Fish Sci (2010) 76:305–313
DOI 10.1007/s12562-010-0224-8

ORIGINAL ARTICLE

Aquaculture

Production of androgenetic amago salmon *Oncorhynchus masou ishikawae* with dispermy fertilization

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Received: 30 October 2009 / Accepted: 11 January 2010 / Published online: 20 February 2010

Abstract With the aim of improving artificial androgenesis in teleost fishes, we tested two methods for producing androgenetic diploids of amago salmon (*Oncorhynchus masou ishikawae*), namely, fertilization of gamma-ray irradiated eggs with fused spermatozoa (sperm-fusion method) and the fertilization of irradiated eggs with untreated sperm followed by the blocking of cell division (mitosis-inhibition method). Our results showed that the optimal condition for sperm fusion was to treat the sperm with 50% polyethylene glycol (molecular weight 7500) for 100 s. The efficiency of the two methods of androgenesis was compared in terms of fertilization rate, hatching rate, and larval survival after hatching. The rate of fertilization was lower with the sperm-fusion method than with the mitosis-inhibition method, but the reverse was true for the hatching rate. The survival rate of hatched larvae was the same with the two methods. Androgenesis was confirmed with a recessive albino color marker, and all viable offspring were found to be heterozygous based on analysis of the microsatellite markers. Our results suggest that androgenesis with the sperm-fusion method is a promising approach with potential applications in both aquaculture breeding programs and the preservation of endangered freshwater fishes.

Keywords Amago salmon · Androgenesis · Fused spermatozoa · Heterozygous · *Oncorhynchus masou ishikawae*

Introduction

Androgenesis is the development of an organism containing only the paternal genome. In teleost fishes, artificial androgenesis can be induced by treating eggs with X-ray or gamma irradiation prior to fertilization [1, 2], but this approach is associated with the inevitable death of the hatched larvae due to haploidy. Consequently, diploidization of the paternal genome is necessary for the survival of the larvae. To this end, earlier studies on artificial androgenesis in fishes [3–7] exclusively achieved diploidization of the paternal genome by inhibiting cell division, producing "double haploid" fish larvae [8].

In recent years, the need of gene banks for endangered wild or important commercial species has increased. The combination of sperm cryopreservation and androgenesis may enable the development of a new technique for preserving species. The method of producing androgenetic diploids by inhibiting cell division is now technically established; however, low production yields are still an issue. With this method, androgenetic offspring are homozygous, meaning a loss of genetic diversity. Therefore, this method would appear to be unsuitable for the conservation of endangered fish. One potential approach to

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avoiding any loss of genetic diversity is artificial androgenesis by fusion of two spermatozoa prior to fertilization. However, successful heterozygous androgenesis using this method has only been reported in a few fishes [9–12].

The aim of this study was to develop a method for artificial androgenesis that would enable the preservation of genetic diversity in endangered teleost fishes. We produced androgenetic diploids of amago salmon (*Oncorhynchus masou ishikawae*) using two methods: (1) fertilization of gamma-ray irradiated eggs with fused spermatozoa (sperm-fusion method) and (2) fertilization of irradiated eggs with untreated sperm followed by the blocking of cell division (mitosis-inhibition method). These methods were then compared in terms of fertilization rate, hatching rate, and larval survival after fertilization. We also discuss the optimized condition of artificial androgenesis and review its usefulness in aquatic biology.

Materials and methods

Experimental design for determining optimal conditions for fusing sperm

In Experiment 1, we evaluated the effects of treating spermatozoa with two polyethylene glycol (PEG) solutions (molecular weight 3000 and 7500, respectively) at three concentrations (30, 40, and 50% w/v) and two treatment durations (20 and 60 s) on the motility of the spermatozoa and the induction of cell fusion in the spermatozoa. We also evaluated the effect of treating the spermatozoa with a high pH–high calcium solution (for 20 and 60 s) (Table 1).

In Experiment 2, we examined the effects of longer treatment durations (from 60 to 180 s) on sperm motility and cell fusion of the spermatozoa. In these experiments, diluted (50%) PEG solutions were used (Table 2).

In Experiment 3, we confirmed the fusion of the spermatozoa by inseminating fresh eggs with the 50% PEG-treated spermatozoa for 100 and 150 s and measuring the fertilization rate and percentage of triploid eggs at the eyed stage.

