

# Long-term (5 years) cryopreserved spermatogonia have high capacity to generate functional gametes via interspecies transplantation in salmonids

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1 **Long-term (5 years) cryopreserved spermatogonia have high capacity to generate**  
2 **functional gametes via interspecies transplantation in salmonids**

3

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13 **Highlights**

- 14
- 15 • Rainbow trout spermatogonia were cryopreserved for up to 5 years.
  - 16 • Cryopreserved spermatogonia were transplanted into triploid sterile masu salmon.
  - 17 • Transplanted spermatogonia differentiated into viable sperm and eggs.
  - 18 • Cryostorage is an effective means to preserve threatened fish spermatogonia.

18

19

20 **Abstract**

21

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

35

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

38

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

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

53 In 2007, we developed a new technique for producing only donor-derived eggs and  
54 sperm via germ cell transplantation using rainbow trout (*Oncorhynchus mykiss*)  
55 spermatogonia as donor cells and triploid sterile masu salmon (*O. masou*) as recipients [9].  
56 We also demonstrated that rainbow trout eggs and sperm could be derived from  
57 spermatogonia-cryopreserved in liquid nitrogen for 98 days after transplantation into  
58 allogeneic recipients [4]. Recent studies have also demonstrated the development of  
59 successful cryopreservation protocols using immature germ cells in several teleost species  
60 [6,10]. However, most cryopreservation attempts used relatively short cryopreservation  
61 periods (2–98 days) for gamete derivation [4,6,10]. Thus, the differentiation of long-term  
62 cryopreserved immature germ cells into functional eggs and sperm via xenogeneic recipients  
63 has not been accomplished for any fish species. If effective, this process could facilitate the  
64 establishment of a “fish germ cell cryobank” capable of regenerating endangered or even  
65 extinct fish species by interspecies transplantation whenever the need arises. In the present  
66 study, we tested the feasibility of this hypothesis by transplanting rainbow trout  
67 spermatogonia cryopreserved for 5 years into triploid recipient masu salmon.

68 All fish used in this study were reared using 10 °C spring water at the Oizumi Research  
69 and Training Station of Tokyo University of Marine Science and Technology (Yamanashi,  
70 Japan). Testis samples (testis weight,  $0.021 \pm 0.002$  g) were obtained from 11-month-old  
71 dominant orange-colored (heterozygous, OR/WT) *pvasa-Gfp* (hemizygous, GFP/WT)  
72 rainbow trout (*Oncorhynchus mykiss*) (standard length,  $12.8 \pm 0.3$  cm; body weight,  
73  $27.7 \pm 3.0$  g) whose type A spermatogonia were specifically labeled by bright green  
74 fluorescence [4]. The testes (gonad-somatic index,  $0.077\% \pm 0.002\%$ ) were composed of only  
75 type A spermatogonia. Approximately 12 mm hatchlings of wild-type (WT) diploid and  
76 triploid masu salmon (*O. masou*, WT/WT/WT) at 40 days post-fertilization were used as  
77 recipients for spermatogonial transplantation. Sterile triploids were induced as previously  
78 described [9]. Experiments were conducted in accordance with the Guide for the Care and  
79 Use of Laboratory Animals of the Tokyo University of Marine Science and Technology.

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

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

92 (Worthington Biochemical Corporation, USA) in PBS containing 1 mM Ca<sup>2+</sup> and 5% FBS for  
93 2 h at 20 °C. The resultant cell suspension was filtered through a 42- $\mu$ m-pore nylon screen  
94 (NBC Incorporation, Japan) to eliminate non-dissociated cell clumps. The harvested cells  
95 were counted using a hemocytometer (Neubauer, Darmstadt, Germany) ( $13.7 \pm 2.10 \times 10^5$   
96 spermatogonia/fish in 5-year-cryopreservation group,  $14.5 \pm 2.63 \times 10^5$  spermatogonia/fish in  
97 1-year-cryopreservation group, and  $28.0 \pm 3.06 \times 10^5$  spermatogonia/fish in fresh group), and  
98 observed under a fluorescent microscope (BX-51-34FL, Olympus, Japan). Approximately  
99 20–30 nL of the cell suspension containing 2,000 GFP (+) spermatogonia were transplanted  
100 into the peritoneal cavity of WT diploid masu salmon hatchlings. At 30 and 120 days  
101 posttransplantation (pt), recipient masu salmon were dissected and their gonads were  
102 observed under fluorescent microscopes (BX51-34FL and MVX10, Olympus). At 30 days pt,  
103 the proportion of recipients that possessed transplanted GFP (+) spermatogonia within their  
104 gonads, as well as the number of incorporated GFP (+) spermatogonia were examined.  
105 Colonization efficiencies of GFP (+) spermatogonia within the recipient gonads were  
106 calculated using the following formula: colonization rate (%) = [(number of fish  
107 incorporating GFP-positive cells at 30 days pt)/(number of fish observed)]  $\times$  100.  
108 Transplantation assays were performed using 26–32 recipients in each group and repeated  
109 three times. Data are presented as mean  $\pm$  standard error of the mean values derived from  
110 three independent experiments using different donor individuals ( $n = 3$ ). Further, to determine  
111 whether the 5-year cryopreserved spermatogonia could generate donor-derived sperm and  
112 eggs, a transplantation experiment using 5-year-cryopreserved testes ( $n = 3$ ) and freshly  
113 prepared testes ( $n = 2$ ) was performed. Approximately 10,000 cells of 5-year cryopreserved  
114 spermatogonia (mixture of above mentioned three donor individuals) were transplanted into  
115 the peritoneal cavity of WT triploid masu salmon hatchlings (CP5-3N). Freshly prepared

