Cytogenetic Study of Diploid and Triploid Marine Medaka, *Oryzias dancena*

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ABSTRACT

Triploidy was induced in the marine medaka, *Oryzias dancena* by cold shock treatment (0°C) of fertilized eggs for 30, 45, or 60 min, applied two minutes after fertilization. The triploid genotype was induced by each of 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 using chromosomal and flow cytometer analyses, and erythrocyte measurements. The surface areas and volumes of the erythrocytes of triploid fish were significantly larger than those of diploid fish, and their chromosome number (3N=72) was 1.5 times greater than for the diploids (2N=48). Based on a flow cytometer analysis, the triploid fish had approximately 1.5 times the cellular DNA content (2.40 pg/cell) of the diploid specimens (1.61 pg/cell). Data from this study provide the basis for the development of unique models for studying reproductive confinement in transgenic fish.

Key words: Cold shock, DNA contents, flowcytometry, marine medaka, triploid

INTRODUCTION

The marine medaka, *Oryzias dancena* is a truly euryhaline teleost fish, having a great capacity for hypo- and hyper-osmoregulation. Most of its physiological attributes are similar across a wide spectrum of salinities, ranging from fresh water to normal seawater (Inoue and Takei, 2003; Kang et al., 2008; Cho et al., 2010). Until now, this species was selected by the Institute of Marine Living Modified Organisms (iMLMO) for a living modified organism evaluation project. Consistent with this purpose, detailed information on its biology, especially its early gonadogenesis, sexual differentiation, early ontogenesis, embryogenesis and and exceptional capacity for hyperosmoregulation and hypoosmoregulation, and is becoming available (Kim et al., 2009a, b). In addition, Kim et al. (2009a) suggested that this species has a short interval between generations with spawning possible only 60 days after hatching. Much attention has been directed at extending the utility of functional transgenic marine medaka strains for ornamental purposes, because they can be used at most naturally occurring salinities (Cho et al., 2011). In addition, in a recent study of transgenic marine medaka containing the myosin light chain-2 (mlc2f) promoter, the expression of a vivid red fluorescent color in their fast skeletal muscles suggested great potential for these as novel ornamental fish for both freshwater and seawater 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 if unwanted transgenic animals are released (Maclean and Laight, 2000; Devlin et al., 2006). For these reasons, much recent scientific research has focused on risk assessment in relation to transgenic fish, with particular emphasis on the repro-
ductive confinement of transgenic stocks (Wong and Van Eenennaam, 2008; Thresher et al., 2009; Hu et al., 2010). Triploidization involving blocking of the second meiotic division has been proposed as one approach to the generation of transgenic fish having 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).

Triploidization is a technique used to generate sterile aquatic animals by taking advantage of the incompatibility in pairing the three homologous chromosomes during meiosis I (Don and Avtalion, 1986). This technique has also been used to enhance the productivity of several fish species because of its assumed ability to increase yield by channeling the energy required from gonadal development to somatic growth (Tave, 1993). More importantly, it generates fish that are unable to breed and contribute to the local gene pool if they were to accidentally escape from confinement. By conferring sterility of exotic fish for a limited purpose, triploidy can serve as an effective method for reducing or eliminating the environmental risks of genetically modified organisms (Kim et al., 1994). Triploidy was confirmed by the 1.5-fold increase in nuclear volume, cellular DNA content and chromosome number as estimated by erythrocyte counting, respectively (Seol et al., 2008).

There are numerous studies in the literature which have investigated various aspects of triploid fish identification methodology including analysis of chromosome sets (Thorgaard, 1986), the microfluorimetry of nuclear DNA content (Komaru et al., 1988), the nuclear DNA content by flowcytometry (Allen and Stanley, 1978), the measurement of erythrocyte and nuclear size (Thorgaard, 1986; Park and Kim, 1994; Park et al., 1994), the distinction of nucleolar number (Philips et al., 1986), the measurement of cell number (Park and Park, 1994), and the measurement of cell and nuclear size in different tissues (Aliiah et al., 1990). Park and Kim (2000) reported that characteristics of the same tissues of retina, optic tectum and trunk kidney in triploid and diploid hybrid between female mud loach, Misgurnus mizolepis and male cyprinid loach, M. andguillicaudatus.