In Experiment 4, we compared two methods for inducing androgenesis in practical situations: the sperm-fusion method and mitosis-inhibition method.

Table 1 Buffers and conditions in Experiment 1

Buffer	Concentration (%)	Treatment time (s)
Control (ASP[a])		
PEG3000[b]	30	20
		60
	40	20
		60
	50	20
		60
PEG7500[c]	30	20
		60
	40	20
		60
	50	20
		60
High pH–high Ca[d]		20
		60

[a] ASP: 110 mM NaCl, 30 mM KCl, 1.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM $NaHCO_3$, buffered with 20 mM TAPS-NaOH at pH 8.0

[b] Polyethylene glycol of a molecular weight of 3000 (PEG3000; Polyethylene Glycol 4000, Wako, Osaka, Japan)

[c] Polyethylene glycol of a molecular weight of 7500 (PEG7500; Polyethylene Glycol 6000, Wako)

[d] High pH–high calcium solution: 750 mg NaCl, 220 mg KCl, 1110 mg $CaCl_2$ dissolved in 100 ml of DDW, buffered with 20 mM 3-cyclohexylaminopropanesulfonic acid (CAPS)-NaOH, pH 10.0

Table 2 Durations of treatment times examined in Experiment II

Treated buffer	Concentration (%)	Treatment time (s)
Control (ASP)		
PEG3000	50	60
		80
		100
		120
		180
PEG7500	50	60
		80
		100
		120
		180

Broodstock and gametes

Wild amago salmon were cultured in outdoor ponds at the Inland Station of the National Research Institute of Aquaculture (NRIA), Mie prefecture, Japan. We also obtained albino amago salmon from a commercial trout farm in Yamanashi Prefecture in 1993, and these have been reared them in outdoor ponds at the same facility. The albinos have a uniformly yellowish color with red eyes. The inheritance of albinism in amago salmon is known to be recessive [13]. We used semen and eggs from 2-year-old amago and albino amago salmon. The semen was collected by gently pressing the abdomen of ripe males. We checked the females twice each week to determine the timing of ovulation and collected eggs soon after ovulation through an incision in the abdomen. The collected semen was

stored in microtubes placed on ice, while the eggs were stored in a Tupperware container and stored in a refrigerator at 4°C until insemination.

Treatment with fusing solution

We diluted 10 μl of semen with 990 μl ASP for the wild amago salmon (ASP: 110 mM NaCl, 30 mM KCl, 1.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM $NaHCO_3$, buffered with 20 mM TAPS-NaOH at pH 8.0; [14]). The diluted milt was placed into a 50-ml round-bottom glass tube and centrifuged at 800 *g* for 5 min. The supernatant was discarded, and 100 μl of 30, 40, or 50% PEG (PEG3000 or PEG7500) or the high pH–high calcium solution was added to the spermatozoa pellet for 20 or 60 s. After treatment, we added 2.0 ml ASP to the tube and vortexed the mixture for 5 s. The tube was then centrifuged at 800 *g* for 5 min and the supernatant discarded. The pellet was washed with ASP, following which we added 90 μl ASP and mixed the solution using a vortex mixer. We then measured sperm motility and the percentage of fused spermatozoa.

Measurement of sperm motility rate

We prepared sperm samples as described in the treatment with fusing solution. The samples were then diluted 1:49 with artificial coelomic fluid (ACF: 152.6 mM NaCl, 3.5 mM KCl, 2.3 mM $CaCl_2$, 0.7 mM $MgCl_2$, and 5.0 mM $NaHCO_3$; [15]) to activate the sperm. An 8-μl aliquot of the mixture was transferred to a glass slide (Teflon Printed Glass Slide: 21 wells, well diameter 4 mm; Funakoshi Co., Tokyo, Japan). We recorded motility using a video camera (Elmo CCD color camera, Aegis Electronic Group, Gilbert, AZ), a video timer (VTG-22; Houei Co, Tokyo, Japan), and a digital camcorder (GV-D1000; Sony, Tokyo, Japan). The spermatozoa were considered to be motile when the sperm head showed forward movement in consecutive video frames for 5–6 s. The percentage motility was determined by assessing the motility of at least 50 randomly selected spermatozoa for each treatment. We repeated the measurements for each treatment twice, and used the average value for the data analysis.

