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Genetic verification of doubled haploid Japanese flounder, *Paralichthys olivaceus* by genotyping telomeric microsatellite loci

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ABSTRACT

Mitotic gynogenetic diploid or doubled haploid Japanese flounder *Paralichthys olivaceus* were produced by activating eggs with UV irradiated sperm of red sea bream (*Pagrus major*), followed by hydrostatic pressure treatment to block the first mitotic division. To verify the doubled haploid, we measured the homozygosity of 21 maternally heterozygous microsatellite loci that are located in the telomeric region of chromosomes. These 21 loci have large marker-centromere distances of 43 to 50 cM estimated from four meiotic gynogenetic diploid lines, suggesting the location at telomeric regions of chromosomes. These 21 loci cover 16 out of the total 24 linkage groups of Japanese flounder. Microsatellite genotyping of these 21 markers in four mitotic gynogentic diploid lines shows that 75% (58 out of 77) individuals are homozygous at all 21 loci, indicating doubled haploids. The other 25% are heterozygous at 1 to 13 loci, probably representing spontaneously occurring meiotic gynogenetic diploids. These results demonstrate the high efficiency of generating doubled haploids in just one generation by mitotic gynogenesis. We have used these doubled haploids to successfully generate clonal lines via a second round of gynogenesis.

1. Introduction

Mitotic gynogenesis is an effective technique to produce all-homozygous inbred or doubled haploid fish in just one generation (Arai, 2001; Komen and Thorgaard, 2007). All-homozygous doubled haploids can be produced by inhibition of the first mitotic cell divisions after the eggs were stimulated by genetically inactivated sperm. The resultant very few surviving double haploids then generate genetically uniform eggs in the species with male heterogametic sex determination system (XX female; XY male). Thus, a second cycle of meiotic gynogenesis will result in the production of a clonal line (Arai, 2001; Komen and Thorgaard, 2007). However, this technique is limited by the extreme low number of survivors of mitotic gynogenetics that are completely homozygous (Arai, 2001; Komen and Thorgaard, 2007). Putative mitotic gynogenetic survivors are often contaminated with a significant fraction of heterozygous individuals that are derived from spontaneous meiotic gynogenesis, where the second polar body releases prior to mitotic cell division. Spontaneous meiotic gynogenesis has been often reported in fish species including rainbow trout (Thorgaard and Gall, 1979), plaice (Thompson et al., 1981), carp (Cherfas et al., 1991, 1995), Nile tilapia (Ezaz et al., 2004) and Siberian sturgeon (Fopp-Batyat, 2007).

Meiotic gynogenetic diploids are often heterozygous at telomeric loci due to a high recombination rate between the telomere region and the centromere. The heterozygous meiotic gynogenetic progenies are unable to produce clonal lines via a second round of gynogenesis. Thus, it is required to separate mitotic gynogenetic progeny from spontaneous meiotic gynogenetic progeny and verify the true doubled haploids before the production of a clonal line. The meiotic gynogenetic progenies of a heterozygous mother will be heterozygous at some loci located on the distal portion of chromosome, i.e. telomeric region, because of the high rates of recombination in relation to the centromere (Estoup et al., 1993; Guyomard, 1984; Lahrech et al., 2007; Lindner et al., 2000; Morishima et al., 2001; Nomura et al., 2006; Thorgaard et al., 1983). The mitotic gynogenetic progenies, however, will be homozygous at all telomeric loci. Therefore, by genotyping multiple telomeric markers, we can distinguish true doubled haploids from spontaneous meiotic gynogenetic diploids.

In previously reported cloning experiments of commercially important Japanese flounder, putative mitotic gynogenotes were identified by the homozygosity at a single locus, the diagnostic isocitrate dehydrogenase (IDH) allozyme locus with high gene-centromere recombination rate before cloning (Tabata and Gorie, 1987). However, this single locus test is insufficient to estimate complete homozygosity. Also, the important homozygous brood stock for cloning was inevitably

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damaged by biopsy. In order to build a better breeding program to improve commercially important traits, we have optimized mitotic gynogenesis protocols to produce relatively large numbers of doubled haploid Japanese flounders. (On average, 20 mitotic gynogenotes are generated per female fish surviving to the adult stage) The doubled haploid individuals of the mitotic gynogenesis progenies will be used to generate clonal lines by a second round of gynogenesis. They can also be used to generate hybrid recombinant lines by crossbreeding between two doubled haploids, since gynogenetic males can be produced by sex-reversal during sex differentiation (Yamamoto, 1999). The key point for making this breeding strategy practical is to reliably generate a relatively large number of true doubled haploids via mitotic gynogenesis.

In the present study, we verified the high efficiency of generating large numbers of doubled haploids by selecting telomeric microsatellite loci based on published Japanese flounder linkage maps (Castaño-Sánchez et al., 2010; Coimbra et al., 2003), estimating their marker-centromere distance using meiotic gynogenetic families, and examining their homozygosity in our mitotic gynogenetic progenies. The verified double haploids were then used to generate clonal lines via a second cycle of gynogenesis.

