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Highlights

- Rainbow trout spermatogonia were cryopreserved for up to 5 years.
- Cryopreserved spermatogonia were transplanted into triploid sterile masu salmon.
- Transplanted spermatogonia differentiated into viable sperm and eggs.
- Cryostorage is an effective means to preserve threatened fish spermatogonia.

Abstract

Although sperm cryopreservation is a powerful tool widely applicable in biodiversity conservation and broodstock management, cryopreservation of teleost eggs and embryos remains challenging. In the present study, we demonstrated that spermatogonia of rainbow trout (*Oncorhynchus mykiss*) cryopreserved for 5 years possessed the ability to differentiate into functional eggs or sperm in the gonads of triploid recipient masu salmon (*O. masou*). After cryopreservation for 5 years in liquid nitrogen, intraperitoneally transplanted spermatogonia migrated toward, and incorporated into, the gonads of xenogeneic recipients. The transplanted spermatogonia resumed spermatogenesis and oogenesis in male and female recipients, respectively, and differentiated into sperm or eggs within the gonads of male and female recipients at 2 years posttransplantation. The differentiated sperm and eggs generated normal rainbow trout representative of donor phenotypes. Thus, cryopreservation of spermatogonia is a powerful and reliable method for long-term preservation of fish genetic resources.

Keywords: Rainbow trout; Masu salmon; Testis cryopreservation; Slow freezing; Spermatogonial stem cells; Interspecies transplantation

Abbreviations: WT, Wild-type; pt, posttransplantation; FBS, fetal bovine serum; CP5-2N, Diploid masu salmon recipients that received rainbow trout spermatogonia cryopreserved for 5 years; CP5-3N, Triploid masu salmon recipients that received rainbow trout spermatogonia cryopreserved for 5 years; SEM, standard error of the mean

Cryopreservation of sperm promises great potential for maintaining the integrity and functionality of fish genetic resources, and is widely applicable in biodiversity conservation, broodstock management in aquaculture, and the preservation of biomedical model fishes. However, it is extremely difficult to cryopreserve teleost oocytes and embryos [3], owing to their large size, high yolk content, and sensitivity to cold [3]. Cryopreservation of immature germ cells may be an effective alternative, which will overcome the limitations of cryopreservation of fish oocytes and embryos, because immature germ cells are more amenable to the process [4,6,10] and can differentiate into eggs and sperm via germ cell transplantation [4,5,9].

In 2007, we developed a new technique for producing only donor-derived eggs and sperm via germ cell transplantation using rainbow trout (*Oncorhynchus mykiss*) spermatogonia as donor cells and triploid sterile masu salmon (*O. masou*) as recipients [9]. We also demonstrated that rainbow trout eggs and sperm could be derived from spermatogonia-cryopreserved in liquid nitrogen for 98 days after transplantation into allogeneic recipients [4]. Recent studies have also demonstrated the development of successful cryopreservation protocols using immature germ cells in several teleost species [6,10]. However, most cryopreservation attempts used relatively short cryopreservation periods (2−98 days) for gamete derivation [4,6,10]. Thus, the differentiation of long-term cryopreserved immature germ cells into functional eggs and sperm via xenogeneic recipients has not been accomplished for any fish species. If effective, this process could facilitate the establishment of a “fish germ cell cryobank” capable of regenerating endangered or even extinct fish species by interspecies transplantation whenever the need arises. In the present study, we tested the feasibility of this hypothesis by transplanting rainbow trout spermatogonia cryopreserved for 5 years into triploid recipient masu salmon.

