growth rate as well as to add desirable traits (Bartley et al., 2001). Moreover, hybridization could be used as a means to prevent biological contamination, because sterile hybrid fishes are not risky to contaminate an indigenous gene pool by escaping from cages to natural environment (Bartley et al., 2001). However, some artificial hybrids are fertile or semi-fertile and are able to produce viable progeny. For example, hybrid catfish between Thai walking catfish (*Clarias macrocephalus*) female and African catfish (*C. gariepinus*) male are reported to be fertile (Senanan et al., 2004). The undesirable fertility of hybrid catfish is controlled by combination of hybridization and triploidization, i.e. allotriploidization (two genome sets of maternal species and one genome set of paternal species) (Na-Nakorn et al., 2004). Such allotriploid (triploid hybrid) catfish cannot produce any viable progeny in reciprocal back crosses between triploid hybrids and parental species but they generate functional gametes (Na-Nakorn et al., 2004). Thus, it is suggested that triploid hybrids should be more infertile than diploid hybrids and could be used as more reliable sterile animals for aquaculture.

In *Misgurnus* loach species, *M. anguillicaudatus* and *M. mizolepis*, polyploid and interspecific hybridization have been investigated to evaluate their reproductive capacity. In autotriploid *M. anguillicaudatus* artificially induced by inhibiting the second polar body exclusion, 1-year-old males are sterile (Suzuki et al., 1985), but 6-year-old triploid males form a small quantity of fertile spermatozoa with various aneuploidies (Zhang and Arai, 1999a). Diploid hybrid male progeny obtained from mating *M. anguillicaudatus* with *M. mizolepis* are reported to have fertility based on histological analysis, showing the presence of spermatozoa (Park et al., 2006). On the other hand, triploid hybrid males are concluded to be sterile based on histological analysis (Nam et al., 2004; Park et al., 2006). Furthermore, no survivors appear from a fertilization experiment using testicular sperm obtained from dissected and minced testes of allotriploid hybrid males comprising two *M. anguillicaudatus* genomes and one *M. mizolepis* genome (Nam et al., 2004). Thus, reproductive capacity of diploid and triploid *Misgurnus* hybrid males was not conclusive and experimental results were fragmentary.

In the present study, we examined ploidy status, motility and fertilization capacity of spermatozoa taken from diploid and triploid *Misgurnus* hybrid males. We also examined microsatellite genotyping in larvae arisen from crosses of diploid and triploid hybrid males to identify allelic segregation during spermatogenesis.

Materials and Methods

Generation of diploid and triploid hybrid

Adults of both sexes of a loach species, *Misgurnus anguillicaudatus* and adult males of another loach species, *M. mizolepis* were subjected to induce maturation as described by Suzuki (1983). The collection of gametes and the artificial fertilization of eggs were performed as described previously (Fujimoto et al., 2004). Diploid interspecific hybrids (genomic constitution: AM) were produced by fertilizing *M. anguillicaudatus* eggs (A) with *M. mizolepis* sperm (M). Triploid interspecific hybrids (AAM), i.e. allotriploids, were produced by the same combination followed by an inhibition of the second polar body extrusion with a cold shock (0°C for 30 min duration) at 5 min after the hybridization. Diploid (AA) and autotriploid (AAA) control purebreds were also produced by the same procedure.

Collection of semen

One-year-old mature diploid control (range of standard length (SL): 86.0 to 89.2 mm), autotriploid (range of SL: 78.0 to 88.1 mm), diploid hybrid (range of SL: 82.2 to 92.4 mm) and triploid hybrid (range of SL: 88.2 to 90.6 mm) males were injected with 20 IU/g body weight of hCG (Asuka Seiyaku Co., Tokyo, Japan) to induce spermiation. Squeezing the abdomen of males, semen was collected in haematocrit tube (Terumo Co., Tokyo, Japan) and semen volume was

measured. Then, the collected semen was immediately mixed with 50 μL of immobilizing solution (IS) (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$: Kurokura et al., 1984) in 0.2 mL volume microtubes. Subsequently, the diluted semen was stored at 4°C.

Measurement of DNA content

The relative DNA content of somatic cells from caudal fin, spermatozoa and larvae was measured by flow cytometry according to the previous study (Fujimoto et al., 2007). The coefficient of variation (CV%) of the major peak showing the DNA content of the G0/G1 phase was automatically calculated by flow cytometer (Ploidy Analyzer, Partec, Mönster, Germany). Ploidy status was determined by comparing relative DNA content against a standard diploid DNA content (2C) obtained from somatic cells of four control diploid fish.