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

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

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

133 To evaluate transplantation efficiency after long-term cryopreservation, GFP (+)  
134 spermatogonia (Fig. 1C) recovered from 5-year cryopreserved testes (Fig. 1A and B) were  
135 transplanted into WT diploid masu salmon (CP5-2N). CP5-2N recipients were dissected at 30  
136 and 120 days pt, and the behavior of transplanted spermatogonia was observed. At 30 days pt,  
137 transplanted spermatogonia had migrated toward, and incorporated into, the gonads of 63 of  
138 92 CP5-2N recipients ( $68.5\% \pm 8.1\%$ ) (Fig. 1E and G). The mean number of spermatogonia  
139 incorporated into gonads of CP5-2N recipients was  $24.7 \pm 4.6$  (Fig. 1E and H). At 120 days

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

147 To determine the production of functional sperm and eggs derived from long-term  
148 cryopreserved testes, 5-year cryopreserved spermatogonia were transplanted into WT  
149 triploid masu salmon (CP5-3N). Triploid masu salmon that had not received spermatogonia  
150 were sterile, except for two males that produced small amounts of aneuploid sperm (Table  
151 1). However, three out of 22 (13.6%) and nine out of 19 (47.4%) CP5-3N males that received  
152 5-year cryopreserved spermatogonia reached sexual maturity at 1 and 2 years pt, respectively  
153 (Table 1). A similar tendency was observed in Fresh males that received freshly prepared  
154 spermatogonia (Table 1). Milt volumes ( $1.1 \pm 0.2$  and  $5.8 \pm 1.3$  mL at 1 and 2 years pt,  
155 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  
156 years pt, respectively) obtained from CP5-3N males did not differ significantly from those  
157 obtained from Fresh control recipients or WT diploid masu salmon of the same age (Table  
158 2). Moreover, nine of 25 (36.0%) CP5-3N females that received 5-year-cryopreserved  
159 spermatogonia reached sexual maturity at 2 years pt (Table 1), similar to results for females  
160 that received freshly prepared spermatogonia (Table 1). The number of eggs ( $147 \pm 21$  at 2  
161 years pt) ovulated by CP5-3N females did not differ significantly from those ovulated by  
162 Fresh control recipients and WT diploid masu salmon (Table 2). To determine whether the  
163 gametes obtained from CP5-3N female and male recipients were donor-derived, eggs



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

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

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

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

207         Although 5-year cryopreserved spermatogonia and xenogeneic recipients were used in  
208 this study, the efficiency of obtaining mature recipients (40.9% at 2 years pt) was much  
209 higher than that (9.8% at 2 years pt) in our previous study using 98-day cryopreserved  
210 spermatogonia and allogeneic recipients [4]. This could be caused by the number of  
211 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.  
222

223

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227

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- 260

## Figure Legends

261

262

263 **Fig. 1. Incorporation, proliferation, and differentiation of long-term cryopreserved**  
264 **spermatogonia in the gonads of interspecific recipients. (A,B)** Rainbow trout testis  
265 cryopreserved for 5 years in the bright-field (A) and fluorescent microscopic view (B). (C)  
266 Testicular cells dissociated from testis, cryopreserved for 5 years. (D–F) Fresh (D) and 5-year  
267 cryopreserved *Gfp*-labeled type A spermatogonia (E) incorporated into recipient gonads at 30  
268 days post-transplantation, and the gonad of a non-transplanted control (F). (G,H) Percentage  
269 of recipients that contained donor spermatogonia within their gonads at 30 days post-  
270 transplantation (G) and number of spermatogonia incorporated into the recipient gonad (H)  
271 were not significantly different between spermatogonia cryopreserved for 5 years ( $n = 3$ ) and  
272 1 year ( $n = 3$ ), as well as freshly prepared spermatogonia ( $n = 3$ ). Columns represent  
273 mean  $\pm$  standard error of the mean values derived from three independent experiments using  
274 different donor individuals ( $n = 3$ ). Columns with different letters are significantly different  
275 with  $P < 0.05$ . (I,J) Five-year cryopreserved *Gfp*-labeled donor ASG proliferated within a  
276 testis of male recipient (I) and trans-differentiated into oocytes within an ovary of female  
277 recipient (J). Arrows indicate the recipient gonads (D–F,I,J). Scale bars, 1 mm (A,B), 50  $\mu$ m  
278 (C–F,I,J).