All fish used in this study were reared using 10 °C spring water at the Oizumi Research and Training Station of Tokyo University of Marine Science and Technology (Yamanashi, Japan). Testis samples (testis weight, 0.021 ± 0.002 g) were obtained from 11-month-old dominant orange-colored (heterozygous, OR/WT) p*vasa-Gfp* (hemizygous, GFP/WT) rainbow trout (*Oncorhynchus mykiss*) (standard length, 12.8 ± 0.3 cm; body weight, 27.7 ± 3.0 g) whose type A spermatogonia were specifically labeled by bright green fluorescence [4]. The testes (gonad-somatic index, 0.077% ± 0.002%) were composed of only type A spermatogonia. Approximately 12 mm hatchlings of wild-type (WT) diploid and triploid masu salmon (*O. masou*, WT/WT/WT) at 40 days post-fertilization were used as recipients for spermatogonial transplantation. Sterile triploids were induced as previously described [9]. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology.

Slow freezing of rainbow trout testes was performed as described previously [4]. Testes isolated from one individual were transferred to each 1.2-mL cryotubes (TPP, Switzerland) containing 500 μL of cryomedium (pH 7.8) containing 1.3 M dimethyl sulfoxide (Sigma-Aldrich, USA), 0.1 M trehalose dehydrate (Sigma-Aldrich), and 10% (v/v) hen egg yolk. Samples were equilibrated for 60 min on ice and cooled at −1 °C/min for 90 min using a Bicell freezing container (Nihon Freezer Company, Japan) located in a −80 °C deep freezer before being plunged into liquid nitrogen. Cryotubes were then cryopreserved for 1 or 5 years. Cryotubes containing testes were thawed quickly in a 10 °C water bath for 1−2 min and then rehydrated in a three-step procedure to minimize osmotic stress, as previously described [4].

Spermatogonial transplantation was performed as previously described [9]. Testes cryopreserved for 5 years (n = 3) and 1 year (n = 3) as well as those that were freshly prepared (n = 3) were chopped with scissors and incubated with 1 mL of 0.5% trypsin

(Worthington Biochemical Corporation, USA) in PBS containing 1 mM Ca^{2+} and 5% FBS for 2 h at 20 °C. The resultant cell suspension was filtered through a 42-μm-pore nylon screen (NBC Incorporation, Japan) to eliminate non-dissociated cell clumps. The harvested cells were counted using a hemocytometer (Neubauer, Darmstadt, Germany) ($13.7 \pm 2.10 \times 10^5$ spermatogonia/fish in 5-year-cryopreservation group, $14.5 \pm 2.63 \times 10^5$ spermatogonia/fish in 1-year-cryopreservation group, and $28.0 \pm 3.06 \times 10^5$ spermatogonia/fish in fresh group), and observed under a fluorescent microscope (BX-51-34FL, Olympus, Japan). Approximately 20−30 nL of the cell suspension containing 2,000 GFP (+) spermatogonia were transplanted into the peritoneal cavity of WT diploid masu salmon hatchlings. At 30 and 120 days posttransplantation (pt), recipient masu salmon were dissected and their gonads were observed under fluorescent microscopes (BX51-34FL and MVX10, Olympus). At 30 days pt, the proportion of recipients that possessed transplanted GFP (+) spermatogonia within their gonads, as well as the number of incorporated GFP (+) spermatogonia were examined. Colonization efficiencies of GFP (+) spermatogonia within the recipient gonads were calculated using the following formula: colonization rate (%) = [(number of fish incorporating GFP-positive cells at 30 days pt)/(number of fish observed)] × 100. Transplantation assays were performed using 26−32 recipients in each group and repeated three times. Data are presented as mean ± standard error of the mean values derived from three independent experiments using different donor individuals (n = 3). Further, to determine whether the 5-year cryopreserved spermatogonia could generate donor-derived sperm and eggs, a transplantation experiment using 5-year-cryopreserved testes (n = 3) and freshly prepared testes (n = 2) was performed. Approximately 10,000 cells of 5-year cryopreserved spermatogonia (mixture of above mentioned three donor individuals) were transplanted into the peritoneal cavity of WT triploid masu salmon hatchlings (CP5-3N). Freshly prepared

spermatogonia (mixture of above mentioned two donor individuals) transplanted into the WT triploid masu salmon (Fresh) was used as a control.