Measurement of concentration and motility of spermatozoa

The diluted spermatozoa in IS were diluted into sperm fix solution (1% formalin, 5% $NaHCO_3$) and were counted three times using Thoma's counting chamber in each sample after sedimentation of spermatozoa for 5minutes. Then average value of concentrations was also calculated. The dilution using sperm fix solution was 200-fold in the case of control groups, but some fish required a lower dilution, since the sperm concentration was lower than usual. In all the cases these dilutions were adjusted in order to provide a total count of more than 150 cells per cytometer field. To estimate the sperm motility, spermatozoa activated with a 70-fold amount of distilled water were recorded with a VHS video recorder (model A-J1; Toshiba Co., Tokyo, Japan) and a digital camera (C3040, Olympus) coupled to a microscope. The slides were covered by a 0.2% BSA solution as suggested by Billard and Zhang (2001). After the observation of each sample, the proportion of total motility corresponding to the percentage of cells with any type of movement, we measured the proportion of progressive motility corresponding to the percentage of motile cells with a straight movement along a linear track and the duration of motility of spermatozoa.

Evaluation of the fertility of spermatozoa from diploid and triploid hybrid

Spermatozoa obtained from diploid and triploid hybrid males were used for artificial fertilization. We used normal diploid and triploid males as control. All the collected sperm samples were diluted and immediately placed on crushed ice. After collecting all the samples, an aliquot of the sperm was immediately utilized for fertilization trials. Other aliquot of the sperm sample was used to evaluate sperm motility and sperm concentration. When we stored the spermatozoa diluted in IS, we did not observe any decrease in sperm motility even after 4 days of storage at 4°C (unpublished data). Artificial fertilization was performed two times using two females (Trial 1 and 2). Each trial was single repetition. Eggs were inseminated with spermatozoa of each male and then activated using dechlorinated tap water. Fertilized eggs were incubated at 20°C. Dead eggs were removed and incubation water was changed every 8 to 12 hours. The survival rate at early somite stage, corresponding to 18 to 22 hours after fertilization, with incubation at 20°C (see Fujimoto et al., 2006) was estimated. The number of hatched larvae was counted. Hatched larvae were divided to two groups, normal and abnormal larvae, according to their external appearance and the percentage of larvae (normal and abnormal) was calculated.

Genetic analysis of the larva from diploid and triploid hybrids

Larvae obtained from the fertilization experiments were subjected to genetic analysis using polymorphic microsatellite loci developed by Morishima et al. (2001; 2008). We used four microsatellite loci *Mac 37*, *60*, *73* and *87*, mapped to different linkage groups. Extraction of genomic DNA, PCR condition and microsatellite genotyping were performed according to Morishima et al. (2008).

Statistical analysis

The parameters of concentration, total motility, progressive motility and duration of motility of spermatozoa and the mean value and CV% of larvae from diploid control, diploid hybrid and triploid hybrid males were subjected to Kruskal-Walis tests. Statistical significance was evaluated by means of a post-hoc multiple comparison using the Scheffé test ($P < 0.05$).

Results

Ploidy status of somatic cells and spermatozoa

Ploidy of somatic cells and spermatozoa of control and hybrid fishes were summarized in Table 1. In four diploid control males, somatic cells were 2n and spermatozoa were 1n, respectively (Fig. 1A, B). Somatic cells of all the five autotriploid males were 3n (Fig. 1C), but 1.5n, 3n, 4.5n and 6n spermatozoa were detected (Fig. 1D). The proportion of spermatozoa with different ploidies varied among these autotriploid males.

In five diploid hybrid males, somatic cells were 2n (Fig. 1E). Three males exhibited 1n, 2n and 4n, but the proportion of the three kinds of spermatozoa varied among individuals (Fig. 1F). On the other hand, spermatozoa derived from the other two males consisted of large population of 4n cells and small population of 2n cells (Fig. 1G). Besides 2n and 4n spermatozoa, a very small proportion of 1n cells was also detected as a fluorescent peak in the flow cytometrical histograms.

In four triploid hybrid males, somatic cells were 3n (Fig. 1H). These males could be categorized to three different types based on the ploidy status of their spermatozoa. Type 1: In the first and second males, sperm samples mainly comprised 6n spermatozoa (Fig. 1I). Type 2: The third male had a large proportion of 6n cell population as well as a small proportion of 1n cell population (Fig. 1J). Type 3: In the fourth male, only 1n spermatozoa were detected (Fig. 1K).

Concentration and motility of spermatozoa

Physiological characteristics of spermatozoa from diploid control, autotriploid, diploid hybrid and triploid hybrid males are shown in Table 2. The average concentrations of spermatozoa were 4612.7×10^6 cells/mL in diploid control and 38.0×10^6 cells/mL in autotriploid. Spermatozoa obtained from diploid control exhibited a vigorous total motility (90.2 %), an active progressive motility (88.1 %) and a long motility time (138.2 s). However, spermatozoa from autotriploid significantly decreased in percentages of total (1.5 %) and progressive motility (22.7 %) and its duration (48.0 s) in comparison with the diploid control.

In diploid hybrid males, concentrations of spermatozoa exhibited large variations among individuals. Average spermatozoa concentration of diploid hybrid males (13.2×10^6 cells/mL) was significantly lower than that of the diploid control. Spermatozoa from diploid hybrids exhibited significantly lower total motility (4.1 %) when compared with the diploid control. However, an average progressive motility (21.9 %) was observed within motile spermatozoa as in the autotriploid. The average duration of motility in spermatozoa obtained from the three males was not significantly different from that of the diploid control. Spermatozoa of the other two males did not show any motility.

