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Full Length Research Paper

The homozygosity verification for doubled haploid Japanese flounder, *Paralichthys olivaceus*, by microsatellite DNA markers

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A total of 845 doubled haploids Japanese flounder, *Paralichthys olivaceus* from three spawns were produced by utilizing hydrostatic pressure treatments on eggs fertilized with ultraviolet (UV)-irradiated sperm of red sea bream (*Pagrus major*). 481 polymorphic microsatellite markers were used to identify the homozygosity of these doubled haploids, of which, only 31% (265 out of 845) individuals were fully homozygous at all loci tested, while, the rest 580 offspring were homozygous at 183 to 320 loci. Additionally, the embryonic development and external morphology of doubled haploids were also observed and recorded. The whole process of embryonic development was divided into 19 stages based on the morphological characteristics of the developing embryo. Hatching took place 73 to 74 h after fertilization. The means of body weight, body length and body depth in 360 days old fishes were always small in doubled haploids and large in normal diploids, but, the standard deviations and coefficients of variation of doubled haploids were significantly higher than those of normal diploids.

Key words: Doubled haploids, gynogenesis, Japanese flounder, *Paralichthys olivaceus*.

INTRODUCTION

Mitotic gynogenesis in fish has been used to produce completely homozygous progenies (Thorgaad, 1986; Ihssen et al., 1990). In such a gynogenesis, eggs are firstly fertilized with sperms genetically inactivated by UV irradiation, and then, gynogenetic diploids can be produced by suppressing the first cleavage of eggs with thermal or pressure shocks. These diploids themselves are usually termed doubled haploids (DHs) when they are produced by inhibiting the first mitotic division. Up to now, the production of DH has been successfully performed in many freshwater and marine fish species (Streisinger et al., 1981; Naruse et al., 1985; Komen et al., 1991; Han et al., 1991; Kobayashi et al., 1994; Suwa et al., 1994; Lin and Dabrowsk, 1998; Galbusera et al., 2000; Paschos et al., 2001; Kato et al., 2002; Bertotto et al., 2005; Waldbieser et al., 2010). The fully homozygous DHs can not only be reproduced by a second cycle of meiotic gynogenesis to induce clonal line, but also be used to detect the rare recessive mutations (Komen and Thorgaard, 2007).

The Japanese flounder, *Paralichthys olivaceus*, is one of the major marine cultured species in Japan, Korean and China, and some breeding programs have been established for improving some important commercial traits in this species (Shikano, 2007). The first mitotic gynogenesis in Japanese flounder was conducted by Yamamoto (Yamamoto, 1999). Subsequently, Liu et al. (2012) obtained 77 mitotic gynogenetic offspring from four families, of which, 58 were verified to be completely homozygous, but few putative individuals survived until adulthood.

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Table 1. Genotype distribution in 481 markers in three mitotic gynogenetic lines.

MIT-1 progeny	MIT-2 progeny	MIT-3 progeny	Total	Homozygous loci
214 (32%)	29 (34%)	22 (25%)	265 (31%)	481 (100%)
105 (16%)	13 (15%)	15 (17%)	133 (16%)	320 (67%)
97 (14%)	7 (9%)	12 (13%)	116 (14%)	251 (52%)
123 (18%)	12 (14%)	19 (22%)	154 (18%)	206 (43%)
133 (20%)	24 (28%)	20 (23%)	177 (21%)	183 (38%)

In this study, we produced large numbers of DHs Japanese flounder and indentified their homozygosity using a good deal of microsatellite markers. Additionally, the embryonic development and external morphology of DHs were also compared with those of normal diploids (NDs).

MATERIALS AND METHODS

Production of DH

Production of DH in Japanese flounder was carried out at Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences, Qinhuangdao, Hebei Province. Eggs and sperm were obtained from three females (F1 to F3) and one male (M1) at the age of four to five years were used. The parents (F1 and M1) were used to produce the normal diploid family as control group. The female parents F1, F2 and F3 were used to produce three mitotic gynogenetic lines (MIT-1, MIT-2 and MIT-3). The procedure of producing DHs was adapted from a previous standard protocol (Yamamoto, 1999). In brief, eggs and sperm were obtained from Japanese flounder and red sea bream. Hand-stripped eggs were inseminated with the sperm that had been genetically inactivated by ultraviolet light (UV) with a dose of approximately 73 mJ/cm^2. The treatment for the suppression of the first cleavage was done with a hydrostatic pressure shock of 650 kg/cm^2 for 6 min at 60 min after insemination. Thereafter, the eggs were incubated under running seawater (17°C).