CP5-3N and Fresh triploid recipients were reared to sexual maturity. During the autumn spawning season at 1 and 2 years pt, recipients were anesthetized using 2-phenoxyethanol (Wako Pure Chemical Industries, Japan), and gametes were collected by massaging the abdomen. Milt volume, and sperm and egg numbers were counted as previously described [4]. At 2 years pt, eggs obtained from female recipients were inseminated with milt obtained from male recipients in the same experimental group. If donor phenotypes of 5-year cryopreserved testes (OR/WT, GFP/WT) were successfully transmitted to the F1 generation, approximately 75% of F1 offspring should show the donor phenotypes (OR and GFP), following Mendelian inheritance. Genotypes of F1 offspring were confirmed by RAPD analysis [12]. DNA contents of F1 juveniles were analyzed using a Guava PCA-96 flow cytometer (Millipore, USA) as previously described [4].

All data are presented as means ± standard error. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, using a statistical significance level of $P < 0.05$. All analyses were performed using GraphPad Prism version 5.0 (GraphPad, USA).

To evaluate transplantation efficiency after long-term cryopreservation, GFP (+) spermatogonia (Fig. 1C) recovered from 5-year cryopreserved testes (Fig. 1A and B) were transplanted into WT diploid masu salmon (CP5-2N). CP5-2N recipients were dissected at 30 and 120 days pt, and the behavior of transplanted spermatogonia was observed. At 30 days pt, transplanted spermatogonia had migrated toward, and incorporated into, the gonads of 63 of 92 CP5-2N recipients (68.5% ± 8.1%) (Fig. 1E and G). The mean number of spermatogonia incorporated into gonads of CP5-2N recipients was 24.7 ± 4.6 (Fig. 1E and H). At 120 days

pt, the incorporated spermatogonia proliferated rapidly in the testes of CP5-2N male recipients (Fig. 1I) and began to form a colony of oocytes in the ovaries of CP5-2N female recipients (Fig. 1J). Colonization rate of 5-year cryopreserved spermatogonia in the recipient gonads and the number of incorporated spermatogonia were comparable to those of freshly prepared (Fig. 1D, G, and H) and 1-year cryopreserved spermatogonia (Fig. 1G and H). However, the donor-derived GFP (+) spermatogonia were not detected in the gonads of non-transplanted recipients (Fig. 1F, G, and H).

To determine the production of functional sperm and eggs derived from long-term cryopreserved testes, 5-year cryopreserved spermatogonia were transplanted into WT triploid masu salmon (CP5-3N). Triploid masu salmon that had not received spermatogonia were sterile, except for two males that produced small amounts of aneuploid sperm (Table 1). However, three out of 22 (13.6%) and nine out of 19 (47.4%) CP5-3N males that received 5-year cryopreserved spermatogonia reached sexual maturity at 1 and 2 years pt, respectively (Table 1). A similar tendency was observed in Fresh males that received freshly prepared spermatogonia (Table 1). Milt volumes (1.1 ± 0.2 and 5.8 ± 1.3 mL at 1 and 2 years pt, respectively) and sperm numbers ($5.04 \pm 0.97 \times 10^9$ and $232.83 \pm 35.65 \times 10^9$ at 1 and 2 years pt, respectively) obtained from CP5-3N males did not differ significantly from those obtained from Fresh control recipients or WT diploid masu salmon of the same age (Table 2). Moreover, nine of 25 (36.0%) CP5-3N females that received 5-year-cryopreserved spermatogonia reached sexual maturity at 2 years pt (Table 1), similar to results for females that received freshly prepared spermatogonia (Table 1). The number of eggs (147 ± 21 at 2 years pt) ovulated by CP5-3N females did not differ significantly from those ovulated by Fresh control recipients and WT diploid masu salmon (Table 2). To determine whether the gametes obtained from CP5-3N female and male recipients were donor-derived, eggs