Average concentrations of spermatozoa of type 1 and type 2 triploid hybrid males were significantly less than that of the diploid control. No spermatozoa of these males exhibited any motility. Characteristics of spermatozoa of the type 3 male were not significantly different from those of the diploid control.

Fertilization capacity of spermatozoa

Fertilization capacity of spermatozoa as assessed by survival potential of larvae is shown in Table 3. When normal eggs were fertilized with spermatozoa of diploid control, embryonic development initiated and normal larvae hatched. No fertilized eggs appeared after the fertilization with the spermatozoa from autotriploid.

The spermatozoa from four out of five diploid hybrid males were able to fertilize eggs and three males generated larvae in both trials. The male which showed the lowest concentration of spermatozoa could not fertilize eggs. Only a few percentages of normal larvae arose from only one cross in trial 2.

In triploid hybrid males, eggs inseminated with 6n spermatozoa from type 1 males (No. 3 and 4) did not initiate embryogenesis. When eggs were fertilized with haploid spermatozoa from type 2 and 3 males, embryogenesis was initiated. Although motility of the spermatozoa was not observed in type 2 male (No. 2), three larvae hatched. In fertilization with spermatozoa from type 3 male (No. 1), a large number of normal larvae hatched.

Ploidy and genetic analysis in the larvae from diploid and triploid hybrids

All the normal larvae from control diploid males were flow cytometrically diploid (Table 4). Their genotypes included only alleles originated from *M. anguillicaudatus* parents (data not shown).

Crosses using spermatozoa of diploid hybrid males produced a total of 17 larvae. Their ploidy was diploid (Table 4). However, CV% of diploid hybrid was significantly higher than that of

other groups. Microsatellite genotypes of 17 larvae at four independent microsatellite loci are summarized in Table 5. Genotypes including *M. anguillicaudatus* allele and those including *M. mizolepis* allele appeared almost equally. These results indicated that alleles of *M. anguillicaudatus* and those of *M. mizolepis* in genotypes of diploid hybrid male parents were randomly segregated to spermatozoa (Table 5). In three larvae, however, both paternal alleles, one from *M. anguillicaudatus* and the other from *M. mizolepis* were transmitted to spermatozoa at *Mac 37* and *Mac 73* loci (Table 5). On the other hand, two larvae did not have any paternal alleles at *Mac 37* or *Mac 73* loci (Table 5).

In larvae arisen from crosses using two triploid hybrid males, 20 larvae randomly selected from the type 3 male (No. 1 in table 3) and total three larvae from the type 2 male (No. 2 in table 3) were all diploids (Table 4). In a total 23 larvae from these two males, alleles originated from *M. anguillicaudatus* in the genotype of male hybrids were exclusively transmitted to their haploid spermatozoa (Table 6).

Discussion

Sperm motility of diploid hybrids and triploid hybrids except for the type 3 male were lower than those of diploid control, but similar to those of autotriploids. Sterility or low fertility was already observed in induced triploids of *M. anguillicaudatus* (Suzuki et al., 1985; Zhang and Arai, 1999a). Thus, fertility of spermatozoa in these diploid and triploid hybrids decreased when compared with those of diploid control.

In contrast, some diploid hybrid males were concluded to produce small quantity of functional haploid spermatozoa, because they generated a few diploid larvae when crossed with normal eggs from *M. anguillicaudatus* females. Natural diploid hybrid of the Iberian minnow was reported to produce fertile unreduced 2n spermatozoa, genetically identical to somatic cells of the father (Alves et al., 1999). On the other hand, artificial diploid hybrids between green sunfish male and bluegill female produced not only a small quantity of 1n cells, but also a large volume of 4n cells in testes which suggested an aberrant meiosis during spermatogenesis (Wills et al., 2000). In diploid hybrid males of *Misgurnus* loach, cell populations of three ploidy types, 1n, 2n and 4n spermatozoa were observed. Haploid (1n) spermatozoa of diploid hybrids could contribute to fertilization and the resultant fertilized eggs initiated normal development, as diploid larvae appeared. In contrast, 2n and 4n spermatozoa had no fertility and they might be matured and spermiated without completion of the meiosis.

According to the present genetic analysis using microsatellite, the chromosomes of diploid hybrid males were distributed to spermatozoa by random segregation between the homologous chromosomes because most larvae had one allele derived from both *M. anguillicaudatus* and *M. mizolepis* at all loci examined. Thus, haploid spermatozoa of hybrid males were presumably produced by normal meiotic process as in normal diploid. Large interspecific karyological differences generally give rise to a disruption in meiotic pairing between non-homologous chromosomes with heterospecific origin (Coyne and Orr, 1998). *M. mizolepis* has 2n = 48 chromosomes (12 metacentric (m) + 4 submetacentric (sm) + 32 acrocentric (a)), while *M. anguillicaudatus* has 2n = 50 chromosomes (10 m + 4 sm + 36 a) (Kim et al., 1995). Both species have the same arm number of 64. Because such an interspecific difference is caused by Robertsonian translocation, spermatogenesis may successfully proceed due to meiotic division between balanced chromosomes in hybrids. However, aberrant marker segregations such as transmittance of both alleles and no transmittance of allele from male parent were observed in a few larvae at *Mac 37* and *Mac 73* loci. These aberrant segregations may be caused by unequal crossing-over between heterospecific homologues from different species or abnormal chromosome disjunction during the first meiotic division due to karyological differences between these species.