Identifying homozygosity

The homozygosity of the DHs was confirmed by multiple micro-satellite markers. After six months, fin-clips samples were collected from surviving offspring. The detailed method for isolation of genomic DNA was reported by Blin and Stafford (1976). A set of 481 microsatellite loci covering 24 linkage groups were chosen from the second generation genetic linkage map of Japanese flounder (Castaño-Sánchez et al., 2010). All markers were genotyped for the dam and progenies of three mitotic gynogenetic lines. Polymerase chain reactions (PCR) were carried out in 15 µl total volume containing 30 to 50 ng template DNA, 1×PCR buffer (50 mM of KCl, 10 mM of Tris-HCl, 1.5 mM of $MgCl_2$, pH 8.3), 200 µM of each dNTP, 1U Taq polymerase (Takara), and 2 pmol of each primer. After amplification, the PCR products were electrophoresed in 8% (wt/vol) denatured polyacrylamide gel (19:1 acrylamide: bis-acrylamide and 7 M urea). Fragments were visualized by silver nitrate staining and UV transillumination (Liao et al., 2007).

Embryonic development and external morphology

The fertilized eggs samples of DHs were collected immediately after fertilization and monitored each day at the same time till hatching. The developmental stages were observed under a light microscope taken photos by using digital camera. In MIT-1 and normal diploid families, 360 days old fishes were used for growth analysis. The traits including body weight, body length and body depth were measured and recorded. Means, standard deviations (SD) and coefficients of variation (CV) of these traits were calculated and compared between DHs and NDs group.

RESULTS

A total of 845 DHs were produced from three females by mitotic gynogenesis, and then, 481 microsatellite markers were used to verify their homozygosity. The three dams used in the induction were highly heterozygous at all markers tested. The summary of genotypes in progenies is given in Table 1. As shown, only 25 to 32% offspring from three mitotic gynogenetic lines were completely homozygous, other 15 to 17% offspring were homozygous at 320 loci each, 9 to 14% at 251 loci each, 14 to 22% at 206 loci each, and 20 to 28% at 183 loci each.

The embryonic development of Japanese flounder from fertilization to hatching was classified into 19 stages. When comparing different embryonic developmental process, attaining time for the same stage in DHs was always larger than that in NDs. The fertilized eggs of DHs took 73 h and 45 min for hatching, but for NDs, the hatching duration time was 72 h and 20 min (Table 2).

Means, SD and CV of body weight, body length and body depth in DHs and NDs are shown in Table 3. The means of three traits in DHs were significantly lower than those in NDs, while, the CV and SD were the highest.

DISCUSSION

From three females, 845 surviving doubled haploids Japanese flounder were produced by chromosome manipulation. Actually, in many fish species, the offspring from mitotic gynogenesis have been successfully obtained with hydrostatic pressure treatment, but the overall embryo survival rate is still very low (Komen and Thorgaard, 2007). Several reasons have been put forward to explain this phenomenon, such as develop-mental damage caused by the pressure shock, expression of the genetic load and inbreeding depression

Table 2. Attaining time for different embryonic developmental stages in doubled haploids and normal diploids of Japanese flounder.

Stage	Time	
	Normal diploid	Doubled haploid
1. Zygote	0 min	0 min
2. Blastodisc formation stage	32 min	33 min
3. Two celled stage	1 h 50 min	1 h 55 min
4. Four celled stage	2 h 30 min	2 h 35 min
5. Eight celled stage	3 h 40 min	3 h 50 min
6. Sixteen celled stage	4 h 30 min	4 h 45 min
7. Thirty-two celled stage	5 h 10 min	5 h 30 min
8. Multiple celled stage	6 h 10 min	6 h 40 min
9. High blastula stage	7 h 50 min	8 h 15 min
10. Low blastula stage	13 h 10 min	13 h 50 min
11. Early gastrula stage	17 h 50 min	18 h
12. Mid gastrula stage	23 h 30 min	23 h 50 min
13. Late gastrula stage	30 h 20 min	31 h 10 min
14. Neurula stage	33 h 30 min	35 h 40 min
15. Kupffers vesicle formation stage	39 h 10 min	41 h 20 min
16. Tailbud formation stage	50 h 50 min	52 h 40 min
17. Heartbeating stage	65 h 10 min	66 h 40 min
18. Prehatching stage	70 h 10 min	72 h
19. Hatching stage	72 h 20 min	73 h 45 min

Table 3. Morphometrics of offspring (360 days old); means, standard deviation (SD) and coefficient of variation (CV) of body weight, body length and body depth in doubled haploids and normal diploids of Japanese flounder.