The objectives of this study were: 1) to develop the optimal conditions for induction of the triploid genotype in marine medaka; and 2) to evaluate the cytogenetic of the triploid form of this species.

MATERIALS AND METHODS

The specimens of marine medaka, Oryzias dancena 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). Breeding occurred in brackish water (5 psu), 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. Triploid and diploid specimens were fed brine shrimp (Artemia nauplii; INVE, Salt Lake City, Utah, USA) and microparticle feed (150~500 μm diameter; Ewha Oil Co., Busan, Korea).

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 females immediately following 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. Following treatment the eggs were placed in a 25°C incubator until they hatched. The hatching success and the incidence of abnormal larvae were assessed based on stereoscopic microscope (C-DS; Nikon Co., Tokyo, Japan) examination of at least 23 eggs per group. The values for these parameters in the experimental treatments were expressed as percentages of treated eggs.

Flow cytometer analysis was undertaken in the Fishery Genetics and Breeding Sciences Laboratory, Korea Maritime and Ocean University, Korea. To assess the incidence of triploidy using flow cytometry, 22 individuals were randomly chosen from each treatment and control group. The caudal fin was excised from each fish using scissors, and the cells were dissociated in nucleic acid extraction buffer, and stained for 15 min in DNA staining buffer (CyStain DNA 2 step; Partec, Germany). To determine their ploidy, the cells were analyzed using a Ploidy Analyzer II flow cytometer (Partec, Germany). 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 was performed using a direct method involving kidney cells (Kim et al., 1995). Metaphase spreads were prepared from randomly selected diploid and triploid fish (n = 10) that had been identified using flow cytometry, as described above. At least 12 countable metaphases were examined per slide, and distinct metaphase chromosomes were photographed using a digital camera (ARTCAM-300MI; Artray Co., Tokyo,
Japan) attached to an optical microscope.

For the erythrocyte and hormone measurements, six diploid and six triploid fish (three of each gender per ploidy group) were selected. Blood samples were taken by cutting the caudal fin and collecting the blood, or by inserting a heparin-coated syringe (Sigma, USA) into the heart and withdrawing blood. 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 \((1/4 \times ab\pi)\) and volume \((4/3 \times \pi(a/2) \times (b/2)^2)\) were calculated; in these formulae, ‘a’ is the major axis of the cell or nucleus, and ‘b’ is the minor axis of the cell or nucleus (Lemoine and Smith, 1980; Park and Kim, 2000).

The hatching success, incidence of abnormal larvae, and early survival rates were assessed using ANOVA followed by Duncan’s multiple range test at the significance level \(P=0.05\). Differences in erythrocyte size between the diploid and triploid groups were assessed using the Student’s t-test. Difference was considered to be significant when \(P<0.05\).

**RESULTS AND DISCUSSION**

Cold shock treatment (0°C) reduced the hatching success of embryos of marine medaka, *Oryzias dancena* (Table 1). The hatching rate was significantly different between the control and treatment groups, and decreased significantly \((P<0.05)\) with increased treatment period. The occurrence of abnormal larvae in the treatment groups was slightly higher than in the control group, but was similar among the treatment groups, where it ranged from 6.5% to 7.5% \((P<0.05)\). The incidence of triploidy was 90.9% in the 30 min treatment group, and 100% in the 45 and 60 min treatment groups (Table 1). The production rate was the highest in the treatment involving cold shock for 45 min, applied 2 min after fertilization.