Measurement of sperm fusion rate

We prepared sperm samples as described above. The sperm was then diluted 1:24 with ASP, and a 10-μl aliquot was transferred to a glass slide and covered with a glass slip. We counted the number of fused spermatozoa under a transmission microscope (×40 objective lens). The spermatozoa were considered to be fused when two to four spermatozoa were touching. Fused spermatozoa, single sperm, and spermatozoa in contact with more than four spermatozoa were counted as a single sample. We observed a total of 100 randomly selected samples in each trial. The percentage of fused sperm was calculated as the number of fused sperm in the 100 samples.

Optimization of treatment duration with PEG

We treated the milt with 50% PEG solution (PEG3000 or PEG7500) for 60, 80, 100, 120, or 180 s in Experiment 2 (Table 2). Following the specific treatment, we processed the spermatozoa according to the protocols described for Experiment 1 and evaluated sperm motility and the percentage of fused spermatozoa.

Evaluation of cross between eggs and fused sperm

We evaluated the percentage of triploids among the hatched larvae following insemination with spermatozoa treated with PEG7500 for 100 of 150 s in Experiment 3. We determined the percentage of triploid offspring by counting the number of nuclear bodies as follows. The eyed-egg embryos were fixed in Carnoy's solution, transferred to a 45% acetic acid solution for 10 min, and then vortexed. The mixture was transferred to a glass slide containing a gelatin solution and nitric acid silver salt and dried at 60°C. The samples were then washed and sealed with a glass cover slip. The number of nuclear bodies was counted under a microscope.

Preparation of gamma ray-irradiated eggs

We obtained eggs from several wild-type amago salmon at the National Research Institute of Aquaculture, Inland Station. After stripping, the eggs were washed with ACF and placed in a container with a lid. The container were exposed to ^{60}Co gamma ray irradiation for 1 h at a final dosage of 350 Gy at room temperature [7].

Androgenetic diploids produced using fused sperm

Milt was obtained from the albino amago salmon. We then diluted 10 μl of semen with 990 μl ASP. The diluted milt was placed into a 50-ml round-bottom glass tube and centrifuged at 800 *g* for 5 min. The supernatant was discarded and 100 μl of 50% PEG7500 was added to the spermatozoa pellet for 100 s. We then added 2.0 ml ASP to the tube and vortexed the mixture for 5 s, following which the tube was centrifuged at 800 *g* for 5 min and the supernatant subsequently discarded. The pellet was then washed with ASP, after which 90 μl ASP was added and the solution was mixed using a vortex mixer. The PEG-treated spermatozoa were added to the irradiated eggs as described above, and the fertilized eggs were kept in 15°C running water.

Androgenesis by inhibiting cell division

We used eggs from the wild amago salmon and sperm from the albino amago salmon. The irradiated eggs were inseminated with untreated spermatozoa from the albino amago salmon. The fertilized eggs were then hydropressure-shocked (650 kgf/cm^2, 6 min) using a French press (Ohtake Works Co, Tokyo, Japan) after 7.5 h at 10°C. Following the hydropressure treatment, the eggs were held in running water at 15°C. After fertilization, we measured the fertilization, hatching, and survival rates of the juveniles at 2 months after fertilization.

Using microsatellite markers to confirm the creation of heterozygous androgenetic amago salmon fry

We used microsatellite markers to measure the heterozygosity of androgenetic fry produced by sperm fusion. We extracted genomic DNA from pectoral fin tissue using a phenol–chloroform method [7]. A total of 16 microsatellite loci were surveyed, namely, *Ots*3, FGT5, *Ots*4, *Ots*1, *Ssa*197, *One*μ7, μ*Sat*73, MST85, *One*μ18, *One*μ21, *One*μ8, MST28, *One*μ13, Omy0002DIAS, *Omy*325, and *One*μ11 [16–23], using the PCR cycling conditions described in the respective papers for each primer set. PCR fragments were analyzed on an ABI 310 automated DNA sequencer according to the manufacturer's recommendations. Allele sizes were determined with Genescan 3.1 and Genotyper 2.5 software (Applied Biosystems, Foster City, CA).

