Thesis for the Degree of Master of Fisheries

Production of transgenic triploid marine medaka (*Oryzias dancena*) carrying red fluorescence protein transgene driven by myosin light chain 2 promoter

by

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(미오신 라이트 사슬-RFP 형질전환 유전자가 이식된 형질전환 바다송사리의 3배체 생산)

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A dissertation

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August 23, 2013

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Abstract

Triploidy was induced in the marine medaka (*Oryzias dancena*) by the cold shock treatment (0 °C) of fertilized eggs two minutes after fertilization for 30, 45, or 60 min. The triploid genotype was induced by all the thermal shock regimes tested. The best result was obtained when the eggs were treated for 45 min, which induced triploidy in all the resulting fish. Triploidy was confirmed with chromosomal and flow-cytometric analyses and erythrocyte measurements. The erythrocyte surface areas and volumes of the triploid fish were significantly larger, and their chromosome numbers (3N = 72) were 1.5 times greater than those of diploid fish (2N = 48). Based on a flow-cytometric analysis, the triploid fish had approximately 1.5 times the cellular DNA content (2.40 pg per cell) of the diploid specimens (1.61 pg per cell). Analysis of the gonads of one-year-old triploid fish showed that the induction of triploidy probably causes sterility in this species, and the effect was apparently greater in females than in males. Data from this study could provide the basis for the development of unique models for studying reproductive confinement in transgenic fish.

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Introduction

The transgenic manipulation of small model fish species has become an established technique in various theoretical research fields. Transgenesis in such laboratory fish models has been considered a useful way to generate novel fluorescent fish for ornamental applications (Gong et al., 2003; Kinoshita, 2004) and to develop pollution-responsive fish strains for environmental purposes (Zeng et al., 2005; Chen et al., 2010). Fluorescent transgenic strains developed in the two most popular models, zebrafish *Danio rerio* and Japanese medaka *Oryzias latipes*, have already entered global markets through aquarium trade (www.glofish.com, www.azoo.com.tw). Although a number of important works have highlighted the functional utility of these transgenic strains, most previous studies have used freshwater fish species for their transgenic studies.

The marine medaka *Oryzias dancena* is a truly euryhaline teleost, with a great capacity for hypo- and hyperosmoregulation. Most of its physiological attributes are similar across a wide spectrum of salinities, ranging from complete freshwater to normal seawater (Inoue and Takei, 2003; Kang et al., 2008; Cho et al., 2010). Therefore, much attention has been directed toward extending the utility of functional transgenic marine medaka strains for ornamental purpose because they can be used at most naturally occurring salinities (Cho et al., 2011). In addition, a recent study on transgenic *O. dancena* expressing a vivid red fluorescent color in their fast skeletal muscles by myosin light chain-2 (*mlc2f*) promoter, suggested their great potential as novel ornamental fish in both freshwater and sea water aquaria

(Cho et al., 2012).

However, the practical application of transgenic fish has raised public and scientific concern about the ecological risks involved especially, those associated with the adverse consequences for natural gene pools, which can be genetically contaminated with unwantedly released transgenic animals (Maclean and Laight, 2000; Devlin et al., 2006). For these reasons, much recent activity in scientific domains has focused on the risk assessment of transgenic fish, with particular emphasis on the reproductive confinement of transgenic stocks (Wong and Van Eenennaam, 2008; Thresher et al., 2009; Hu et al., 2010). Triploidization by blocking the second meiosis has been proposed as one approach to the generation of transgenic fish with depressed reproductive capacities (Piferrer et al., 2009). To date, transgenic triploid fish have been reported among several growth-hormone-transgenic fish strains and the effects of triploidy on the functions and reproductive attributes of transgenic fish are known to be species specific (Razak et al., 1999; Nam et al., 2001; Devlin et al., 2004; Yu et al., 2011).

The objectives of this study were: 1) to develop the optimal conditions for the induction of triploid genotype in this species; 2) to evaluate the cytogenetic and reproductive characteristics of the triploid marine medaka; 3) to generate of triploidy in transgenic marine medaka expressing red fluorescent protein (RFP) and 4) to examine the effects of triploidy on RFP level.

Materials and Methods

Breeding and laboratory maintenance of experimental fish

The marine medaka specimens used in this study were from a laboratory stock maintained at the Institute of Marine Living Modified Organisms (IMLMO), Pukyong National University, Busan, Korea. The general maintenance of the experimental fish was according to the method of Song et al. (2009). Brackish water (5 psu) was used as the breeding water, as described by Cho et al. (2010). The breeding conditions included a temperature of 25 ± 1 °C and a 16 h light:8 h dark cycle. The fish were fed with brine shrimp (*Artemia nauplii*; INVE, Salt Lake City, Utah, USA) and micro-particle feed (150 – 500 μm in diameter; Ewha Oil Co., Busan, Korea).

Induction of triploidy

Fertilized eggs were obtained each day, by mating male and female broodfish in a glass tank containing 30 L of well-aerated water. The eggs were collected from the egg-laying females immediately after fertilization. At 2 min after fertilization, the fertilized eggs were subjected to a cold shock treatment at 0 °C for 30, 45, or 60 min. After treatment, the eggs were placed in a 25 °C incubator until they were hatched.

The hatching success and the incidence of abnormal larvae were measured based on an examination of at least 23 eggs per group under a stereoscopic microscope (C-DS; Nikon Co., Tokyo, Japan). The values of these parameters in the experimental treatments were expressed as percentages

of treated eggs.