Triploidization is considered to be a potential method for preventing unwanted reproduction in fish (Piferrer et al., 2009). The hatching rates for the eggs of marine medaka were lower in the temperature shock treatment groups than in the control group \((P<0.05)\), with the exception of eggs treated for 30 min \((P>0.05)\). A reduction in the hatching rate following a temperature shock has been reported in previous studies (Kavumpurath and Pandian, 1990; Da Silva et al., 2007; Karami et al., 2010). The occurrence of abnormal larvae in the treatment groups was also significantly different from the control group \((P<0.05)\). The lower hatching rate and greater incidence of abnormal larvae in triploid fish compared with that of diploid fish may be related to the adverse effects of thermal shock on membrane fluidity, RNA/DNA synthesis, cell morphology, and protein function (Hildebrandt et al., 2002; Al-Fageeh and Smales, 2006). Our cold shock treatment protocol for the induction of triploidy in marine medaka was 100% effective, but the hatching rate was lower for eggs treated for 60 min than for those treated for 45 min. Therefore, the optimal conditions for the induction of triploidy in marine medaka in this study was a cold shock treatment at 0°C for 45 min. The yield of triploidy in this study was very similar to that previously reported in other fish species (Felip et al., 1999; Piferrer et al., 2000; da Silva et al., 2007).

The number of chromosomes in the diploid and triploid marine medaka were 48 and 72, respectively (Fig. 1). The karyotype of diploids comprised 24 acrocentric chromosome pairs, while the triploids contained three sets of 24 acrocentric chromosomes. Two nucleoli were observed in the metaphase stage of diploids, and three were observed in the triploids. The chromosome number in the cells of triploid fish was 1.5 times greater than that of the diploid fish. The flow cytometry assessment showed that triploidy was successfully induced in all treatment groups. The DNA content of the diploid and triploid fish is shown in

| Table 1. Effects of cold shock (0°C) treatment 2 mins after fertilization at fertilized eggs of marine medaka, *Oryzias dancena* |
|---|---|---|---|---|---|
| Duration of shock (min) | Number of eggs used | Hatching rate (%)\(^a\) | Abnormal rate of juvenile (%) \(^b\) | Induction rate of triploidy (%) \(^c\) | Early survival (%) \(^d\) | Production rate of triploidy (%) \(^e\) |
| No treatment | 164 | 91.7 ± 1.8\(^a\) | 1.7 ± 1.7\(^b\) | 0 | 97.8 ± 0.8\(^a\) | 0 |
| 30 | 315 | 82.2 ± 1.6\(^ab\) | 7.5 ± 1.2\(^a\) | 90.9 | 94.8 ± 3.7\(^a\) | 88.1 ± 1.4\(^a\) |
| 45 | 419 | 71.4 ± 5.9\(^ac\) | 6.5 ± 1.1\(^a\) | 100 | 95.1 ± 2.6\(^a\) | 97.2 ± 1.2\(^b\) |
| 60 | 391 | 61.0 ± 12.9\(^c\) | 7.0 ± 1.1\(^a\) | 100 | 93.8 ± 4.5\(^a\) | 95.9 ± 1.7\(^b\) |

\(^a\)This experiment was performed in triplicate.

\(^b\)Hatching rates of each group were analyzed until 24 hours after first hatching observed.

\(^c\)Induction rates of triploid were analyzed until 50 days after hatched. Each value is mean percentage of triplicate experiments. Induction rates of triploid were analyzed in survived experimental animals.

\(^d\)Early survival rates of each group were analyzed at 96 hours after hatched.

\(^e\)Production rate of triploid = ([early survival rate of treated group]/[early survival rate of control group]) × (induction rate of triploid).
The mean quantity of DNA in the diploid and triploid groups was $1.64 \pm 0.019$ pg/nucleus and $2.45 \pm 0.026$ pg/nucleus, respectively. The DNA content of the triploid group was a factor of 1.5 higher than that of the diploid group (Table 2 and Fig. 2).