Generally, fertility of triploid hybrids was reported to be lower than that of diploid hybrids (Lincoln, 1981; Lilyestrom et al., 1999; Wills et al., 2000; Na-Nakorn et al., 2004). The meiosis in triploid hybrids is arrested at the first division (Wills et al., 2000), although autotriploids generally performed abnormal pairing of chromosomes to produce aneuploid gametes (Allen et al., 1986; Zhang and Arai 1999a; Linhart et al., 2006). In the present study, spermatozoa of two triploid hybrids were infertile and never produced any viable larva. Therefore, these triploid hybrid males were concluded to be sterile.

On the other hand, the other two triploid hybrids produced fertile haploid spermatozoa. In the present study, these haploid spermatozoa of the triploid hybrid males included the haploid

genome (chromosome set) exclusively derived from *M. anguillicaudatus*. This suggests that meiotic division presumably occurred after synapsis between two sets of homologous chromosomes from *M. anguillicaudatus* in triploid hybrid genome and the unmatched set of chromosomes from *M. mizolepis* was eliminated. Such a transformation of meiosis is similar to the reproductive system named meiotic hybridogenesis, which was first recognized in triploid hybrid frog *Rana esculenta* (Günther et al., 1979) and later defined in the Iberian minnow (Alves et al., 1998). In this system, the elimination of the unmatched set of chromosomes may permit a random segregation and recombination between the two homologous chromosomes from the same species. However, such system is acted in the oogenesis of triploid females, in most cases. Formation of haploid eggs by triploid females has been observed in synthetic triploid (normal diploid female × natural tetraploid male) loach (Matsubara et al., 1995), natural triploid loach (Zhang and Arai, 1999b; Oshima et al., 2005), triploid Iberian minnow (Alves et al., 2004), triploid *Phoxinus eos-neogaeus* (Goddard and Schultz, 1993) and *Cobitis* (Kim and Lee, 2000; Saitoh et al., 2004). In allotriploid fish, production of haploid spermatozoa was only found in triploid hybrids between Atlantic salmon and brown trout (Castillo et al., 2007). The meiotic hybridogenesis is different from the typical hybridogenesis defined in *Poeciliopsis* fish, in which only maternal haploid genome is hemiclonally transmitted to eggs without any recombination and paternal haploid genome is excluded during oogenesis (Schultz, 1969). The other source of fertile spermatozoa in triploid hybrid male is unreduced spermatogenesis. Fertile triploid spermatozoa with genetic uniformity were found in triploid hybrid males of the Iberian minnow (Sousa-Santos et al., 2007), but such unreduced spermatozoa were not recognized in the present study. Unreduced spermatogenesis in the loach was reviewed by Yoshikawa et al. (in press).

In conclusion, diploid hybrid males between *M. anguillicaudatus* and *M. mizolepis* were considered to be semi-fertile in the case of artificial fertilization. On the contrary, triploid hybrid males were sometimes fertile, because 1n spermatozoa were able to be produced by the meiotic hybridogenesis.

Acknowledgements

We are thankful to Dr. K. Morishima and Ms. Z. Lahrech for their assistance. This study was supported in part by a Grant-in-Aid for the 21st Century COE program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, that for Scientific Research (B) (No. 18380108) from Japan Society for the Promotion of Science (JSPS) to K.A. and that for Young Scientists (B) (No. 18780138) from MEXT to T.F.

References

Allen, S.K.J.; Thiery, R.G.; Hagstrom, N.T., 1986: Cytological evaluation of the likelihood that triploid grass carp will reproduce. T. Am. Fish. Soc. **115**, 841-848.

Alves, M.J.; Coelho M.M.; Collares-Pereira, M.J., 1998: Diversity in the reproductive modes of females of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): a way to avoid the genetic constraints of uniparentalism. Mol. Biol. Evol. **15**, 1233-1242.

Alves, M.J.; Coelho, M.M.; Próspero, M.I.; Collares-Pereira, M.J., 1999: Production of fertile unreduced sperm by hybrid males of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): An alternative route to genome tetraploidization in unisexuals. Genetics **151**, 277-283.

Alves, M.J.; Gromicho, M.; Collares-Pereira, M.J.; Crespo-López, E.; Coelho, M.M., 2004: Simultaneous production of triploid and haploid eggs by triploid *Squalius alburnoides* (Teleostei: Cyprinidae). J. Exp. Zool. **301A**, 552-558.

Bartley, D.M.; Rana, K.; Immink, A.J., 2001: The use of inter-specific hybrids in aquaculture and fisheries. Rev. Fish. Biol. Fisher. **10**, 325-337.