Flow cytometry

Twenty-two individuals were randomly chosen for each treatment and control group to assess the incidence of triploidy with flow cytometry. The caudal fin was excised from each fish; the cells were dissociated with scissors in nucleic acid extraction buffer and then stained with DNA staining buffer (CyStain DNA 2 step, Partec Co., Münster, Germany) for 15 min. The prepared samples were analyzed with a PAII flowcytometer (Partec Co.) to determine their ploidy. The DNA content per cell was measured based on a reference standard prepared from the caudal fin of a diploid mud loach (*Misgurnus mizolepis*; 2.8 pg/cell) (Nam et al., 1999).

Chromosome analysis and erythrocyte measurements

The chromosome analysis was performed with a direct method using kidney cells (Kim et al., 1995). Metaphase spreads were prepared from randomly selected diploid and triploid fish (n = 10 each) that had been identified with flow cytometry, as described above. At least 12 countable metaphases were examined per slide, and distinct metaphase chromosomes were photographed with a digital camera (ARTCAM-300MI; Artray Co., Tokyo, Japan) attached to an optical microscope.

For the erythrocyte measurements, six diploid and six triploid fish (three males and three females per ploidy group) were selected. The major and minor axes of each cell and nucleus of at least 30 erythrocytes per fish were measured using a micrometer. The surface area was calculated with the following formula: surface area = major axis (a) x minor axis (b) x $\pi/4$

(Sezaki and Kobayashi, 1978). The cellular and nuclear volumes were calculated with the formula: volume = $4(a/2) \times (b/2)^2 \times \pi/3$ (Lemoine and Smith, 1980).

Morphological and histological analyses of the gonads

The gonad morphology was observed in one-year-old diploid and triploid males and females. The gonads were surgically removed and fixed in buffered 4% formaldehyde solution for histological analysis after the morphological analysis. Conventional histological techniques were used to assess gonad development, including embedding in paraffin wax, sectioning to 6 μm thickness, and staining with Mayer's hematoxylin and eosin.

Transgenic fish

Transgenic founders were generated by microinjection of a 7.03-kilobase pair (kb) linear DNA fragment consisting of the RFP gene driven by the *O. dancena mlc2f* regulatory region. Based on a recent study (Cho et al., 2012), transgenic line (TG#008) displaying a strong hot pink-reddish phenotype was selected for production of transgenic triploid groups. This line stably transmitted transgenic locus and the brilliant red fluorescent phenotype through numerous generations with the Mendelian inheritance pattern.

Identification of transgenic homozygous fish

F_2 transgenic fish were obtained by sister-brother mating F_1 heterozygous broodfish. Ten F_2 transgenic fish individuals (five female and five male) were crossed to non-transgenic controls. After hatching, the

incidence of RFP-positive larvae was measured with fluorescence microscopy, using an AZ100 fluorescence microscope (Nikon Corporation Instruments Company, Japan) in order to screen the homozygous transgenic fish.

Generation of transgenic triploidy

Abbreviations of ploidy groups are shown in Table 1. Broodfish were used F_3 transgenic homozygous and non-transgenic marine medaka (MM and DD). Testis from transgenic homozygous males or non-transgenic males were kept in approximately 0.3 ml saline (0.85% NaCl), and sperm suspensions without testicular debris were prepared on ice until fertilization. Sexually mature females of transgenic homozygous genotype or non-transgenic genotype were used for collecting the unfertilized ripe eggs, which were stored in the saline.

To produce one, two and three homologous chromosomes with transgenic locus in transgenic triploid marine medaka (DDM, MMD and MMM), eggs of DD or MM were fertilized with individual sperm sample from either DD or MM. To blocking the second meiosis, the cold shock (0°C for 45 min) was applied at 2 min post-fertilization, and then, the eggs were placed in a 25°C incubator until hatched.

The hatching success and the incidence of abnormal larvae were measured as percentages by examining at least 15 eggs per group under a stereoscopic microscope (C-DS; Nikon Co., Tokyo, Japan). Early survival rate was evaluated as percentage of hatched larvae, after 7 days post hatching.

The ploidy of each individual was determined using flow cytometry and chromosome count.

RFP level assay

We measured RFP per fish weight and RFP phenotype in order to evaluate the RFP levels. 12 fish (10-week-old; weight = 32.9 ± 3.1 mg) were randomly chosen from each transgenic ploidy line, which were reared to the same condition of maintenance of broodfish.

To assessed the relative RFP levels with a fluorescence measurements per fish weight, method of protein extraction was carried as described by Cho et al. (2012). Whole body fish samples were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.6), 0.5 % Triton X-100, 30 mM NaCl] supplemented with a protease inhibitor cocktail (Roche Applied Science, Germany). The prepared homogenates were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatants were collected and then, the supernatants were diluted 1:20 with phosphate buffered saline. The fluorescence intensity was measured with a Tecan Polarion microplate fluorescence reader (Tecan Austria GmbH, Salzburg, Austria) using 485 nm excitation and 595 nm emission wavelengths. A standard curve was constructed for calculating the amount of RFP per fish weight, by DsRed2 Fluorescent Protein (Clontech Laboratories Inc., Mountain View, CA, USA)

To compare the RFP phenotype among transgenic ploidy groups, caudal muscle was photographed with a digital camera (DS-Ri1; Nicon Co., Tokyo, Japan) attached to an AZ100 fluorescence microscope.

Histology of transgenic gonads

To examine the gonad development of transgenic ploidy groups, histological analysis on the gonads obtained from 4-month-old females and

males each group were performed.

Statistical analysis

The hatching success, incidence of abnormal larvae, early survival rate and RFP per fish weight were assessed with ANOVA followed by Duncan's multiple range test at the level of $P= 0.05$. Erythrocyte sizes between diploidy and triploidy were assessed by Student's t-test. Difference was considered to be significant when $P< 0.05$.