Flow cytometer and chromosomal analyses are accurate methods for assessing the ploidy of fish. Estimating the average DNA content per cell in triploid fish cells, and observations of the metaphase chromosome stage clearly showed that an extra haploid chromosome set was present relative to normal diploid nuclei; the average cellular DNA content (2.40 pg/cell) and modal chromosome number ($3n = 72$) were factors of 1.5 greater than the diploid values; these values are typical for induced triploidy in fish (Kavumpurath and Pandian, 1990; Kim et al., 1994; Felip et al., 1999; Piferrer et al., 2000; Karami et al., 2010). Two and three sets of 24 acrocentric chromosomes were observed in the karyotype of diploid and triploid marine medaka, respectively. The karyotype of Japanese medaka, *O. latipes*, is the same as that of marine medaka. However, Uwa and Ojima (1981) showed that the karyotype of Japanese medaka comprises 48 chromosomes consisting of 2 metacentric pairs, 8 submetacentric pairs, 1 subtelocentric pair, and 13 acrocentric pairs. Therefore, karyotype analysis can be used to distinguish the marine and Japanese medaka.

Table 2. DNA contents of diploid and triploid in marine medaka, *Oryzias dancena*.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>DNA contents (pg/nucleus)</th>
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<tbody>
<tr>
<td></td>
<td>Diploid</td>
</tr>
<tr>
<td>1</td>
<td>1.61</td>
</tr>
<tr>
<td>2</td>
<td>1.65</td>
</tr>
<tr>
<td>3</td>
<td>1.64</td>
</tr>
<tr>
<td>4</td>
<td>1.62</td>
</tr>
<tr>
<td>5</td>
<td>1.65</td>
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<tr>
<td>6</td>
<td>1.63</td>
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<td>7</td>
<td>1.64</td>
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<tr>
<td>8</td>
<td>1.64</td>
</tr>
<tr>
<td>9</td>
<td>1.64</td>
</tr>
<tr>
<td>10</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Mean ± SD: $1.64 \pm 0.019$ (diploid) and $2.45 \pm 0.026$ (triploid).

*DNA contents of mud loach, *Misgurnus mizolepis* (2.81 pg/nucleus) were used as standard references of diploid and triploid Korean rose bitterling’s DNA contents measurement (Nam et al., 1999).

The values are means ± standard deviation of each group.

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mosomes (Uwa et al., 1983). Therefore, the gender of each ploidy in this species can be investigated by analyzing sexual dimorphism.

Overall, the erythrocytes of the induced triploids were larger than those of the diploids (Fig. 3). The ratio of the major axis to the minor axis of erythrocytes for the diploids and induced triploids was 1.40 and 1.27, respectively, and the surface area to volume ratio (based on the major and minor axes) was 1.77 and 2.26, respectively. Similarly, the ratio of the major axis to the minor axis of the nucleus of erythrocytes for the diploids and induced triploids was 1.25 and 1.19, respectively, and the corresponding surface area to volume ratio, was 1.49 and 1.79, respectively (Table 3). The measurements showed that the surface area and volume of both the erythrocyte cells and nuclei of triploid marine medaka were larger than those of the diploid fish.

As expected, the triploids of marine medaka had significantly larger erythrocyte cell and nuclear dimensions than the diploids. For both the cells and nuclei, the increase in length was more pronounced in the major axis than in the minor axis, which is a feature commonly observed in induced triploid fish (Kavumpurath and Pandian, 1990; Kim et al., 1994; Peruzzi et al., 2005; Gao et al., 2007). In a previous study, Seol et al. (2008) reported that the erythrocyte count for diploids was higher than that for triploids, and they found that in triploids the nucleus of red blood cells had major and minor axes that were factors of 1.33 and 1.26 larger than in the diploids, re-

Table 3. Erythrocyte size of marine medaka, *Oryzias dancena* between diploid and triploid*