Billard, R.; Zhang, T., 2001: Techniques of genetic resource banking in fish, In: Cryobanking the genetic resource. Wildlife conservation for the future? Eds.: Watson, P.F.; Holt, W.V., London, Taylor and Francis, pp. 156-170.

Castillo, A.G.F.; Beall, E.; Moran, P.; Martinez, J.L.; Ayllon, F.; Garcia-Vazquez, E., 2007: Introgression in the genus *Salmo* via allotriploid. Mol. Ecol. **16**, 1741-1748.

Cherfas, N.B.; Gomelsky, B.I.; Emelyanova, O.V.; Reconbratsky, A.V., 1994: Induced diploid gynogenesis and polyploidy in crucian carp, *Carassius auratus gibelio* (Bloch) × common carp, *Cyprinus carpio* L., hybrids. Aquacult. Fish. Manage. **25**, 943-954.

Chevassus, B., 1983: Hybridization in fish. Aquaculture **33**, 245-262.
Coyne, J.A.; Orr, H.A., 1998: The evolutionary genetics of speciation. Philos. Trans. R. Soc. Lond. B Biol. Sci. **353**, 287-305.
Dawley, R.M., 1989, An introduction to unisexual vertebrates, In: Evolution and ecology of unisexual vertebrates. Eds.: Dawley, R.M.; Bogart, J.P., Albany, NY: New York State Museum, pp. 1-18.
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.* Zool. Sci. **21**, 747-755.
Fujimoto, T.; Kataoka, T.; Sakao, S.; Saito, T.; Yamaha, E.; Arai, K., 2006: Developmental stages and germ cell lineage of the loach (*Misgurnus anguillicaudatus*). Zool. Sci. **23**, 977-989.
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. J. Exp. Zool. **307A**, 449-462.
Goddard, K.A.; Schultz, R.J., 1993: Aclonal reproduction by polyploid members of the clonal hybrid species *Phoxinus eos-neogaeus* (Pisces; Cyprinidae). Copeia **1993**, 650-660.
Günther, R.; Uzzell, T.; Berger, L., 1979: Inheritance patterns in triploid *Rana* "*esculenta*" (Amphibia, Salientia). Mitt. Zool. Mus. Berl. **55**, 35-57.
Kim, D.S.; Nam, Y.K.; Park, I.-S., 1995: Survival and karyological analysis of reciprocal diploid and triploid hybrids between mud loach (*Misgurnus mizolepis*) and cyprinid loach (*Misgurnus anguillicaudatus*). Aquaculture **135**, 257-265.
Kurokura, H.; Hirano, R.; Tomita, M.; Iwahashi, M., 1984: Cryopreservation of carp sperm. Aquaculture **37**, 267-273.
Lilyestrom, C.G.; Wolters, W.R.; Bury, D.; Rezk, M.; Dunham, R.A., 1999: Growth, carcass traits, and oxygen tolerance of diploid and triploid catfish hybrid. N. Am. J. Aquacult. **61**, 293-303.
Lincoln, R.F., 1981: Sexual maturation in triploid male plaice (*Pleuronectes platessa*) and plaice × flounder (*Platichthys flesus*) hybrid. J. Fish Biol. **19**, 415-426.
Linhart, O.; Rodina, M.; Flajshans, M.; Mavrodiev, N.; Nebesarova, J.; Gela, D.; Kocour, M., 2006: Studies on sperm of diploid and triploid tench, *Tinca tinca* (L.). Aquacult. Int. **14**, 9-25.
Liu, S.; Liu, Y.; Zhou, G..; Zhang, X.; Luo, C.; Feng, H.; He, X,; Zhu, G.; Yang, H., 2001: The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture **192**, 171-186.
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.; Nakayama, I.; Arai, K., 2001: Microsatellite-centromere mapping in the loach, *Misgurnus anguillicaudatus*. Genetica **111**, 59-69.
Morishima, K.; Nakayama, I.; Arai, K., 2008: Genetic linkage map of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). Genetica, in press.
Na-Nakorn, U.; Rangsin, W.; Boon-ngam, J., 2004: Allotriploidy increases sterility in the hybrid between *Clarias macrocephalus* and *Clarias gariepinus*. Aquaculture **237**, 73-88.
Nam, Y.K.; Park, I.-S.; Kim, D.S., 2004: Triploid hybridization of fast-growing transgenic mud loach *Misgurnus mizolepis* male to cyprinid loach *Misgurnus anguillicaudatus* female: the first performance study on growth and reproduction of transgenic polyploid fish. Aquaculture **231**, 559-572.
Oshima, K.; Morishima, K.; Yamaha, E.; Arai, K., 2005: Reproductive capacity of triploid loaches obtained from Hokkaido Island, Japan. Ichthyol. Res. **52**, 1-8.
Park, I.-S.; Nam, Y.K.; Kim, D.S., 2006: Growth performance, morphometric traits and gonad development of induced reciprocal diploid and triploid hybrids between the mud loach (*Misgurnus mizolepis* Günther) and cyprinid loach (*Misgurnus anguillicaudatus* Cantor). Aquac. Res. **37**, 1246-1253.
Saitoh, K.; Kim, I.S.; Lee, E.H., 2004: Mitochondrial gene introgression between spined loaches via hybridogenesis. Zool. Sci. **21**, 795-798
Schultz, R.J., 1969: Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. Amer. Natur. **103**, 605-619
Senanan, W.; Kapuscinski, A.R.; Na-Nakorn, U.; Miller, L.M., 2004: Genetic impacts of hybrid catfish farming (*Clarias macrocephalus* × *C. gariepinus*) on native catfish populations in