Table 1. Abbreviation

Abbreviation	Ploidy level	Breeding
NN	2N	NTG[1] *Oryzias dancena* ♀ x NTG *O. dancena* ♂
NNN	3N	
NT	2N	NTG *O. dancena* ♀ x TG[2] *O. dancena* ♂
NNT	3N	
TN	2N	TG *O. dancena* ♀ x NTG *O. dancena* ♂
TTN	3N	
TT	2N	TG *O. dancena* ♀ x TG *O. dancena* ♂
TTT	3N	

[1]NTG, non-transgenic.

[2]TG, transgenic.

Ⅲ. Results

Induction of triploidy

Cold shock treatment at 0°C reduced the hatching success of embryos (Table 2). The hatching rates were decreased as treatment period increased significantly ($P< 0.05$) among the treatment groups, and also between control and treatment groups. The occurrence rates of abnormal larvae in the treatment groups was slightly higher than in the control group, while those among the treatment groups, were similar, ranging from 6.5% to 7.5% ($P< 0.05$).

The flow-cytometric assessment of ploidy showed that triploid fish were successfully induced in all treatment groups. The incidence of triploidy was 90.9% in the 30 min treatment group and the other two groups (45 and 60 min treatment groups) were showed 100% (Table 2).

Cytogenetic analyses

The DNA contents of the diploid and triploid *O. dancena* individuals were 1.61 ± 0.05 pg/cell and 2.40 ± 0.07 pg/cell, respectively, based on the reference standard of mud loach cells (2.8 pg/cell; Nam et al., 1999), indicating that the genome size of the triploid *O. dancena* was 1.5-fold that of its diploid counterpart. The chromosome number in the cells of the triploid fish was 3N = 72, which was 1.5 times greater than those of the diploid fish (2N = 48) (Fig. 1). Our measurements show also that the surface areas and volumes of both the erythrocyte cells and nuclei of the triploid marine medaka were larger than those of the diploid fish (Table 3).

Gonad development

Morphological and histological analyses of the gonads of one-year-old fish showed that the triploid genotype caused a significant depression of gonad development in *O. dancena*. The ovaries of the diploid females were well developed, with fully yolk-laden eggs, whereas those of the triploid females were poorly developed and significantly smaller than the diploid ovaries (Fig. 2a and 2b). There was no clear difference, on the other hand, in the testicular morphology of the diploid and triploid male, as judged with the naked eye, although the testes of the triploid fish were slightly smaller, suggesting that the gonad somatic index of the triploid males differed from that of their diploid counterparts (Fig. 2c and 2d).

Histological analysis of the gonads showed clear differences between the diploid and triploid females. Unlike the diploid fish, with highly developed ovaries filled with mature yolk-containing oocytes, the ovaries of the triploid females contained considerable numbers of oogonia, with very few oocytes at the chromatin - nucleolus stage in the mesenchymal tissue (Fig. 3a and 3b). Beside, the triploid male showed depressed gonad development compared with their diploid counterparts, even though the gross morphologies of the diploid and triploid testes were not significantly different. Normal spermatids and sperm were present in the testes of the diploid fish, whereas the testes of the triploid males had few spermatids or no sperm (Fig. 3c and 3d).

Table 2. Effects of cold shock (0℃) treatment of fertilized eggs of marine medaka *Oryzias dancena*. Means of hatching rate and incidence of abnormal larvae are based on minimum of three observations.

Treatment duration (min)	No. of eggs used	Hatching rate (%)	Incidence of abnormal larvae (%)	Incidence of triploidy (%)
Control	164	91.7 ± 1.8[a]	1.7 ± 1.7[b]	0.0
30	315	82.2 ± 1.6[ab]	7.5 ± 1.2[a]	90.9
45	419	71.4 ± 5.9[bc]	6.5 ± 1.1[a]	100.0
60	391	61.0 ± 12.9[c]	7.0 ± 1.1[a]	100.0

Means with different superscripts within a column indicate significant differences at $P< 0.05$.

Fig. 1. Metaphase chromosome spreads from *Oryzias dancena*: (a) diploid 2n = 48 and (b) triploid 3n = 72.

Table 3. Erythrocyte dimensions (mean ± SD) of diploid and triploid *Oryzias dancena*

Dimension	Diploidy	Triploidy	Ratio
Erythrocyte			
Major axis (μm)	8.29 ± 0.33[a]	11.61 ± 0.61[b]	1.40: 1
Minor axis (μm)	5.28 ± 0.12[a]	6.71 ± 0.30[b]	1.27: 1
Surface area (μm^2)	34.43 ± 1.79[a]	61.06 ± 3.21[b]	1.77: 1
Volume (μm^3)	121.90 ± 8.64[a]	276.07 ± 21.35[b]	2.26: 1
Erythrocyte nucleus			
Major axis (μm)	5.06 ± 0.05[a]	6.32 ± 0.36[b]	1.25: 1
Minor axis (μm)	2.66 ± 0.07[a]	3.17 ± 0.13[b]	1.19: 1
Surface area (μm^2)	10.55 ± 0.30[a]	15.72 ± 1.29[b]	1.49: 1
Volume (μm^3)	18.82 ± 1.16[a]	33.70 ± 3.90[b]	1.79: 1

Means with different superscripts within a line indicate significant differences at $P< 0.05$.

Fig. 2. External morphology of gonads from diploid and triploid *Oryzias dancena*. (a) diploid ovary; (b) triploid ovary; (c) diploid testis; (d) triploid testis. Bar indicates 1 mm.