Trait	Statistic	Normal diploid (150)*	Doubled haploid (160)*
Body weight (g)	Mean	310.97$^{a\&}$	289.41^{b}
	SD	53.05^{a}	92.61^{b}
	CV	0.17^{a}	0.32^{b}
Body length (cm)	Mean	34.83^{a}	28.73^{b}
	SD	2.14^{a}	3.73^{b}
	CV	0.06^{a}	0.13^{b}
Body depth (cm)	Mean	15.32^{a}	11.09^{b}
	SD	0.94^{a}	1.55^{b}
	CV	0.06^{a}	0.14^{b}

*Number of fish; means within a line not marked by the same superscript letter are significantly different (P<0.05).

(Bertotto et al., 2005; Komen and Thorgaard, 2007; Bongers et al., 1997a).

In this study, we applied 481 polymorphic markers to identify the homozygosity of DHs, and each of the 24 linkage groups was covered by at least eleven markers. Similar studies have been conducted in many fish species, such as African catfish (Galbusera et al., 2000), European sea bass (Bertotto et al., 2005) and Channel catfish (Waldbieser et al., 2010). For Japanese flounder, Liu et al. (2012) choose 21 microsatellite markers that were located in the telomeric region of chromosomes to verify the homozygosity of mitotic gynogenetic diploids. Different from the above mentioned studies, the markers used here are the most numerous, and furthermore, covered the entire genome. Current results strongly confirm the homozygous status of DHs Japanese flounder.

The DH theoretically should be completely homozygous, but, in this study, the percentages of heterozygous individuals were up to 66 to 75%. Similar results have

also been observed in African catfish (Galbusera et al., 2000) and European sea bass (Bertotto et al., 2005). Inferring the possible reason for forming heterozygous individuals, developmental asynchrony among eggs may be one reason for spontaneous production of diploid gynogens by inhibiting the second polar body release. When applying hydrostatic pressure to inhibit cleavage, some eggs with delayed development might have still been in the period of meiosis, and thus, the pressure shock might have blocked the delayed second meiotic division rather than the first zygotic cleavage, causing the appearance of individuals with heterozygous loci (Libertini et al., 2002). Additionally, a prevalent mortality of DHs and the occurrence of spontaneous polocyte retention in the surviving gynogenetic fish could be another hypothesis for demonstrating this phenomenon.

The embryonic development pattern of DH is the same as those of ND. Of DH, the hatching started after 73 h and 45 min of fertilization, which is higher than that of ND. Analyzing the reason, pressure shocks may have wide and ranging undesirable side-effects on embryo development. The CVs of body weight, body length and body depth of DHs were 2.0-fold larger than that of NDs. Large CVs in DHs may have been due to the effects of segregation of polygenes for quantitative characters, as well as the effects of deleterious genes and developmental instability in the inbred group. For ayu and red sea bream, Taniguchi et al. (1990) and Kato et al. (2001) reported the expansion of CVs of some quantitative traits in DHs, respectively. However, growth performance was significantly lower in DHs than in NDs. Poor growth of DHs in Japanese flounder have also been observed in Tabata and Gorie (1988) study. From this, it can be seen that the first generation of DHs cannot be directly used as a commercial strain. As good material for fish breeding and culture, F_1 hybrids need to be created by crossing DH individuals, which are groups of genetically identical, heterozygous animals.

Based on the present research, large number of DHs Japanese flounder was successfully produced. These DHs developed normally like ND fish and will be maintained to the adult stage using them as broodstocks for the production of homozygous or heterozygous inbred lines. In addition, mapping of some special trait loci controlling lethal allele or filiform gonad in Japanese flounder will also be carried out.

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