Fertilization, hatching, and survival rates

We designed four experiments. Experiment 1 (intact control) was the cross between untreated wild amago salmon eggs and untreated albino amago salmon sperm. Experiment 2 (androgenesis control) was the cross between the gamma ray-irradiated wild amago salmon eggs and untreated albino amago salmon. Experiment 3 (fusion method) was the cross between the gamma-ray irradiated wild amago salmon eggs and the fused albino amago salmon. Experiment 4 (mitosis-inhibition method) consisted of the treatment in which cell division was inhibited after gamma ray-irradiated wild amago salmon eggs were fertilized with untreated albino amago salmon sperm.

To evaluate the success of fertilization, we collected 30 eggs at 15 h after fertilization and fixed these in Bouin's fixative. We then examined the eggs under a microscope to determine whether cleavage had occurred. The hatching rate was calculated as the number of hatched larvae relative to the number of eggs that were successfully fertilized. The survival rate was calculated as the number of surviving fry 2 months after fertilization relative to the number of eggs that were successfully fertilized.

Statistical analysis

Each treatment for the determination of the optimal condition was replicated four times, and the mean values were calculated. Differences between the treatments were considered to be significant when $P < 0.05$ or 0.01 by the paired t test. The number of fertilized eggs, the number of hatched eggs and the number of surviving fry were compared between the two methods (sperm-fusion method and mitosis-inhibition method). Differences were considered to be significant at $P < 0.05$ or $P < 0.01$ by Fisher's exact test.

Results

Effect of treatment solution and of treatment duration on sperm motility

Sperm motility was high in the control (ASP only) group (87.0 ± 6.7%, $n = 4$) (Fig. 1). In the PEG3000 treatment, sperm motility decreased significantly with increasing concentration of PEG3000 and increasing duration of treatment. Motility was lowest (52.6 ± 9.8%) at a concentration of 50% PEG3000 for 60 s (Fig. 1). Similarly, in the PEG7500 treatment, motility decreased with increasing concentration of PEG7500 and increasing time of treatment. There was no difference in motility of sperm treated with PEG3000 and PEG7500 for the same period of time and same concentration. For both the 20- and 60-s treatments, the lowest sperm motility was seen with the high pH–high calcium solution, namely, 36.1 and 15.2%, respectively.

Effect of the treatment solution and of treatment duration on sperm fusion

We observed some degree of cell fusion in the control treatment group (2.4 ± 0.8%). The frequency of cell fusion tended to increase as the concentration of PEG3000 increased; this tendency was also observed when the concentration of PEG7500 increased. At the highest concentration (50% w/v), the percentage of fused cells increased with the duration of treatment (Fig. 2). Thus, treatment with 50% PEG7500 for 60 s resulted in the highest level of cell fusion (12.6 ± 1.5%). The high pH–high calcium solution increased the percentage (5.4–5.7%) of fused cells relative to the controls, but the levels were lower than those in the groups treated with 50% PEG. Figure 3 is a photomicrograph of the fused sperm following treatment with PEG. We did not observe any morphological difference between the fused and control sperm, with the exception of the partial lack of a tail in the former.