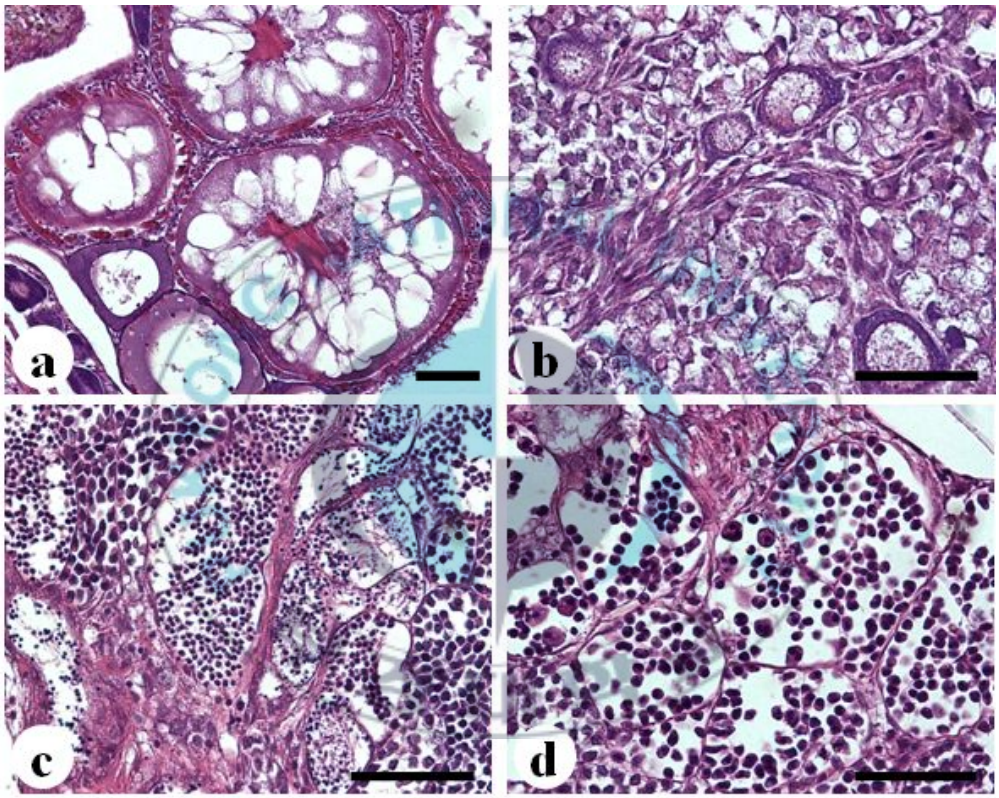

Fig. 3. Transverse sections of diploid and triploid gonads. (a) diploid ovary; (b) triploid ovary; (c) diploid testis; (d) triploid testis. Scale bars indicate 50 μm.

Progeny test of homozygous fish

Approximately 75% of the F_2 generation, produced by sister-brother mating was transgenic (Cho et al., 2012). To distinguish homozygous transgenic fish from hemizygous fish, ten F_2 transgenic fish were crossed with non-transgenic control fish. Based on the expected Mendelian transmission rates, 100% of the offspring of homozygous fish were transgene-positive, while those of the hemizygous fish were close to 50%. As the result, three individuals (TG ♀-2, TG ♂-3 and TG ♂-4) transmitted RFP to their F_3 offspring by 100% of transmission rate, and others (7/10) transferred transgene by about 50% rate of their progeny ().

To produce F_3 homozygous fish for generation of transgenic triploidy, three F_2 homozygous transgenic fish were mated with their sister-brother mating.

Production of transgenic triploid O. dancena

The results of production of transgenic triploid lines are shown in . Hatching success was significantly lower in cold-shocked groups than in any diploid groups. Depending on using transgenic or non-transgenic eggs, hatching rates in treatment groups (NNN, NNT, TTN and TTT) were significantly different at $P< 0.05$, however there were no differences in the means of the hatching rates of diploidy groups (NN, NT, TN and TT). The larvae in the triploid groups showed a significantly higher incidence of abnormalities (12.0 to 22.7 %) than in the diploid groups (1.5 to 5.6 %). The early survival rates were not significantly different among groups, except

for the DD NN and TTT survival rate.

Incidence of triploidy was 100% in all the treated groups. when subjected to flow cytometry and chromosome count, the triploid *O. dancena* showed a 1.5-fold increase in the DNA content (1.6 pg/cell for 2N vs. 2.4 pg/cell for 3N; Fig. 4), metaphase chromosome count (2N = 48 vs. 3N = 72; Fig. 5). This evidence clearly confirms the successful induction of triploidy in the transgenic *O. dancena*.

Evaluation of RFP level

The levels of RFP in transgenic ploid lines were estimated by using microplate fluorescence reader. Total fish proteins were extracted from the whole body and analyzed. To calculate the protein concentration (μg/mg) of the extract from each fish species, we used the DsRed2 Fluorescent Protein as a standard (Fig. 6). Most of RFP levels were related to ratio of homologous chromosomes with transgenic locus among whole homologous chromosomes. There were no statistical differences between TT and TTT, and TN and NT, respectively, and also two groups (TT and TTT) were highest RFP per fish weight among all transgenic treatment groups. In transgenic diploidy, RFP level of homozygous transgenic fish (TT; 25.25 μg/mg) was 1.7 times higher than that of hemizygous fish (NT and TN; 15.10 μg/mg). Besides, RFP levels of transgenic triploid lines were associated with the homologous chromosomes including transgene. RFP per fish weight of the TTT and TTN (17.48 μg/mg) were 2.5-fold and 1.7-fold, respectively, greater than those of the NNT (10.23 μg/mg). The order of the amounts of RFP per fish weight was as follows: TT and TTT> TTN> NT and TN> NNT (Fig.