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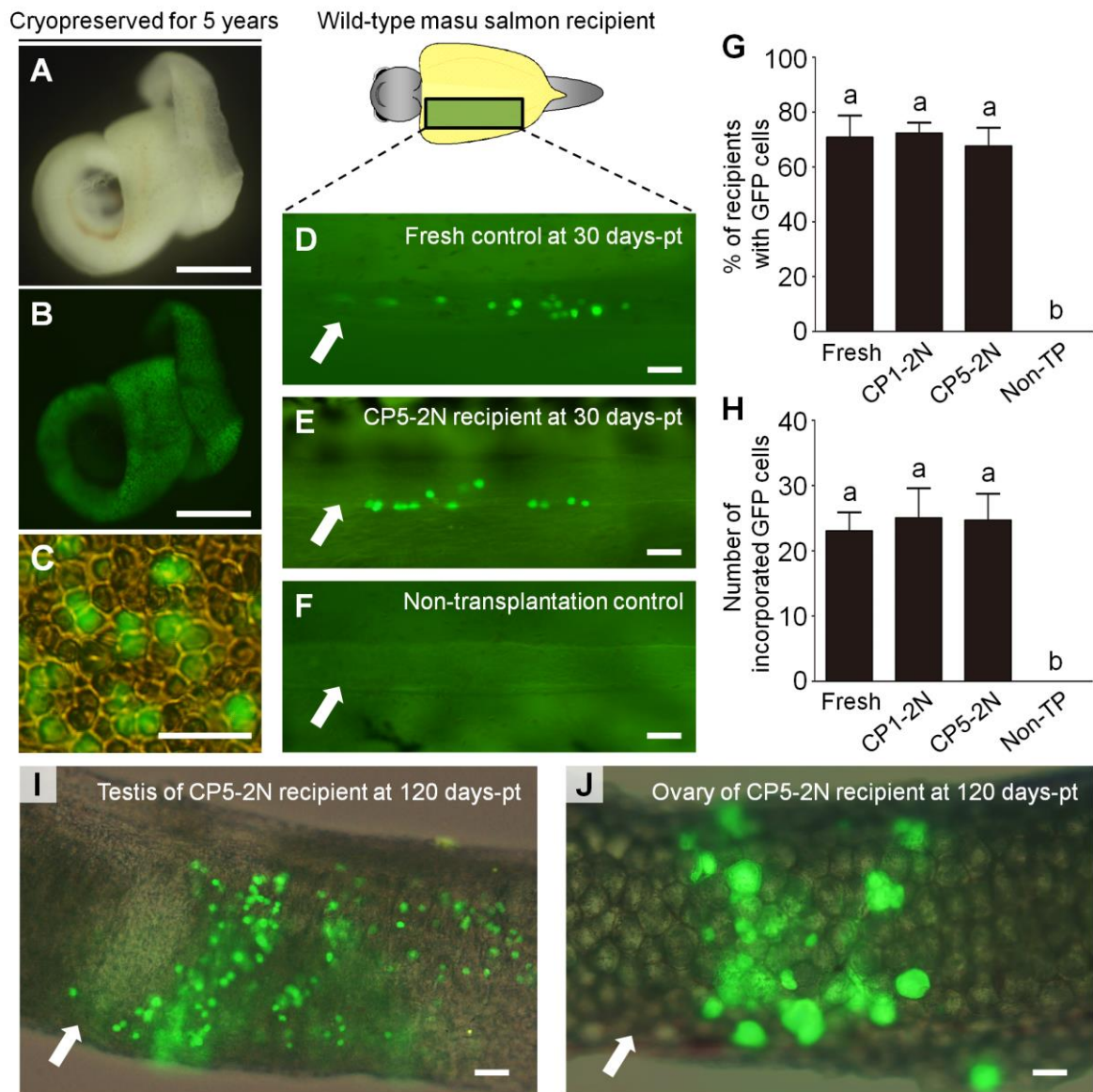
280 **Fig. 2. Rainbow trout offspring produced by interspecies transplantation of**  
281 **spermatogonia cryopreserved for 5 years. (A)** F1 juveniles produced by masu salmon  
282 recipients that received rainbow trout spermatogonia cryopreserved for 5 years. Scale bar,  
283 5cm (A). (B–E) Approximately 75% F1 juveniles derived from masu salmon recipients  
284 displayed the donor-derived phenotypes of orange body color (B) and *vasa-Gfp*-labeled germ

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

294



295 Fig. 1



296

297