<table>
<thead>
<tr>
<th></th>
<th>Diploid</th>
<th>Triploid</th>
<th>Ratio of triploid to diploid</th>
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<tbody>
<tr>
<td><strong>Cell</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major axis</td>
<td>8.29 ± 0.33</td>
<td>11.61 ± 0.61</td>
<td>1.40 : 1</td>
</tr>
<tr>
<td>Minor axis</td>
<td>5.28 ± 0.12</td>
<td>6.71 ± 0.30</td>
<td>1.27 : 1</td>
</tr>
<tr>
<td><strong>Nucleus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major axis</td>
<td>5.06 ± 0.05</td>
<td>6.32 ± 0.36</td>
<td>1.25 : 1</td>
</tr>
<tr>
<td>Minor axis</td>
<td>2.66 ± 0.07</td>
<td>3.17 ± 0.13</td>
<td>1.19 : 1</td>
</tr>
<tr>
<td><strong>Area (μm²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell</td>
<td>34.43 ± 1.79</td>
<td>61.06 ± 3.21</td>
<td>1.77 : 1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>10.55 ± 0.30</td>
<td>15.72 ± 1.29</td>
<td>1.49 : 1</td>
</tr>
<tr>
<td><strong>Volume (μm³)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell</td>
<td>121.90 ± 8.64</td>
<td>276.07 ± 21.35</td>
<td>2.26 : 1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>18.82 ± 1.16</td>
<td>33.70 ± 3.90</td>
<td>1.79 : 1</td>
</tr>
</tbody>
</table>

*Six samples for each ploidy were used in this experiment. Thirty erythrocytes for each sample were analyzed. Mean ± S.D. Means in superscript letter are significantly different.

*Area = (a · b · π)/4; a: major axis of cell and nucleus; b: minor axis of cell and nucleus (Seol et al., 2008).

*Volume was calculated by formulas of Sezaki et al. (1988). Volume = 4/3π (a/2) · (b/2)²; a: major axis of cell and nucleus; b: minor axis of cell and nucleus.
spectively. Differences in erythrocyte size is commonly used as the sole criterion for determining the ploidy in fish (Benfey, 1999).

Despite the greater size of their erythrocytes, triploid fish typically have lower hematocrit values (i.e. lower cell numbers) than diploid fish, because of the compensatory increase in cell volume in polyploidy (Benfey, 1999; P eruazzi et al., 2005; Gao et al., 2007). Therefore, it will be useful to investigate whether respiratory performance and energy metabolism differ between diploid and induced triploid marine medaka (Stillwell and Benfey, 1996; Hyndman et al., 2003; Lemieux et al., 2003; Shrimpton et al., 2007). So, further studies will be necessary to comparative study of respiratory ability between diploid and triploid marine medaka. In particular, long-term observations of growth and maturation is necessary in triploid marine medaka.

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REFERENCES


해산송사리, *Oryzias dancena* 유도 3배체의 세포유전학적 연구

박인석, 김현우, 이태호, 남윤권, 고민균, 김동수

한국해양대학교 해양생명과학부, 부경대학교 해양바이오신소재공학과

요약: 본 연구는 3배체 해산송사리, *Oryzias dancena*를 생산하기 위해서 다양한 조건에서 실험을 실시하였다. 저온처리(0°C)에서 30, 45, 60분간 처리하여 3배체를 유도 생산하였다. 여러 유도조건 결과, 45분간 처리할 경우 가장 높은 3배체 생산율이 나타났다. 3배체 판별은 Chromosome 관찰, flow cytometer 분석 및 적혈구 측정을 통해서 판별하였다. 3배체 해산송사리의 적혈구 표면적과 부피는 2배체 해산송사리보다 크게 나타났고, 3배체 Chromosome number는 72개, 2배체는 48개가 관찰되었다. Flow cytometer 분석에서도 3배체가 2.40 pg/cell 그리고 2배체가 1.61 pg/cell 측정되어 DNA contents도 3배체가 2배체보다 1.5배 정도 크게 관찰되었다. 본 연구 결과는 불임 형질전환 어류를 위한 실험동물로서의 3배체 해산송사리의 유용성 및 가치성을 제공한다.

 찾아보기 낱말: 저온처리, DNA 함량, 유세포분석기, 해산송사리, 3배체