obtained from CP5-3N females were inseminated with milt obtained from CP5-3N males. The number of F1 hatchlings (123 ± 18 at 2 years pt) derived from CP5-3N recipients was comparable to that derived from Fresh control recipients and WT diploid masu salmon (Table 3). In the F1 juveniles produced by CP5-3N recipients, the percentage of orange-colored (73.0% ± 1.7%) and *vasa-Gfp* (+) (75.7% ± 2.9%) fish was approximately 75%, following Mendelian inheritance (Table 3, Fig. 2A-E). Analyses of the DNA content of 24 F1 juveniles produced by CP5-3N recipients revealed that all F1 juveniles were diploid and none exhibited any sign of aneuploidy (Fig. 2H). RAPD analysis of the F1 offspring also revealed that the DNA fingerprinting patterns of the F1 offspring were similar to those of WT rainbow trout and were distinct from those of WT masu salmon and the hybrid (Fig. 2I).

Cryopreservation and interspecific transplantation of spermatogonial stem cells provides a novel strategy for effectively conserving and utilizing intact genetic resources [3]. Since numerous fish species face the threat of extinction, it is imperative to understand whether long-term cryopreserved spermatogonia can differentiate into functional eggs and sperm. In the present study, we demonstrated that rainbow trout spermatogonia cryopreserved for 5 years possessed a high ability to differentiate into functional eggs and sperm in the gonads of xenogeneic triploid recipient masu salmon. The F1 offspring produced by the recipients exhibited the donor phenotypes of orange body color and *vasa-Gfp*-labeled germ cells, normal ploidy level, and the DNA fingerprint of the donor rainbow trout. As all triploid masu salmon that did not receive spermatogonia were functionally sterile, our results indicate that all the gametes produced by the recipients were surely derived from the 5-year cryopreserved spermatogonia.

Theoretically, storage in liquid nitrogen does not affect cell viability and function because of the cessation of all thermally driven chemical reactions [8]. However, Lee et al. [7]

showed that culture potential of mouse spermatogonial stem cells gradually decreased during the first 3 months storage in liquid nitrogen. Previous studies using Persian sturgeon and red seabream [1,2] also shown that fertilization rates of frozen-thawed sperm decreased significantly in relation to the storage duration in liquid nitrogen. Furthermore, Riesco and Robles [11] demonstrated that cryopreservation caused genetic and epigenetic changes in zebrafish genital ridges containing primordial germ cells. Thus, long-term cryopreservation raises concerns regarding unwanted genetic and epigenetic changes that can be transmitted to the next generation and decrease cell viability and function. Further, it remains unclear whether the differentiation ability of transplanted immature germ cells into oocytes remained essentially constant between long-term-cryopreserved and freshly prepared spermatogonia. The present study demonstrated that the number and developmental potency of gametes derived from xenogeneic recipients as well as the differentiation efficiencies of spermatogonia into functional eggs did not change significantly with cryopreservation period up to 5 years. Our previous study using allogeneic trout recipients [4] revealed that viability of spermatogonia did not decrease over a 728-day cryopreservation period and the proliferation efficiencies of transplanted spermatogonia did not decrease with up to 939 days of cryopreservation in liquid nitrogen. Collectively, these results suggest that long-term cryopreservation in liquid nitrogen does not affect the stability and functionality of fish spermatogonia and is, therefore, suitable for use in the “fish germ cell cryobank”.