7).

The RFP phenotype, in common with RFP per fish weight, was related to ratio of transgene copies present in a cell. In transgenic diploidy, RFP expression of homozygous trasngenic fish (TT) was greater than that of hemizygous fish (NT and TN). Furthermore, TTT was strongest RFP expression among transgenic triploid lines, and TTN was greater than that of NNT (Fig. 8).

Histology of gonads

Histologically, the gonads of transgenics compared with those of non-transgenics. No notable difference in gonad development was observed between transgenics and non-transgenics. Ovaries of diploid groups were packed with developing oocytes in various stages of development (Fig. 9a and 9c). However, ovaries from triploid transgenic and non-transgenics were small and undeveloped (Fig. 9b and 9d).

The testes of triploids of transgnics and non-transgenics also showed retarded, abnormal development (Fig. 9f and 9h). In contrast, diploid testes, spermatogonia completing mitotic division were easily observed (Fig. 9e and 9g).

Table 4. Progeny test of the presumptive homozygous transgenic fish

Fish	Generation of progeny	No. of progeny analyzed	No. of transgenic progeny (%)	Transgenic state
TG ♀-1	F_3	30	15 (50.0)	Heterozygote
TG ♀-2	F_3	36	36 (100.0)	Homozygote
TG ♀-3	F_3	31	17 (54.8)	Heterozygote
TG ♀-4	F_3	34	17 (50.0)	Heterozygote
TG ♀-5	F_3	37	21 (56.8)	Heterozygote
TG ♂-1	F_3	71	36 (50.7)	Heterozygote
TG ♂-2	F_3	46	22 (47.8)	Heterozygote
TG ♂-3	F_3	54	54 (100.0)	Homozygote
TG ♂-4	F_3	52	52 (100.0)	Homozygote
TG ♂-5	F_3	57	27 (47.4)	Heterozygote

Table 5. Mean (± SD) percent hatching success, incidence of abnormal larvae, early survival rate (after 7 days post hatching) and incidence of triploidy of experimental groups. All values are based on a minimum of three replications.

Experimental group	No. of eggs used	Hatching success (%)	Incidence of abnormal larvae (%)	Early survival rate (%)	Incidence of triploidy (%)
NN	160	94.2 ± 6.5^{a}	1.5 ± 1.9^{b}	97.5 ± 2.3^{a}	0.0 ± 0.0^{b}
NNN	134	71.3 ± 16.8^{b}	12.0 ± 4.9^{ab}	93.5 ± 2.4^{ab}	100.0 ± 0.0^{a}
NT	179	87.6 ± 3.1^{a}	3.2 ± 2.7^{b}	94.0 ± 6.9^{ab}	0.0 ± 0.0^{b}
NNT	141	64.4 ± 14.3^{bc}	19.0 ± 14.3^{a}	92.8 ± 9.1^{ab}	100.0 ± 0.0^{a}
TN	198	85.0 ± 3.5^{a}	4.0 ± 1.6^{b}	90.7 ± 8.2^{ab}	0.0 ± 0.0^{b}
TTN	141	52.7 ± 6.6^{cd}	19.5 ± 11.6^{a}	92.3 ± 3.4^{ab}	100.0 ± 0.0^{a}
TT	212	84.6 ± 5.0^{a}	3.5 ± 2.3^{b}	86.1 ± 2.4^{ab}	0.0 ± 0.0^{b}
TTT	211	50.1 ± 10.7^{d}	22.7 ± 16.4^{a}	83.6 ± 7.2^{b}	100.0 ± 0.0^{a}

Means within a column with different superscripts are significantly different at $P< 0.05$.

Refer to table 1. for abbreviations of group names.

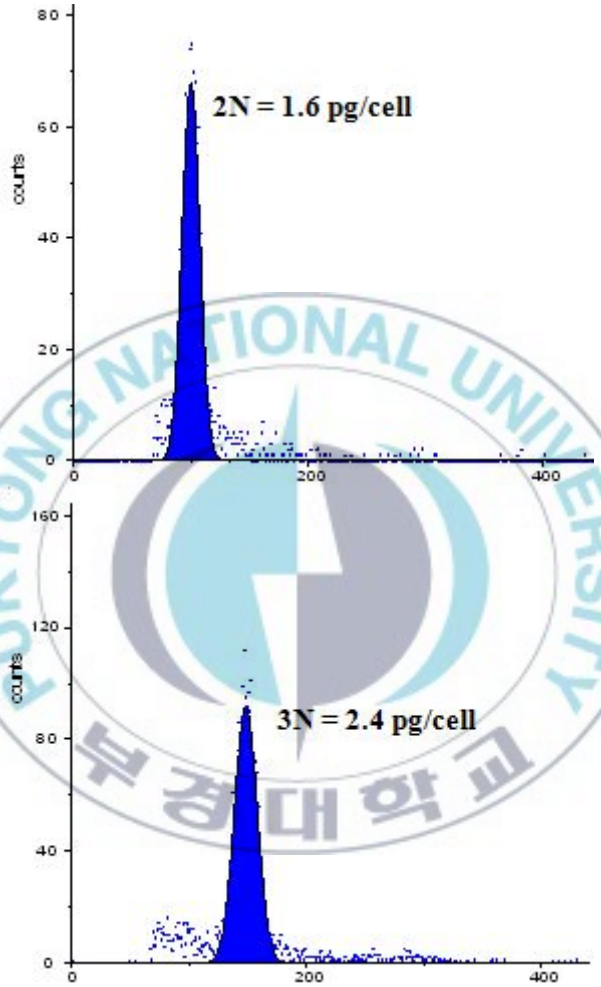

Fig. 4. Representative histograms showing cellular DNA contents of diploid (2N) and triploid (3N) *Oryzias dancena*.