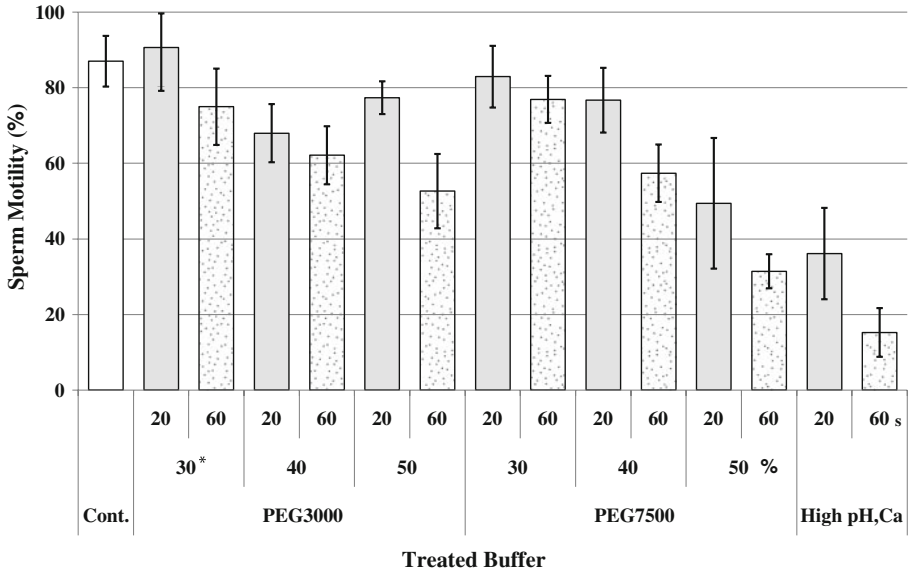

Fig. 1 Percentage of sperm motility following treatment with different concentrations of polyethylene glycol (*PEG*) or a high pH–high calcium solution for different treatment durations (20 and 60 s, respectively). *PEG3000*, *PEG7500* Buffer solutions containing PEG at either a molecular weight of 3000 or 7500, respectively, *high pH,Ca* high pH–high calcium solution (750 mg NaCl, 220 mg KCl, 1110 mg $CaCl_2$ dissolved in 100 ml double-distilled water (DDW), adjusted to pH 10 with NaOH [14]). *Error bars* represent 1 standard deviation (SD). *Asterisk* represents significant differences between the 20- and 60-s treatments at $P < 0.01$ by the paired *t* test. *Cont.* Control

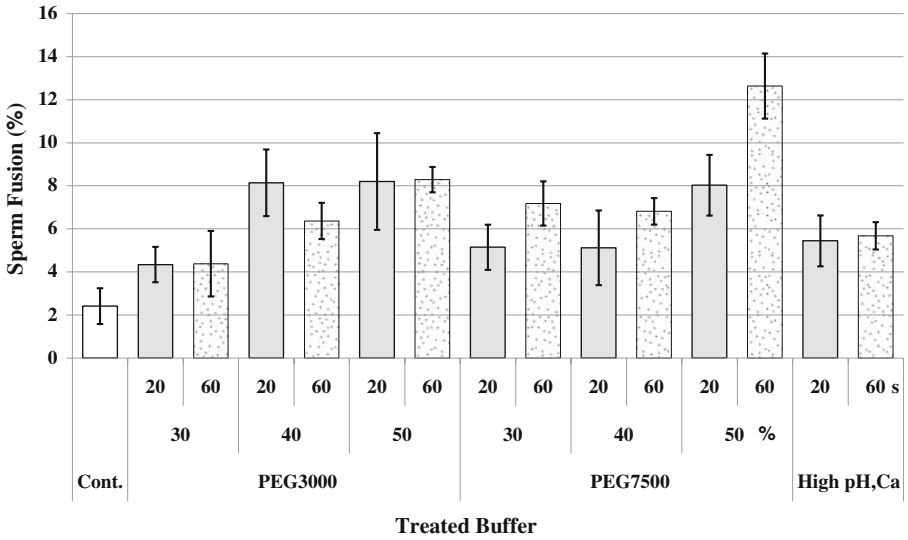

Fig. 2 Percentage of fused sperm following treatment with different concentrations of PEG or with the high pH–high calcium solution for various treatment durations (20 and 60 s, respectively). *Error bars* represent 1 SD

Effect of longer treatment durations on sperm motility and sperm fusion

Treatment with 50% PEG resulted in a high efficiency in terms of cell fusion although sperm motility decreased with increasing concentrations of PEG. This led us to examine the optimal time required for cell fusion. The percentage of motile and fused sperm in the control group was 91.3 ± 3.9 and 1.9 ± 0.3%, respectively (Fig. 4). Sperm motility decreased as the duration of treatment with PEG3000 increased (from 62.5 ± 4.2 to 15.6 ± 6.0%); in contrast, the frequency of cell fusion increased as the duration of treatment increased (from 6.7 ± 0.3 to 18.8 ± 1.9%) (Fig. 4). Sperm motility tended to increase slightly as the duration of treatment with PEG7500 increased up to 100 s (Fig. 4); however, motility drastically decreased thereafter. The percentage of fused cells showed approximately the same pattern of increase as in the treatment with PEG3000, with a maximum at 120 s (Fig. 4).