Although 5-year cryopreserved spermatogonia and xenogeneic recipients were used in this study, the efficiency of obtaining mature recipients (40.9% at 2 years pt) was much higher than that (9.8% at 2 years pt) in our previous study using 98-day cryopreserved spermatogonia and allogeneic recipients [4]. This could be caused by the number of spermatogonia transplanted into each recipient (10,000 cells in this study vs. 5,000 in the

212 previous study [4]) and/or reproductive characteristics of the recipient species including age
213 at full sexual maturity (2 years in masu salmon vs. 3 in rainbow trout [4]). We speculated that
214 the number of transplanted spermatogonia and the reproductive characteristics of the
215 recipient fish species would be more important factors for effective production of donor-
216 derived gametes than the cryopreservation period and genetic distance between donor and
217 recipient fish.
218 We have initiated the building of a Fish Germ Cell Cryobank to preserve
219 spermatogonia of salmonid fish under the threat of extinction. The results of this study
220 strongly support the feasibility of long-term cryostorage of spermatogonia of threatened fish
221 and their regeneration through interspecies transplantation when their habitats are restored.
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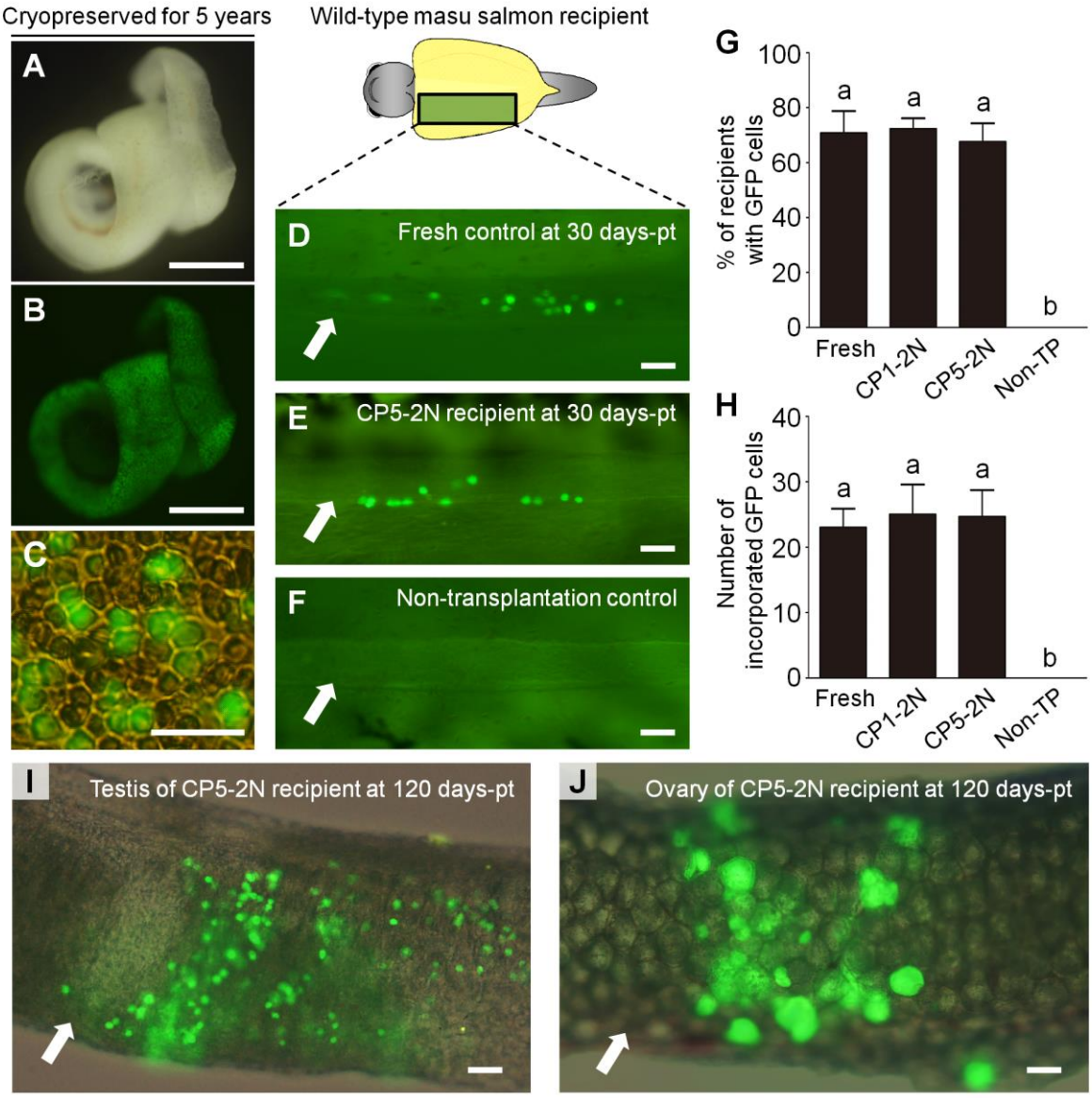
Figure Legends