Fig. 5. Metaphase chromosome spreads: (a) diploid 2n = 48 and (b) triploid 3n = 72 Transgenic *Oryzias dancena.*

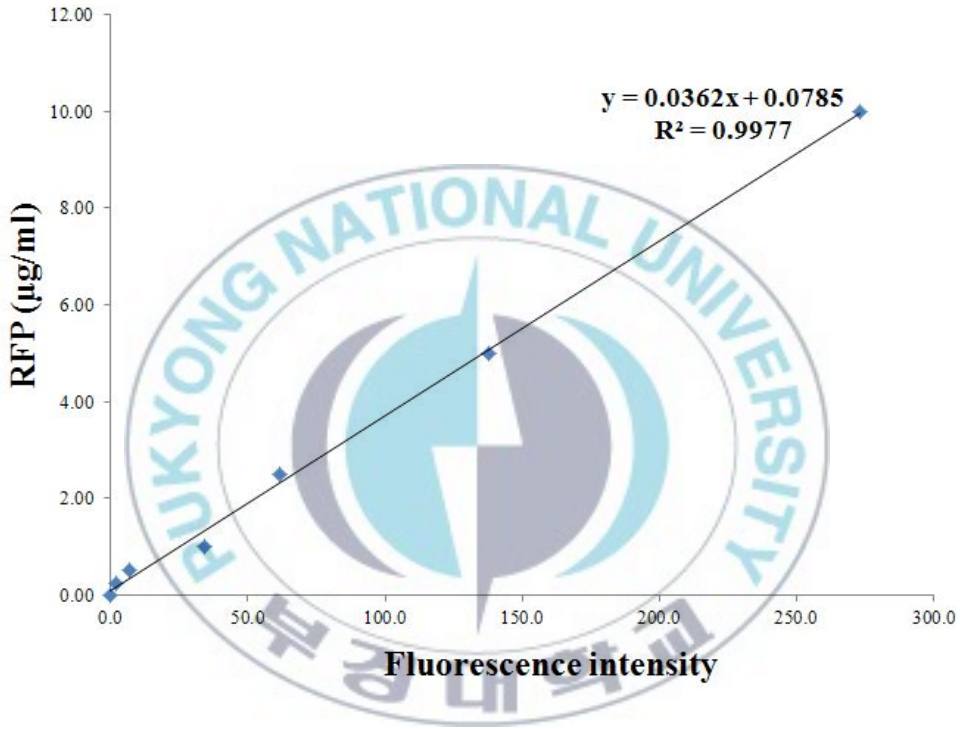

Fig. 6. Standard curve for calculating the amount of RFP per fluorescence intensity by DsRed2 Fluorescent Protein (Clontech Laboratories Inc.).

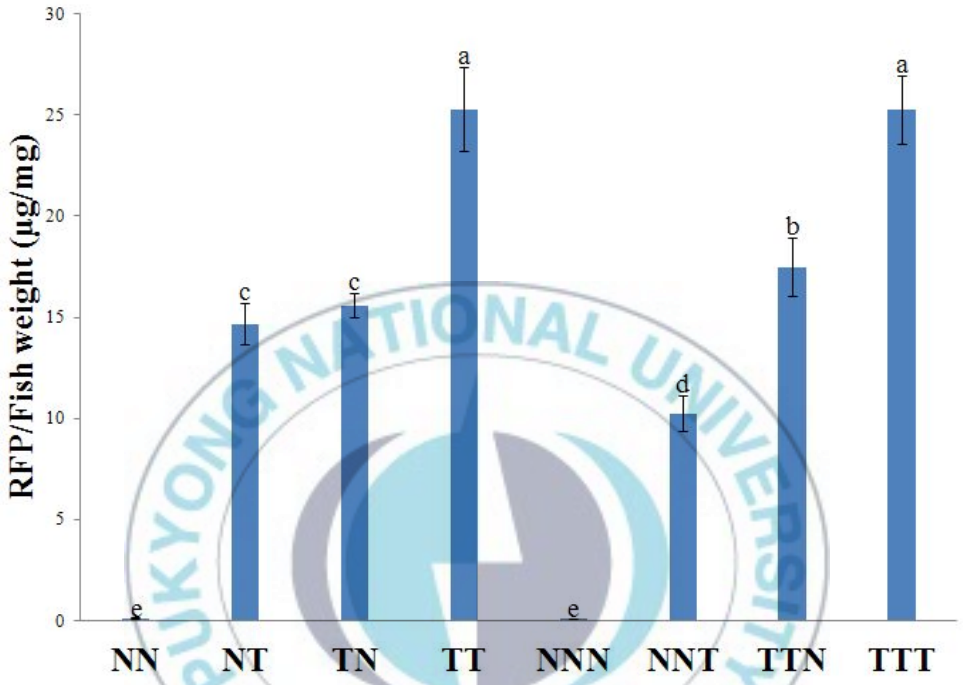

Fig. 7. Quantitative analysis of RFP per fish weight. Error bars indicate the standard error of the mean from twelve experiments. Bars with different letters indicate means with significant difference at $P< 0.05$.

Refer to table 1. for abbreviations of group names.