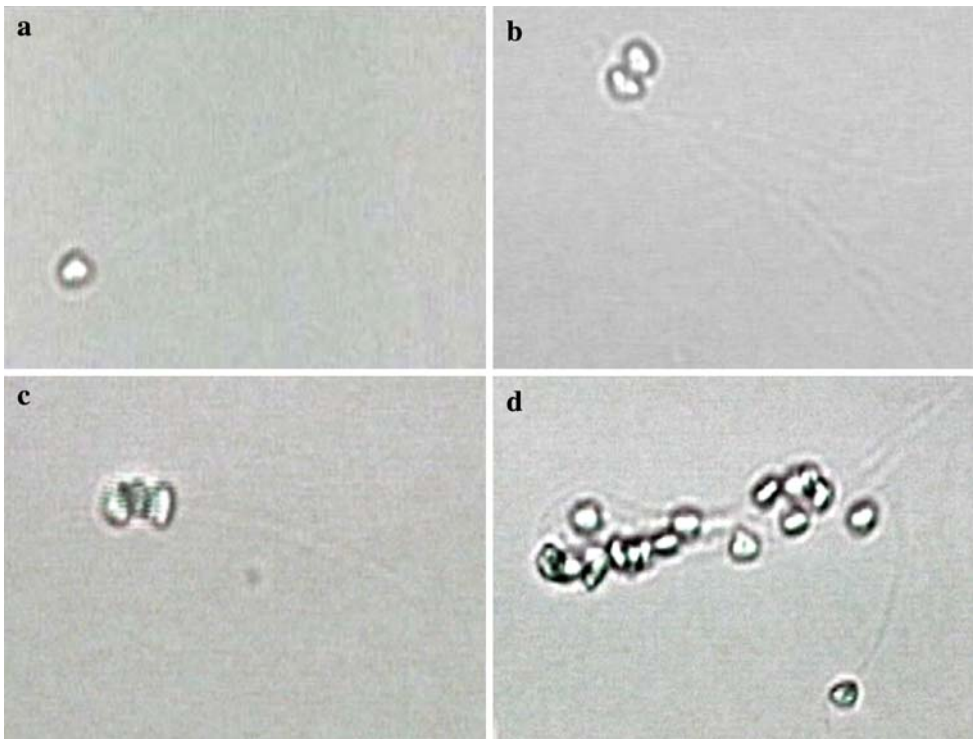

Fig. 3 Photomicrographs of fused sperm. **a** Control, **b** two sperm, **c** three sperm, **d** four or more sperm

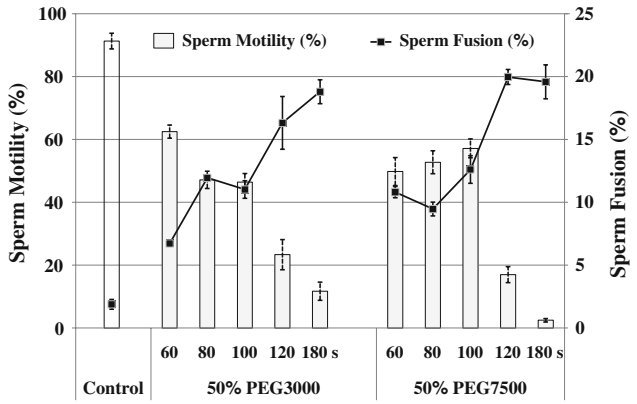

Fig. 4 Sperm motility and percentage of fused sperm according to treatment duration with 50% PEG3000 and 7500, respectively. *Error bars* represent 1 SD

Induction of triploidy with fused sperm

In the treatment with 50% PEG7500 (Fig. 4), sperm motility decreased when the treatment was longer than 100 s, whereas the ratio of sperm fusion increased at treatment times >100 s. Therefore, we treated the spermatozoa for 100 and 150 s using 50% PEG7500 and obtained percentages of motile and fused sperm of 35.2 and 13.2% (100 s) and 19.5 and 12.4% (150 s), respectively. The rate of fertilization was high in both treatment groups (92.3 ± 0.9% for 100 s and 91.0 ± 2.9% for 150 s). In contrast, the rate of triploidy was quite low (0.7 ± 0.25% for 100 s and 0.5 ± 0.5% for 150 s) (Fig. 5).