Fig. 1. Incorporation, proliferation, and differentiation of long-term cryopreserved spermatogonia in the gonads of interspecific recipients. (A,B) Rainbow trout testis cryopreserved for 5 years in the bright-field **(A)** and fluorescent microscopic view **(B). (C)** Testicular cells dissociated from testis, cryopreserved for 5 years. **(D–F)** Fresh **(D)** and 5-year cryopreserved *Gfp*-labeled type A spermatogonia **(E)** incorporated into recipient gonads at 30 days post-transplantation, and the gonad of a non-transplanted control **(F)**. **(G,H)** Percentage of recipients that contained donor spermatogonia within their gonads at 30 days post-transplantation **(G)** and number of spermatogonia incorporated into the recipient gonad **(H)** were not significantly different between spermatogonia cryopreserved for 5 years ($n = 3$) and 1 year ($n = 3$), as well as freshly prepared spermatogonia ($n = 3$). Columns represent mean ± standard error of the mean values derived from three independent experiments using different donor individuals ($n = 3$). Columns with different letters are significantly different with $P < 0.05$. **(I,J)** Five-year cryopreserved *Gfp*-labeled donor ASG proliferated within a testis of male recipient **(I)** and trans-differentiated into oocytes within an ovary of female recipient **(J)**. Arrows indicate the recipient gonads **(D–F,I,J)**. Scale bars, 1 mm **(A,B)**, 50 μm **(C–F,I,J)**.

Fig. 2. Rainbow trout offspring produced by interspecies transplantation of spermatogonia cryopreserved for 5 years. (A) F1 juveniles produced by masu salmon recipients that received rainbow trout spermatogonia cryopreserved for 5 years. Scale bar, 5cm **(A)**. **(B–E)** Approximately 75% F1 juveniles derived from masu salmon recipients displayed the donor-derived phenotypes of orange body color **(B)** and *vasa-Gfp*-labeled germ

cells **(D)** following Mendelian inheritance. Phenotypes of black-pigmented body color **(C)** and gonads containing *vasa-Gfp*-negative germ cells **(E)** in F1 juveniles derived from masu salmon recipients. **(F–H)** Ploidy analyses of donor **(F)**, masu salmon recipients **(G)**, and 24 F1 juveniles produced by masu salmon recipients **(H)** revealed that all the F1 juveniles were normal diploid. **(I)** RAPD analysis of the F1 offspring produced by masu salmon recipients showed that DNA fingerprinting patterns of F1 offspring were same as those of WT rainbow trout and were clearly distinct from those of the WT masu salmon and the hybrid. Lanes are labeled as follows: M, marker; 1–8, F1 offspring of masu salmon recipients; MS, WT masu salmon; RT, WT rainbow trout; HB, hybrid; and DW, distilled water.

