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Genetic verification of induced gynogenesis and microsatellite–centromere mapping in the barfin flounder, *Verasper moseri*

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Abstract

Primer sets were newly developed for 34 polymorphic microsatellite loci in the barfin flounder *Verasper moseri*. Mendelian inheritance was confirmed by examining the genotypic segregation in two normal diploid full-sib families. All the 34 loci showed genotypic segregation according to the Mendelian manner of inheritance; in some cases, null alleles were assumed. The genotypes at 27 loci were also examined in four meiotic gynogenetic diploid lines produced by fertilizing eggs with UV-irradiated sperm, followed by inhibition of the second meiotic division by cold shock. The absence of paternal alleles verified the success of gynogenetic development in all four meiotic gynogenetic diploid lines; the proportion of heterozygous progeny of a heterozygous mother, i.e., the frequency of second division segregation (y), was used to estimate the map distance of each microsatellite locus in relation to the centromere. Marker–centromere distances were estimated to be in the range of 0 to approximately 50 centiMorgan (cM) under the assumption of complete interference. Using eight diagnostic loci located at the telomeric region of the chromosome, complete homozygosity was confirmed in 1 mitotic gynogenetic diploid line that was produced by suppressing the first cleavage via hydrostatic pressure shock.
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1. Introduction

The barfin flounder *Verasper moseri* is a large flatfish species that inhabits cold sea basins near the east coast of Hokkaido, Japan. This species is a promising candidate for aquaculture and stocking due to its high commercial value and stable growth in cold water. Similar to the growth of other flatfishes, the barfin flounder exhibits sexually dimorphic growth and the females grow to adult size faster than males (Mori et al., 1999). Thus, sex manipulation is required in order to achieve an all-female population, especially in aquaculture. The induction of gynogenetic diploids is considered a useful technique to identify the sex determination system in the barfin flounder and to generate an all-female population in a male heterogametic species. Meiotic gynogenetic diploids can be produced by inhibiting the extrusion of the second polar body, while completely homozygous mitotic gynogenetic diploids are produced by the inhibition of

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the first cleavage after the activation of eggs with genetically inert UV-irradiated sperm.

In order to produce an all-female population and for future cloning of the barfin flounder, meiotic and mitotic gynogenetic diploids have been experimentally produced after the optimization of treating conditions to induce gynogenesis and duplicate the chromosomes (Mori et al., 2004). In other species, gynogenesis is usually induced using UV-irradiated sperm of a different species in order to eliminate sporadic or occasionally appearing diploids by inviable hybridization (Arai, 2001). However, in barfin flounders, meiotic gynogenesis has been induced using irradiated sperm from the same species because it is difficult to collect heterospecific sperm during the spawning season (Mori et al., 2004). Thus, the possible contamination of the putative gynogenetic progeny with sporadic diploids due to insufficient genetic inactivation of the irradiated sperm cannot be ruled out. Genetic verification of gynogenesis, i.e., all-female inheritance, is an important step in chromosome manipulation in order to evaluate the success of induction.

At the level of homozygosity, meiotic and mitotic gynogenesis differ genetically (Arai, 2001; Nagy and Csanyi, 1984; Palti et al., 2002). Complete homozygosity can be achieved by mitotic gynogenesis in a single generation. Thus, mitotic gynogenesis is an effective technique for producing inbred lines of fish in a substantially shorter time. Such completely homozygous fish that spawn genetically uniform eggs are indispensable for cloning via a second cycle of gynogenesis. However, contamination by meiotic gynogenetic diploids due to the spontaneous inhibition of the release of the second polar body should be eliminated (Arai, 2001). Therefore, precise separation of the two kinds of gynogenetic diploids is necessary for successful cloning of a target aquaculture species. In the meiotic gynogenetic diploids of a heterozygous mother, the locus at the distal portion of the chromosome is considered heterozygous because of its high gene or marker-centromere recombination rate, as reported for other fish species (Thorgaard et al., 1983; Guyomard, 1984; Estoup et al., 1993; Kauffman et al., 1995; Lindner et al., 2000; Matsuoka et al., 2004). Thus, putative mitotic gynogens must be identified by their complete homozygosity at such diagnostic markers (Suwa et al., 1994; Morishima et al., 2001; Ezaz et al., 2004).

Codominant microsatellite markers are superior for use in parentage studies because of their numerous polymorphisms, ubiquitous distribution in the genome, and simple sampling and preservation requirements (Ferguson et al., 1995). In the present study, new microsatellite markers were developed, and their Mendelian inheritance was examined by observing genotypic segregation in two normal diploid full-sib families generated by normal fertilization. We used 27 microsatellite markers to confirm the success of gynogenetic development in four meiotic gynogenetic diploid lines. The microsatellite–centromere map distance for a polymorphic locus was estimated from the frequency of second division segregation (y) as the proportion of heterozygous meiotic gynogenetic diploid progeny of a heterozygous mother. Microsatellite loci with high marker-centromere recombination frequencies, located in the telomeric region of chromosomes, were then used to verify complete homozygosity of the mitotic gynogenetic diploids.

Table 1
Full-sib families and gynogenetic diploid lines analyzed in the present study

Family/line	Abbreviation	Parental fish		No. of progeny
		Female	Male	
Normal diploid full-sib family	ND-1	F1	M1	48
	ND-2	F2	M2	30
Meiotic gynogenetic diploid line	MEI-1	F1	M1	40
	MEI-2	F2	M2	40
	MEI-3	F3	M3	40
	MEI-4	F4	M4[a]	40
Mitotic gynogenetic diploid line	MIT-1	F5	M5	10

[a] Unknown due to the loss of sample.

2. Materials and methods

2.1. Induction of gynogenetic fish

A barfin flounder broodstock was transported from the Hokkaido Institute of Mariculture, Shikabe (reorganized in 2006 to the Hokkaido Mariculture Fisheries Experiment Station, Muroran), to the Hokkaido Central Fisheries Experiment Station, Yoichi, and was reared in 4000-l tanks. Eggs and sperms were obtained from five different females and males (Table 1), respectively. In this study, the parental fishes and their progenies from two normal diploid full-sib families (ND-1 and ND-2), four meiotic gynogenetic diploid lines (MEI-1, MEI-2, MEI-3, and MEI-4), and one mitotic gynogenetic diploid line (MIT-1) were used for the genetic analyses. Meiotic gynogenetic diploids were obtained by fertilizing the eggs with UV-irradiated sperm (40–45 mJ/cm^2) and subsequent cold shock (−1.5 °C) for a duration of 70 min at 7 min after insemination (Mori et al., 2004). The same set of parents (F1 and M1) were used to produce the normal diploid full-sib family ND-1 and the meiotic gynogenetic line MEI-1. The female F2 and male M2 parents were used to produce the full-sib family ND-2 and the meiotic gynogenetic line MEI-2. The meiotic gynogenetic lines MEI-3 (parents: F3 and M3) and MEI-4 (parents: F4 and M4) comprised only meiotic

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