2. Material and methods

2.1. Production of gynogenetic diploid

Eggs and sperm were obtained from 10 females (F1–F10) and 2 males (M1–M2) at the age of four to five years, in five strains of Japanese flounder which were established in 1999 from five separate regions (Beidaihe, Weihai, Qingdao, Yantai and Fujian). In this study, the parents (F1 and M1, F2 and M2) were used to produce the normal diploid full-sib family ND-A and ND-B. The four meiotic gynogenetic lines (MEI-A, MEI-B, MEI-C, and MEI-D) were produced from females F3, F4, F5 and F6. The female parents F7, F8, F9 and F10 were used to produce the four mitotic gynogenetic diploid lines, i.e., doubled haploids (MIT-A, MIT-B, MIT-C, and MIT-D). Sixty individuals from the Beidaihe wild population were used to test the polymorphism of microsatellite markers, including allele diversity, Hardy–Weinberg equilibrium, and genotypic linkage disequilibrium.

The procedure of chromosome manipulation was adapted from a previous standard protocol (Yamamoto, 1999). Briefly, meiotic gynogenetic diploid lines were produced by fertilizing flounder eggs with ultraviolet (UV)-irradiated red sea bream sperm at the dose of 40–50 mJ/cm^2, followed by inhibition of the extrusion of the second polar body with cold shock (0 °C) for 45 min duration starting 3 min post fertilization. For induction of mitotic gynogenetic diploids, eggs fertilized with UV-irradiated red sea bream sperm were treated to inhibit the first cleavage with hydrostatic pressure (650 kg/cm^2) for 6 min starting 60 min after fertilization.

A fin clip was sampled from each parental fish just after artificial fertilization and from progeny of each group at three months after hatching. Thirty individuals from each of two full-sib families, 30 individuals taken from each of four meiotic gynogenetic families and a total of 77 survivors from four mitotic gynogenetic families were collected. The survival rate of doubled haploid Japanese flounder was about 5%. The survivors included 20 offspring from MIT-A family, 20 offspring from MIT-B family, 15 offspring from MIT-C family and 22 offspring from MIT-D family. Genomic DNA of each sample was isolated using phenol-chloroform extraction (Blin and Stafford, 1976).

2.2. Production of clonal lines

Using eggs of the two completely homozygous gynogens (#3 from MIT-A and #5 from MIT-B) verified by genetic analyses, clonal lines (clones 1 and 2) were induced by fertilizing eggs with UV-irradiated heterospecific red sea bream sperm, followed by cold shock (0 °C, 45 min duration) to inhibit the second polar body release. At three months post hatching, 30 progeny were collected from clonal lines 1 and 2 to verify the complete identity and homozygosity at microsatellite loci.

2.3. Microsatellite genotyping

A set of 24 microsatellite loci located at relatively distal regions of linkage groups in the Japanese flounder linkage maps (Castaño-Sánchez et al., 2010; Coimbra et al., 2003) were selected, whose core sequences, primer sequences, annealing temperatures, number of allele, allele sizes, linkage group and accession number in GenBank were in Table S1. All loci were genotyped for the progenies of the control families and the meiotic and mitotic gynogens. PCR was performed in a reaction mixture (15 μl) containing: 30–50 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, 1 U *Taq* polymerase (Takara), and 2 pmol of each primer. The PCR products were separated by electrophoreses on 8% (wt/vol) denatured polyacrylamide gel (19:1 acrylamide: bis-acrylamide and 7 M urea). After electrophoresis, the gel was stained with silver nitrate as described by Liao et al. (2007).

2.4. Genetic analysis

The Mendelian inheritance at each locus was examined in both control families ND-A and ND-B. The chi-square (χ^2) test was used to test Mendelian segregation pattern, where significant level was considered as 5%. The microsatellite-centromere recombination rate was estimated from the frequency of heterozygous recombinant genotypes in all four meiotic gynogenetic lines (MEI-A, B, C and D) at each locus. Assuming complete chiasma interference, microsatellite-centromere map distance in centiMorgans (cM) may be obtained as $100(r/2)$ with r being recombination rate (Thorgaard et al., 1983). The loci with high recombination rates were chosen to examine the homozygosity of the progeny of the four mitotic gynogenetic lines (MIT-A, B, C and D). The number of alleles and allele sizes of the 24 loci were examined in 60 individuals from the Beidaihe wild population.

3. Results

The number of alleles and allele sizes of 24 microsatellite loci in the 60 individuals from a wild population are listed in Table S1. The selected 24 polymorphic microsatellite loci were distributed in 16 out of the 24 linkage groups of the Japanese flounder linkage map (Castaño-Sánchez et al., 2010; Coimbra et al., 2003). There is only one locus on 13 of 16 linkage groups. Mendelian segregation of alleles at these 24 loci was confirmed in two full-sib families (ND-A and B). No null alleles were detected in the two families.

In the four meiotic gynogenetic diploid lines (MEI-A, B, C and D), the proportions of the heterozygous genotypes at the 24 microsatellite loci are shown in Table 1. The recombination frequency at the 24 loci ranged from 0.05 to 1.00. Almost complete recombination rates (close to 1.00) were observed at 21 loci. Two other loci gave intermediate recombination rates (from 0.43 to 0.51). There is only one locus that showed very low recombination rate of 0.05.

We chose the twenty one microsatellite loci with high recombination rate to examine the complete homozygosity of the progeny from the four mitotic gynogenetic lines (MIT-A, B, C and D). The number of homozygous loci is listed in Table 2 for all 77 mitotic gynogenetic survivors. Of them, fifty-eight were completely homozygous at all 21 loci, three were homozygous at 20 out of the 21 loci and the others were homozygous at eight to 14 loci.

From eggs of the complete homozygotes: #3 female from MIT-A and #5 female from MIT-B, clonal lines were induced by inhibiting