

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**

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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 ± 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 ± 0.3 cm; body weight,
73 27.7 ± 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 μ 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 Ca²⁺ and 5% FBS for
93 2 h at 20 °C. The resultant cell suspension was filtered through a 42- μ 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 ± 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 ± 0.2 and 5.8 ± 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 ± 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 ± 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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- 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 μ m
278 (C–F,I,J).

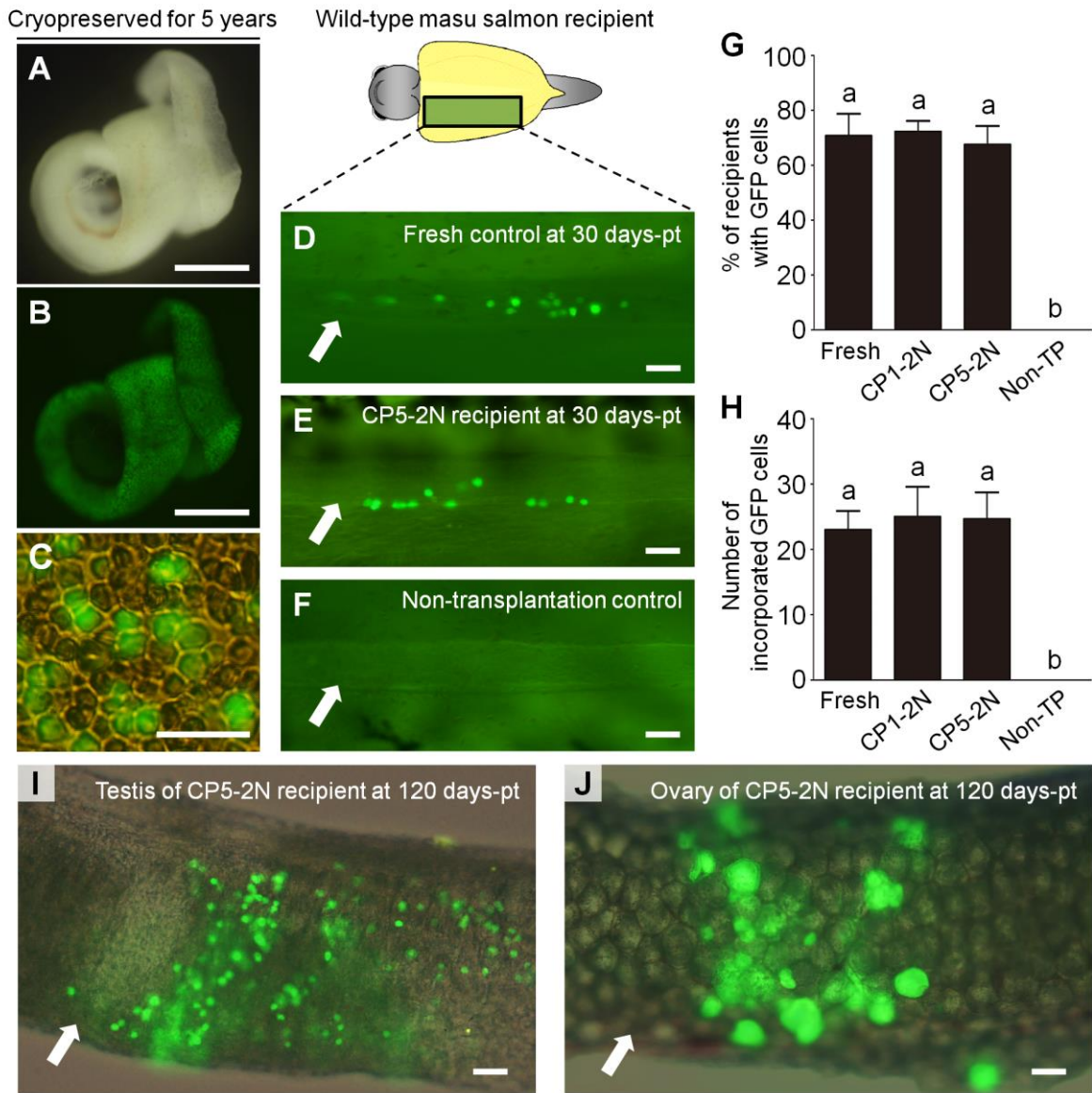
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280 **Fig. 2. Rainbow trout offspring produced by interspecies transplantation of**
281 **spermatogonia cryopreserved for 5 years. (A)** F1 juveniles produced by masu salmon
282 recipients that received rainbow trout spermatogonia cryopreserved for 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

