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Cold-shock induced androgenesis without egg irradiation and subsequent production of doubled haploids and a clonal line in Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

Androgenesis is a useful manipulation to fix male-specific genetic traits as well as to restore the genotypes of cryopreserved sperm, and it has been induced by the genetic inactivation of the egg nucleus with gamma-, X- or UV-ray irradiation before fertilization. Recently, a technique for cold-shock induced androgenesis was developed in a freshwater species, the loach, *Misgurnus anguillicaudatus*, in which viable androgenetic diploids and doubled haploids (DHs) were successfully produced by the fertilization of diploid sperm and chromosome doubling by heat-shock treatment, respectively. This technique was immediately applied for cloning in the zebrafish, *Danio rerio*. Here, we reported the first successful induction of androgenetic development by means of cold-shock treatment (at 0 °C for 15 min) just after the fertilization (within 10 s) of eggs in a marine aquaculture fish species, the Japanese flounder, *Paralichthys olivaceus*. Then, androgenetic embryos thus generated were subjected to hydrostatic pressure treatment (650 kg/cm^2, 6 min) after incubation at 17 °C so as to produce DHs by chromosome doubling. The yield rate of putative DHs, which was estimated as the frequency of diploid larvae at the first feeding stage in relation to the total eggs used, ranged from 0.81% to 1.79%. The complete homozygosity of these putative DHs was genetically verified using 36 microsatellite markers that covered 24 linkage groups of Japanese flounder. Thus, an androgenetic clonal line was produced with the sperm of a mito-gynogenetic DH male by using cold-shock and subsequent hydrostatic pressure-shock treatments.

Statement of relevance: The results of our manuscript confirm the broad applicability of the cold-shock induced androgenesis method in commercially important marine fish species.

1. Introduction

Japanese flounder, *Paralichthys olivaceus*, is an economically important fish species widely cultured along the coasts of China, Japan, and Korea. Females of this species grow much faster than males, and all-female populations have therefore been established by using artificially induced gynogenesis (Yamamoto, 1999). Individuals hatched via induced gynogenesis only have the maternal genome, with the paternal genome being entirely eliminated. However, in some cases, males exhibit better performances in specific traits than females; thus, it is important to develop technology for fixing these traits both quickly and effectively.

Androgenesis is a mode of development in which only the paternal genome genetically contributes to the progeny, without the contribution of maternal chromosomes (Komen and Thorgaard, 2007). Inducing androgenesis can lead to male traits being effectively fixed. The traditional method to induce androgenesis in fish requires the irradiation of unfertilized eggs to genetically inactivate the maternally derived genome. This irradiation has been carried out by using gamma rays, X-rays or UV-rays (Arai, 2001). For all irradiation procedures, specific equipment is needed to ensure the safety of the operators, especially when using gamma- and X-ray irradiation; in addition, protection solutions are also needed to maintain the fertilization potential of the eggs. The most widely used protection solutions are water (Myers et al., 1995), synthetic ovarian fluid (Bongers et al., 1994), natural ovarian fluid (Lin and Dabrowski, 1998; Rothbard et al., 1999), Ringer's solution (David and Pandian, 2006), seminal plasma (Corley-Smith et al., 1996; Fujimoto et al., 2007) or Hank's solution (Yasui et al., 2010). The requirement of specific equipment and protection solutions makes the traditional method of induced androgenesis inconvenient for routine use (Arai, 2001).

In the loach, *Misgurnus anguillicaudatus*, a new method to induce androgenesis was established (Morishima et al., 2011). Activated eggs were immediately cold-shocked at a temperature of 0 to 3 °C for 60 min, which resulted in >30% of the hatched larvae being haploid androgenotes by means of eliminating the maternally derived nucleus. The haploids were non-viable owing to the expression of

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an abnormality widely referred as haploid syndrome. Thus, viable diploid androgenotes were successfully induced by using the cold-shock method together with diploid sperm from the neo-tetraploid male (Hou et al., 2013). Doubled haploid (DH) androgenetic loach progenies were also produced by the cold-shock method from haploid sperm of diploid male, complemented with a heat-shock at the metaphase of the first mitosis, which doubled the chromosomes (Hou et al., 2014). The cold-shock method to induce haploid androgenesis was also proved to be effective in zebrafish, *Danio rerio*, and the first androgenetic clonal zebrafish line was established using a combination of cold-shock androgenesis and heat-shock chromosome doubling treatments (Hou et al., 2015).

Here, we established an androgenetic induction method using cold-shock of just-activated eggs of Japanese flounder. We optimized the cold-shock treatment duration, after which androgenetic DHs were induced by a combination of cold- and hydrostatic pressure-shock treatments. Finally, an androgenetic clonal line was established by cold-shock induction followed by chromosome doubling with hydrostatic pressure in eggs fertilized with the sperm of a mito-gynogenetic DH male, which had been established previously (Liu et al., 2012a). We determined ploidy status based on cellular DNA content by flow cytometry, and then genetically verified the exclusive transmission of paternal genome and complete homozygosities of DHs as well as the genetic identity among clonal progenies by microsatellite genotyping.

2. Materials and methods

2.1. Ethics

This study was performed in accordance with the Guide for Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences (No. 2011-2).