295 Fig. 1



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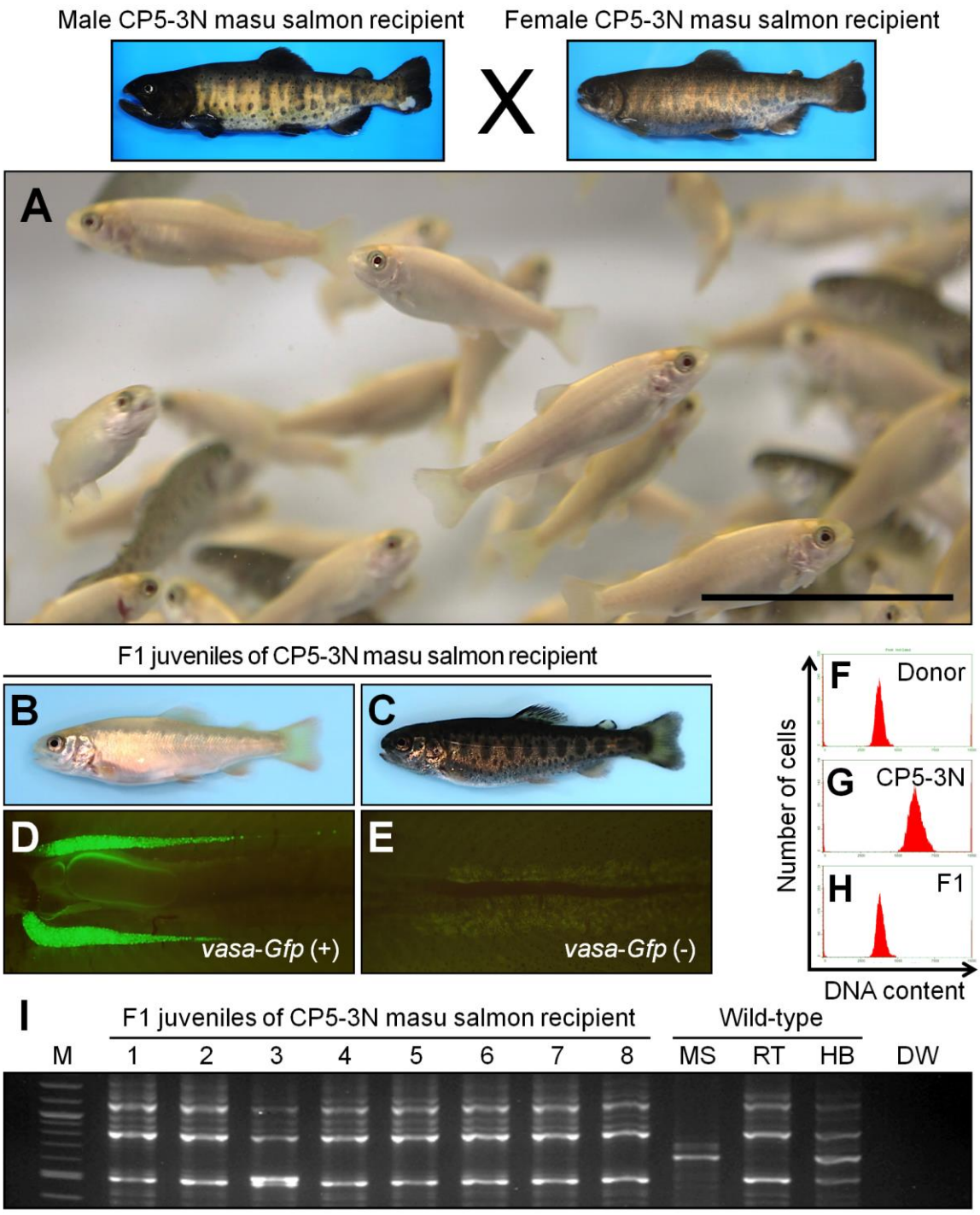
298 Fig. 2


Male CP5-3N masu salmon recipient
Female CP5-3N masu salmon recipient
X
A
F1 juveniles of CP5-3N masu salmon recipient
B
C
D
vasa-Gfp (+)
E
vasa-Gfp (-)
F
Donor
G
CP5-3N
H
F1
Number of cells
DNA content
I
F1 juveniles of CP5-3N masu salmon recipient
Wild-type
M
1
2
3
4
5
6
7
8
MS
RT
HB
DW

299