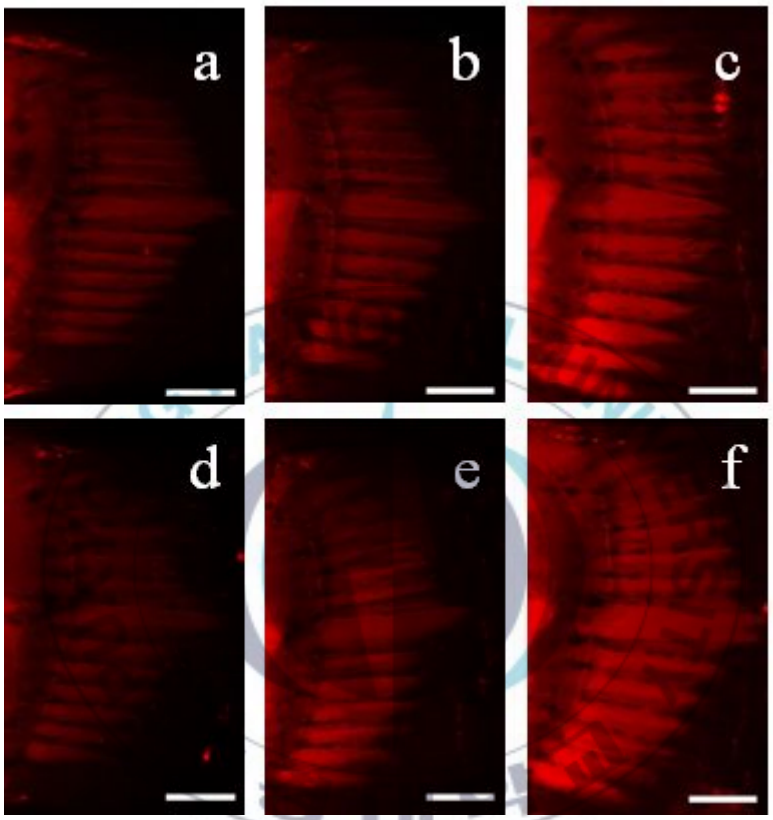

Fig. 8. Representative photographs to show the different expression of RFP over caudal muscles among transgenic ploid groups. (a) NT; (b) TN; (c) TT; (d) NNT; (e) TTN; (f) TTT. Scale bars indicate 300 μm.

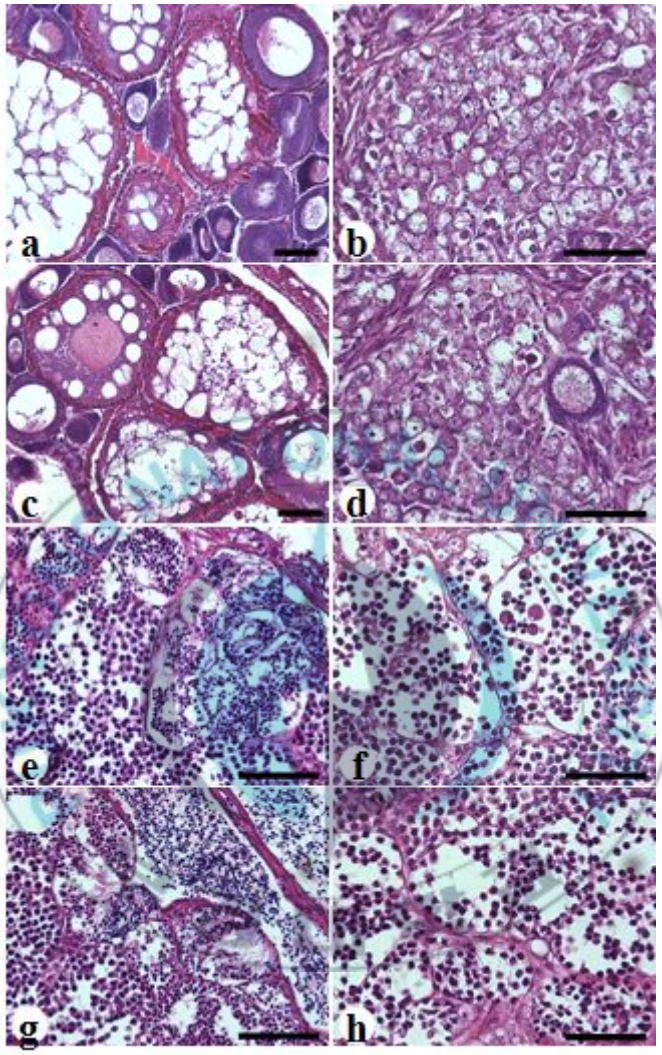

Fig. 9. Transverse sections of diploid and triploid gonads. (a) transgenic diploid ovary; (b) transgenic triploid ovary; (c) non-transgenic diploid ovary; (d) non-transgenic triploid ovary; (e) transgenic diploid testis; (f) transgenic triploid testis; (g) non-transgenic diploid testis; (h) non-transgenic triploid testis. Scale bars indicate 50 μm.