central Thailand. Aquaculture **235**, 167-184.
Sousa-Santos, C.; Collares-Pereira, M.J.; Almada, V., 2007: Fertile triploid males - an uncommon case among hybrid vertebrates. J. Exp. Zool. **307A**, 220-225.
Sun, Y.; Zhang, C.; Liu, S.; Duan, W.; Liu, Y., 2007: Induced interspecific androgenesis using diploid sperm from allotetraploid hybrids of common carp × red crucian carp. Aquaculture **264**, 47-53.
Suzuki, R., 1983: Multiple spawning of the cyprinid loach, *Misgurnus anguillicaudatus*. Aquaculture **31**, 233-243.
Suzuki, R.; Nakanishi, T.; Oshiro, T., 1985: Survival, growth and sterility of induced triploids in the cyprinid loach *Misgurnus anguillicaudatus*. Bull. Japan. Soc. Sci. Fish. **51**, 889-894.
Wills, P.S.; Sheehan, R.J.; Allen, S.K.J., 2000: Reduced reproductive capacity in diploid and triploid hybrid sunfish. T. Am. Fish. Soc. **129**, 30-40.
Yoshikawa, H.; Morishima, K.; Fujimoto, T.; Arias-Rodriguez, L.; Yamaha, E.; Arai, K., 2008: Ploidy manipulation using diploid sperm in the loach, *Misgurnus anguillicaudatus*: A review. J. Appl. Ichthyol., in press
Zhang, Q.; Arai, K., 1999a: Aberrant meiosis and viable aneuploid progeny of induced triploid loach (*Misgurnus anguillicaudatus*) when crossed to natural tetraploid. Aquaculture **175**, 63-76.
Zhang, Q.; Arai, K., 1999b: Distribution and reproductive capacity of natural triploid individuals and occurrence of unreduced eggs as a cause of polyploidization in the loach, *Misgurnus anguillicaudatus*. Ichthyol. Res. **46**, 153-161.

Figure legend

Fig. 1. Relative DNA contents measured by flow cytometry in somatic cells and spermatozoa of diploid control (A, B), autotriploid (C, D), diploid hybrid (E, F, G) and triploid hybrid (H, I, J, K). The number showing DNA content indicates the ploidy status. A: 2n somatic cells in diploid control; B: 1n spermatozoa in diploid control; C: 3n somatic cells in autotriploid; D: 1.5n, 3n, 4.5n and 6n spermatozoa in autotriploid; E: 2n somatic cells in diploid hybrid; F: 1n, 2n and 4n spermatozoa in diploid hybrid; G: 2n and 4n spermatozoa in other diploid hybrid; H: 3n somatic cells in triploid hybrid; I: 6n spermatozoa in type 1 triploid hybrid; J: 1n and 6n spermatozoa in type 2 triploid hybrid; K: 1n spermatozoa in type 3 triploid hybrid.

Table 1. Ploidy status of somatic cells and spermatozoa in diploid control, autotriploid, diploid hybrid and triploid hybrid males.

	N	Somatic cells	Spermatozoa					
Diploid control	4	2n (4)	1n	(4)				
Autotriploid	5	3n (5)	1.5n/3n/4.5n/6n	(5)				
Diploid hybrid	5	2n (5)	2n/4n	(2)	1n/2n/4n	(3)		
Triploid hybrid	4	3n (4)	1n	(1)	1n/6n	(1)	6n	(2)

In parentheses, number of individuals showing each ploidy status.

Table 2. Concentration, total motility, progressive motility and duration of motility of spermatozoa from diploid control, autotriploid, diploid hybrid and triploid hybrid males.

	N	Concentration ($\times 10^6$ cells/mL)		Total motility (%)		Progressive motility (%)		Duration of motility (s)	
		Average Range	SD	Average Range	SD	Average Range	SD	Average (N)* Range	SD
Diploid control	4	4612.7[a] 3728.6 - 6848.1	1364.15	90.2[a] 87 - 94	5.81	88.1[a] 83 - 93	7.29	138.2[a] (4) 106 - 159	21.19
Autotriploid	5	38.0[b] 12.6 - 104.7	35.84	1.5[b] 0 - 3	1.99	22.7[b] 0 - 50	36.74	48.0[b] (1)	7.55
Diploid hybrid	5	13.2[b] 0.0028 - 39.0	17.52	4.1[b] 0 - 8	4.88	21.9[b] 0 - 39	33.94	102.9[ab] (3) 63 - 131	30.06
Triploid hybrid									
Type 1	2	1.3[b] 0.58 - 2.0	0.80	0.0[b] -	0	0.0[b] -	0	- (0) -	
Type 2	1	9.6[b] -	3.21	0.0[b] -	0	0.0[b] -	0	- (0) -	
Type 3	1	6270.3[a] -	516.08	88.0[a] 84 - 94	2.89	85.4[a] 79 - 84	6.7	127.3[a] (1) 116 - 135	10.02

Different alphabet superscripts mean significant difference ($P<0.05$).