2.2. Fish and gamete collection

Mature male and female Japanese flounders were reared at the Beidaihe Central Experimental Station, Chinese Academy of Fishery Sciences, at 14 °C water temperature with an 18 h light/6 h dark photoperiod. Eggs were manually stripped and collected from the females using a 1000-mL glass beaker. Sperm was collected using a 5-mL plastic syringe, with gentle pressure on the abdomen to facilitate collection. At least 2 mL of sperm was collected from each male. The collected eggs and sperm were store at 4 °C in dark before use.

2.3. Optimization of the cold-shock treatment duration

Before use, sperm samples were diluted 50 times with Ringer's solution. Diluted sperm samples were added to the eggs at a ratio of 1:40, mixed well, and activated with a small amount of filtered 17 °C sea-water. Within the first 10 s after activation, activated eggs were transferred to a 0 °C water-bath, and subjected to cold shock for 15, 30, 45 or 60 min. During the cold-shock treatment, water temperature was constantly maintained at precisely 0.5 °C. After the cold-shock treatment, the eggs of each group were transferred to a 1000-mL glass beaker that contained filtered 17 °C sea-water and maintained in an incubator at constant temperature of 17 °C until hatching. The control group was composed of just-fertilized eggs that were directly transferred to the incubator at a constant temperature of 17 °C.

2.4. Chromosome doubling by hydrostatic pressure

Just-activated eggs were first transferred to a 0 °C water-bath for a cold-shock of 15 min, followed by a 60 min incubation at 17 °C in a water-bath. The eggs were then exposed to a 650 kg/cm^2 hydrostatic pressure for 6 min, following the protocol of Yamamoto (1999). After the treatment, eggs were returned to the incubator with 17 °C water-bath until hatching.

2.5. Ploidy, all-male inheritance and homozygosity

The ploidy of all hatched abnormal larvae at 69 h after activation was analyzed by flow cytometry (PA-II, Partec GmbH, Münster, Germany), following the procedure of Fujimoto et al. (2007). Each larva was first digested by using 85 μL of solution A (CyStain DNA 2 step, Cod. 05-5005, Partec GmbH) for 15 min. Then, 15 μL of the digested solution was stained by using 500 μL of solution B (CyStain DNA 2 step, Cod. 05-5005, Partec GmbH), and analyzed by flow cytometry. The remaining 70 μL of digested solution was used for DNA extraction.

To determine all-male inheritance (exclusive paternity), eight haploid larvae and five diploid larvae of the control group, together with female and male parents, were genotyped using two Japanese flounder microsatellite markers: *Poli1359TUF* (Castaño-Sánchez et al., 2010) and *HLJYP38* (Liu et al., 2013). Homozygosity of eight diploid (putative DH) progeny, hatched from the cold-shock and hydrostatic pressure treatment groups, was genetically confirmed by analyzing 36 microsatellite markers that covered 24 linkage groups of the Japanese flounder (Castaño-Sánchez et al., 2010; Liu et al., 2013). DNA was extracted using TIANamp Marine Animals DNA Kit (DP324-02, Tangent). PCR was performed in a 15 μL reaction solution (Liu et al., 2012a) under the following conditions: one cycle of initial denaturation for 3 min at 94 °C, 25 cycles of denaturation for 30 s at 93 °C, annealing for 30 s at 62 °C, extension for 30 s at 72 °C, and one cycle of final extension for 10 min at 72 °C. Electrophoresis of PCR products together with molecular weight markers were performed on 6% denaturing polyacrylamide gel, and after electrophoresis, the gel was stained with silver nitrate (Liao et al., 2007).

2.6. Fertilization, hatch, abnormal and haploid rates

The fertilization rate was calculated as the frequency of gastrula embryos relative to the total eggs used, measured 24 h after activation. The hatching rate was calculated as the proportion of hatched larvae relative to the total eggs used. The abnormality rate was calculated as the rate of externally abnormal larvae relative to the total hatched larvae. The haploid rate was calculated as the rate of haploid larvae relative to the total eggs used. The normal rate of larvae that hatched from eggs subjected to cold-shock and hydrostatic pressure treatments was calculated as the proportion of normal larvae relative to the hatched larvae. Survival rate at first feeding was the frequency of surviving larvae at this stage

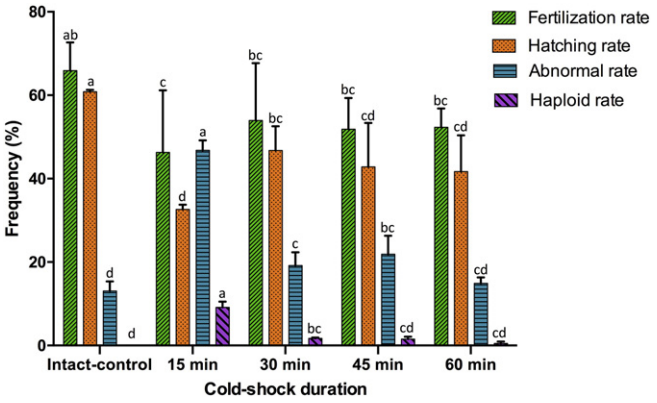

Fig. 1. Fertilization rate, hatching rate, abnormal rate, and haploid rate of just-fertilized eggs of the Japanese flounder, *Paralichthys olivaceus*, cold-shocked at 0 °C for 15, 30, 45 and 60 min. Letters above columns indicate significant differences as determined by one-way ANOVA and Student-Newsman-Keuls multiple comparisons ($P < 0.05$).