IV. Discussion

Triplodization in fish has been considered as a potential method to prevent unwanted reproduction (Piferrer et al., 2009). Hatching rates in the treatment groups were decreased in comparison with control group ($P< 0.05$) except eggs treated for 30 min ($P> 0.05$). This reduction in the hatching rate following a temperature shock treatment is similar to the results of previous studies (Kavumpurath and Pandian, 1990; da Silva et al., 2007; Karami et al., 2010). The occurrence of abnormal larvae was also significantly different with the control group ($P< 0.05$). The lower hatching rate and incidence of abnormal larvae of triploidy compared to that of diploidy could be related to adverse effects of thermal shocks on membrane fluidity, RNA/DNA synthesis, cell morphology, and protein functions (Hildebrandt et al., 2002; Al-Fageeh and Smales, 2006). Our protocol for induction of triploid *O. dancena* using cold shock treatment yielded 100% triploidy. The hatching rate was lower in the eggs treated for 60 min than in those treated for 45 min. Therefore, the optimal conditions for the induction of triploidy in the marine medaka in this study were a cold shock treatment at 0 ℃ for 45 min. The yield of triploidy in this study was very similar to those previously reported in other fish species (Felip et al., 1999; Piferrer et al., 2000; da Silva et al., 2007).

Flow-cytometric and chromosomal analyses are an accurate method for checking the ploidy of fish. By estimating the average DNA content per cell and observing the metaphase chromosome spreads clearly showed that an extra haploid chromosome set was present in the normally diploid nuclei, evidenced by average celluar DNA content (2.40 pg/cell) and their modal

chromosome number (3n = 72), which were 1.5-fold diploid values. These were also typical of induced triploidy in fish (Kavumpurath and Pandian, 1990; Kim et al., 1994; Felip et al., 1999; Piferrer et al., 2000; Karami et al., 2010).

Tiploids had significantly larger erythrocyte cellular and nuclear dimensions than diploids, as expected. In both the cells and nuclei, the increase in length was more pronounced in the major axis than in the minor axis, which is commonly observed in induced triploid fish (Kavumpurath and Pandian, 1990; Kim et al., 1994; Peruzzi et al., 2005; Gao et al., 2007). Despite the greater size of their erythrocytes, triploid fish typically display lower hematocrit values (*i.e.*, lower cell numbers) than diploid fish because of the compensatory increase in cell volume in polyploidy (Benfey, 1999; Peruzzi et al., 2005; Gao et al., 2007). Therefore, it could be valuable to investigate whether the respiratory performance and energy metabolism of the induced triploid marine medaka differ from those of their diploid counterparts (Stillwell and Benfey, 1996; Hyndmanetal., 2003; Lemieuxetal., 2003; Shrimptonetal., 2007).

Morphological and histological analyses of triploid gonads probably causes sterility in this species. The odd chromosome number induced during triploidization precludes homologous chromosomal pairing during meiosis I, which leads to inefficient gamete differentiation and consequently confers sterility on the triploid fish (Zhang et al., 2005). Overall, most of our observations of the gonads in this study were similar to those previously reported in other triploid fish: smaller gonads, significantly delayed gonadal development, and more pronounced sterility in females (Kim et al., 1994;

Felip et al., 1999; Tiwary et al., 2000; Feindel et al., 2011). A recent study also reported that the induction of triploidy lowers the estradiol concentration and changes its internal secretion, resulting in oogenesis disorders (Cal et al., 2010). However, considerable numbers of previous studies have also claimed that triploid fish, especially old triploid males, might have a functional capacity to reproduce, in spite of a lower degree, despite their sterile-like gonadal development (Benfey, 1999). The artificial insemination of normal haploid eggs with sperm from triploid tench (*Tinca tinca* L.) has been reported to produce some abnormal larvae (Linhart et al., 2006). A recent study of triploid African catfish (*Clarias gariepinus*) has also reported that treatment with ovaprim (Syndel Laboratories Ltd., Vancouver, British Columbia, Canada), an ovulating/spermiating agent, induced advanced vitellogenin sequestration in triploid oocytes and promoted the fertilizing capacity of milt from triploid males (Karami et al., 2011). Hence, extended observation of the potential maturation of older triploid marine medaka fish in future research should extend our insight into their reproductive capacity.

국문 요약

미오신 라이트 사슬-RFP 형질전환 유전자가 이식된 형질전환 바다 송사리의 3배체 생산

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고 민 균

본 연구는 바다 송사리(*Oryzias dancena*)의 3배체 유도조건을 확립하고 세포유전학적인 평가를 하며, 이를 토대로 형질전환 어류를 이용한 3배체를 생산함으로써 형광 단백질 발현정도를 측정하여 관상용 어류로서의 가치를 평가하기 위하여 수행하였다.

3배체 유도를 위하여 수정 2분 후 각각 30, 45, 60분간 0°C에서 저온 처리 한 결과 처리 시간에 따라 다양한 빈도의 3배체 출현율을 보였으며, 그 중 45분간 저온 처리에서 모든 개체가 3배체 유도율을 나타내었다. 염색체 분석, 세포크기 및 flow cytometer를 통해 3배체의 세포유전학적 특성을 분석한 결과 염색체 수(3N = 72)는 2배체(2N = 48)보다 1.5배 많았다. Flow cytometer를 통한 genome size에서도 DNA 함량이 3배체(2.40 pg per cell)가 2배체(1.61 pg per cell) 보다 1.5배 더 많았으며, 적혈구 및 핵의 표면적 및 부피에서도 3배체가 2배체 보다 1.5배 증가한 것으로 나타났다. 3배체 유도 후 1년 된 3배체 개체의 생식소 형태를 2배체와 비교한 결과 2배체보다 유의적으로 작게 나타났으며($P<0.05$), 조직학적 분석에서도 불임으로 나타나 불임 3배체 유도를 확인하였다. 본 연구 결과는 형질전환 어류의 3배체 생산을 통한 생식능력을 억제하여 앞으로 형질전환어류를 이용함에 있어 비의도적 방출 시 잠재적인 생태학적 위험이 초래될 문제를 해결하기 위한 방안으로 사용될 수 있을 것이다.

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