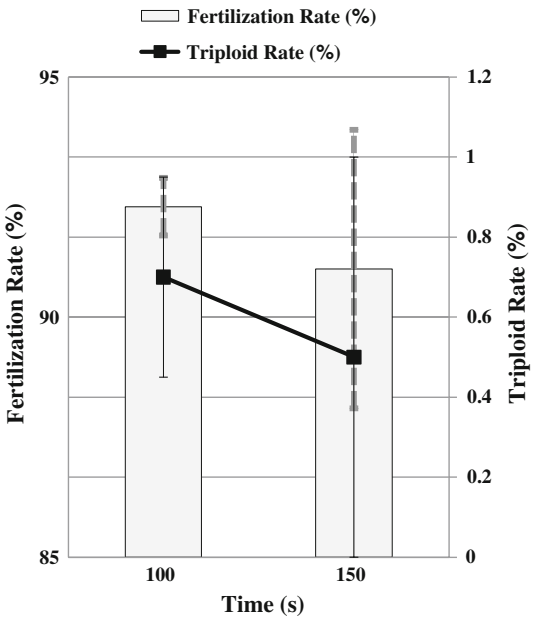

Fig. 5 Fertilization and triploid rates with treatment of 50% PEG7500 for 100 and 150 s. *Bar* Standard deviation

Confirmation of androgenetic diploids produced by both methods

Androgenetic amago salmon produced by both methods showed albinism as a phenotype character. No larvae hatched when the irradiated eggs were inseminated with untreated albino amago salmon sperm.

Table 3 Fertilization rate and survival number (%) at hatching and 2 months after fertilization

Experiment no.	Eggs	Sperm	Treatment	Fertilized rate (%)	Hatched eggs, *n* (%)	Survival, *n* (%)[a]	Number of used eggs
1	Wild amago salmon	Albino amago salmon	No (intact control)	90	173 (96.1)	170 (94.4)	200
2	Irradiated wild amago salmon		No (androgenesis control)	80	0 (0)	0 (0)	208
3			Fused[b]	53.3	38 (0.22)	22 (0.13)	31960
4			Blocking cell division[c]	80	8 (0.09)	8 (0.09)	11034

[a] Survival rates at 2 months after fertilization

[b] Eggs irradiated with ^{60}Co were fertilized with sperm fused with PEG

[c] Cell division was inhibited by hydropressure shock in eggs irradiated with ^{60}Co and fertilized with normal sperm

We were able to amplify six microsatellite loci of 16 loci with the genomic DNA of amago salmon. Among the six PCR-amplified microsatellite loci, three (*μSat*73, *Ots*4, *Omy*325) showed a heterozygous pattern in androgenetic amago salmon produced using the sperm-fusion method.

Comparison of fertilization, hatching, and survival rates with the different methods

The fertilization (90%) and hatching rates (96.1%) were high in Experiment 1 (Table 3; intact control). Survival rates 2 months after fertilization were also high (94.4%) in the crosses. No larvae hatched when the gamma ray-irradiated amago salmon eggs were inseminated with untreated albino amago salmon sperm in Experiment 2 (Table 3; androgenesis control). When irradiated amago salmon eggs were inseminated with PEG-treated albino amago salmon sperm in Experiment 3 (Table 3; androgenesis-fused sperm), the fertilization rate was 53.3%. The hatching and survival rates were low (0.22 and 0.13%, respectively). In the irradiated eggs inseminated with untreated sperm, followed by a hydrostatic pressure shock 7–8 h after fertilization (Experiment 4; Table 3; androgenesis-blocking of cell division), hatching and survival rates were low (0.09 and 0.09%, respectively). The hatching rate in Experiment 1 significantly differed from those in Experiments 3 and 4, respectively at $P < 0.01$, and the hatching rates in Experiments 3 and 5 differed significantly at $P < 0.05$. The survival rates in Experiments 1 and 3 and those in Experiments 2 and 4 differed significantly at $P < 0.01$. However, those in Experiments 3 and 4 did not differ significantly.