*These data indicate duration of motility with regard to the males generating motile spermatozoa and the number of males examined were shown in parentheses.

Table 3. Survival potential of progenies produced by fertilization of eggs from two different *M. anguillicaudatus* females (trial 1 and 2) with spermatozoa from diploid control (1-4), autotriploid (1-5), diploid hybrid (1-5) and triploid hybrid (1-4) males.

	Male No.	No. of egg used	Survival at early somite stage (%)	Hatched larvae (%)	
				Normal	Abnormal
Trial 1					
Diploid control	1	176	81.8	79.5	1.7
	2	187	67.9	64.7	1.6
	3	314	66.2	60.2	4.8
	4	463	66.5	63.5	3.0
Autotriploid	1	300	0.0	0.0	0.0
	2	369	0.0	0.0	0.0
	3	345	0.0	0.0	0.0
	4	298	0.0	0.0	0.0
	5	407	0.0	0.0	0.0
Diploid hybrid	1	295	0.0	0.0	0.0
	2	386	1.0	0.0	0.3
	3	380	0.3	0.0	0.0
	4	399	0.0	0.0	0.0
	5	561	1.1	0.0	0.9
Triploid hybrid	1	455	64.6	60.2	3.5
	2	386	0.3	0.0	0.3
	3	385	0.0	0.0	0.0
	4	289	0.0	0.0	0.0
Trial 2					
Diploid control	1	261	45.2	43.7	1.5
	2	272	41.5	40.1	0.7
	3	814	32.2	31.4	0.5
	4	707	31.7	31.4	0.1
Autotriploid	1	380	0.0	0.0	0.0
	2	382	0.0	0.0	0.0
	3	427	0.0	0.0	0.0
	4	388	0.0	0.0	0.0
	5	588	0.0	0.0	0.0
Diploid hybrid	1	281	0.0	0.0	0.0
	2	433	0.0	0.0	0.0
	3	528	0.0	0.0	0.0
	4	557	0.2	0.0	0.2
	5	459	2.2	1.5	0.7
Triploid hybrid	1	745	25.4	25.2	0.0
	2	706	0.3	0.3	0.0
	3	390	0.0	0.0	0.0
	4	439	0.0	0.0	0.0

Table 4. Ploidy status flow cytometrically determined in larvae from diploid control, diploid hybrid and triploid hybrid males.

Type of males	*N*	Putative ploidy status	Mean value of main fluorescent peak		CV% of main fluorescent peak	
			Average	SD	Average	SD
Diploid control	10	2n	96.7[a]	2.42	2.12[a]	0.371
Diploid hybrid	17	2n	96.4[a]	1.89	2.52[b]	0.586
Triploid hybrid	23	2n	95.4[a]	1.10	2.13[a]	0.261

Different alphabet superscripts mean significant difference (P <0.05).

Table 5. Microsatellite genotyping in diploid larvae between *M. anguillicaudatus* females and diploid hybrid males.

Locus	Diploid hybrid male (M* / A1**)	*M. anguillicaudatus* female (A2*** / A3***)	*N*	Genotypes in larvae			
				M / A2 or A3	A1 / A2 or A3	M / A1 / A2 or A3	A2 or A3
Mac 37	M: 99 or 116 A1: 107 or 127	Trial 1: 112 / 112 Trial 2 : 105 / 109	17	6	8	2	1
Mac 60	M: 116 or 186 A1: 128 or 132	Trial 1: 122 / 128 Trial 2 : 132 / 132	17	9	8	0	0
Mac 73	M: 247 or 349 A1: 275 or 279	Trial 1: 273 / 275 Trial 2 : 271 / 275	17	8	7	1	1
Mac 87	M: 235 or 235 A1: 271 or 275	Trial 1: 253 / 281 Trial 2 : 237 / 281	17	6	11	0	0

*: M means one allele derived from *M. mizolepis* in diploid hybrid genotypes.