Discussion

The production of heterozygous androgenetic diploids is exclusively dependent on the fusion efficiency of spermatozoa. In our experiments, we found that it was highest following treatment of the spermatozoa with 50% PEG7500 for 60 s (Fig. 2). However, under these experimental conditions, the rate of fused sperm, including fusion of more than two sperm, was low (12.6%) (Fig. 2). When eggs were fertilized with fused sperm, the rate of triploids was low, although the rate of fertilization was high (Fig. 5). These results imply that fused spermatozoa may experience difficulties in terms of sperm penetration. Ueda et al. [25] counted seven triploids among 21 rainbow trout that had hatched from eggs inseminated with the sperm treated with PEG4000. The rate of triploidy (33.3%) was very high compared with our results (0.97–2%). However, these authors did not report the procedure for fusion of spermatozoa and the developmental stage in great detail. We treated spermatozoa for 100 s in our fusion protocol, whereas Ueda et al. [24] used a two-step process for a total of 180 s (first a 60-s treatment, followed by a 120-s treatment). In another study, Ueda et al. [25] achieved 33.3% (4/12) triploidy by treating spermatozoa with a high pH–high calcium solution for 20 min. In contrast, we had little success with this method as sperm motility was very low compared with the PEG treatment. However, the percentage of fused sperm was relatively high (26.8%) using the high pH–high calcium solution. In a study aimed at determining the optimal time for sperm, Araki et al. [9] evaluated the fusion rate of sperm from rainbow trout and amago salmon treated with a high pH–high calcium solution. They concluded that a 5-min incubation time is optimal. In contrast, using their method, our results suggest that 20 s is optimal for amago salmon sperm. The high percentage of triploids in the experiment of Ueda et al. [25] may have resulted from a mixture of spontaneous triploids because triploids do exist in the control (no treatment). Taken together, these results suggest that the rate of sperm fusion is important in artificial androgenesis with fused sperm. We found no evidence of morphological abnormalities in fused sperm treated with PEG (Fig. 3).

Our results also suggest that, in terms of productivity, artificial androgenesis using the sperm-fusion method is approximately equal to that using the mitosis-inhibition method. However, the survival rate of androgenetic offspring using the sperm-fusion method has been reported to be much lower than that with the mitosis-inhibition method. In their review, Komen and Thorgaard [8] stated that the success rate (yield) of producing androgenetic or gynogenetic offspring greatly differed among species, ranging from 1 to 20%. Extremely low yields of doubled haploids in experiments with a variety of fish species is still a serious problem. The treatment of doubling chromosomes may have wide-ranging and undesirable side effects on embryo development [8]. It is possible that the low productivity of artificial androgenesis using the sperm-fusion method in our experiments was caused by incomplete gamma ray irradiation of the eggs.

The coupled technique of artificial androgenesis and cryopreservation of sperm hold promise for the recovery of endangered species or subspecies [26, 27]. Although the cryopreservation of spermatozoa is well established in many species, efficient and reliable protocols for teleost eggs and embryos are still lacking. Therefore, androgenesis is a promising approach for stock regeneration because it can regenerate stocks with only sperm as the source of nuclear genomic material. In such a framework, the inhibition of cell division to force a doubling of the chromosome number is not appropriate because the products are completely homozygous, which means a loss of genetic diversity. In contrast, androgenetic offspring produced using the sperm-fusion method are heterozygous, as theoretically expected. However, this latter method is approximately equal to an ordinary mitosis-inhibition method in terms of low productivity. To improve artificial androgenesis using the sperm-fusion method, a technique to isolate two fused sperm before insemination needs to be considered. It is noteworthy that androgenetic offspring produced with this method contain maternally inherited mitochondrial DNA, which is different from genomic DNA in origin. Therefore, androgenesis is not a complete regeneration of species or strains, but a hybrid in the genomic sense. Despite these limitations, artificial androgenesis using the sperm-fusion method coupled with cryopreservation, as proposed in this study, is a promising technique for the preservation of not only endangered fishes but also selected strains in fish breeding programs.

Acknowledgments We are grateful to Shigefumi Imai, ^{60}Co Radiation Facility, Nagoya University, for his technical assistance with the gamma irradiation procedure. We also thank Minoru Haga, Director of Oshino branch, Fisheries Technical Center in Yamanashi prefecture, for the collection of albino amago salmon.

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