**: A1 means one allele derived from *M. anguillicaudatus* in diploid hybrid genotypes.

***: A2 and A3 mean alleles in counterpart *M. anguillicaudatus* females for experimental breeding in each trial.

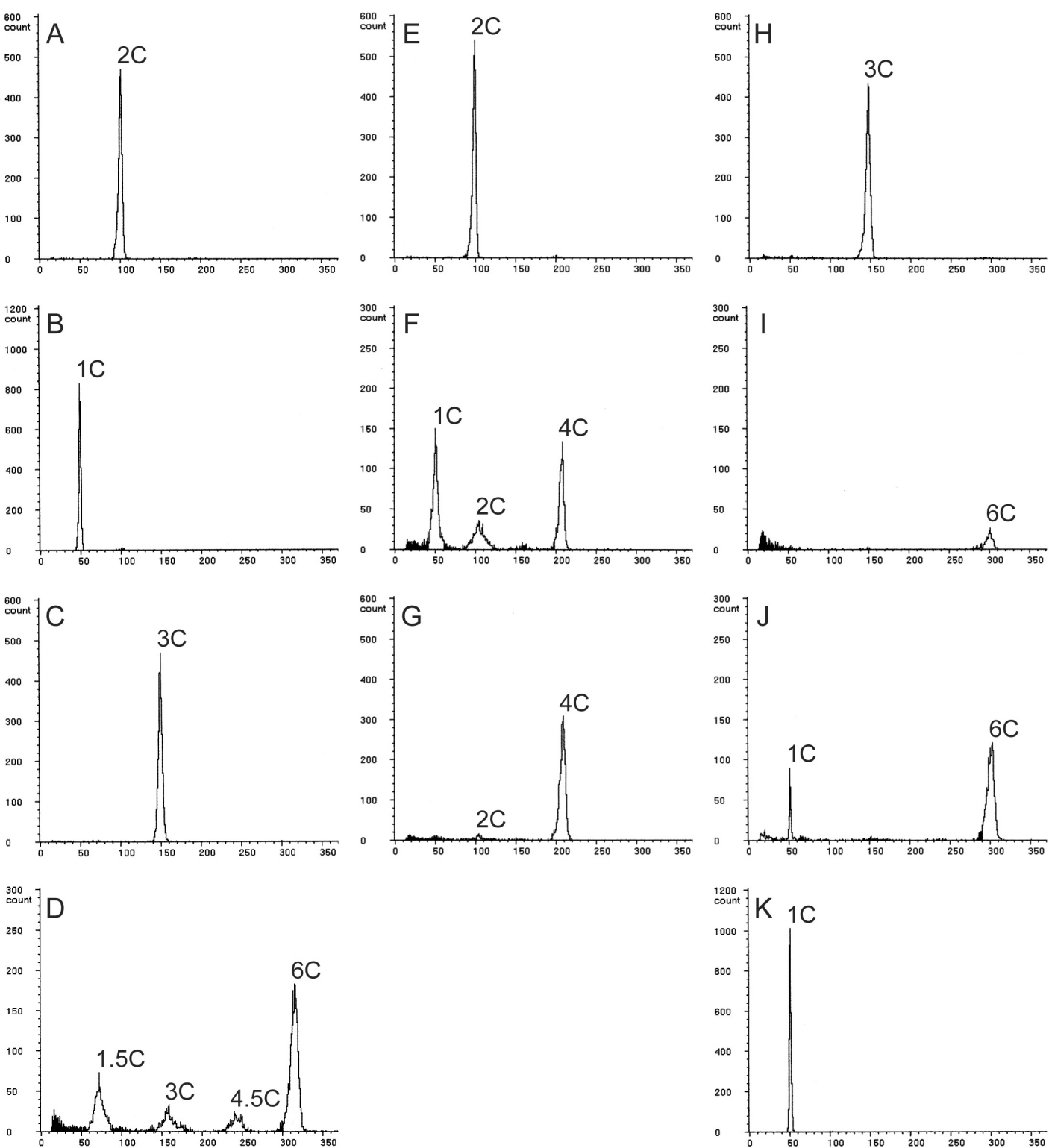
Table 6. Microsatellite genotyping in diploid larvae between *M. anguillicaudatus* females and triploid hybrid males.

Locus	Triploid hybrid male (M* / A1** / A1**)	*M. anguillicaudatus* female (A2** / A3**)	*N*	Genotypes in larvae			
				M / A2 or A3	A1 / A2 or A3	M / A1 / A2 or A3	A2 or A3
Mac 37	M: 99 or 116 A1: 96 and 107	Trial 1: 112 / 112 Trial 2 : 105 / 109	23	0	23	0	0
Mac 60	M: 116 or 186 A1: 124 and 128	Trial 1: 122 / 128 Trial 2 : 132 / 132	23	0	23	0	0
Mac 73	M: 247 or 349 A1: 279 and 313	Trial 1: 273 / 275 Trial 2 : 271 / 275	23	0	23	0	0
Mac 87	M: 235 or 235 A1: 237 and 237	Trial 1: 253 / 281 Trial 2 : 237 / 281	23	0	23	0	0

*: M means one allele derived from *M. mizolepis* in triploid hybrid genotypes.

**: A1 means two alleles derived from *M. anguillicaudatus* in triploid hybrid genotypes.

***: A2 and A3 mean alleles in counterpart *M. anguillicaudatus* females for experimental breeding in each trial.


A
2C
B
1C
C
3C
D
1.5C
3C
4.5C
6C
E
2C
F
1C
2C
4C
G
2C
4C
H
3C
I
6C
J
1C
6C
K
1C
count