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Full Length Research Paper

Production and confirmation of clones using gynogenesis in Japanese flounder

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Two homozygous clones of Japanese flounder were produced by the gynogenesis that suppresses the cleavage of the eggs in the mitosis and then blocks the extrusion of the second polar body in the meiosis. Microsatellite was used to identify the genetic status of maternal parents and their progenies. 21 polymorphic microsatellite markers were chosen, distributed at the distal region of the 16 linkage group. In this experiment, the two used maternal parents were completely heterozygous, while both mitotic gynogenetic diploids and their clone progenies were homozygous for each marker, demonstrating the successful development of cloning.

Key words: Japanese flounder, microsatellite, clone, gynogenesis.

INTRODUCTION

Gynogenesis by chromosome manipulation has been increasingly applied to improve the economic traits of cultured fishes and to produce various useful breeds and strains in aquaculture (Thorgaard, 1983). Using this procedure, completely homozygous clones can be established in only two generations: homozygous diploids are produced in the first generation by mitotic gynogenesis, which suppresses the first mitotic cleavage of the egg. The clones are produced in the second generation by meiotic gynogenesis, which inhibits the release of the second polar body during meiosis (Purdom et al., 1985; Arai, 2001; Komen and Thorgaard, 2007). Therefore, gynogenesis could dramatically shorten the time to produce clonal lines, as compared to the conventional brother-sister mating method (Taguchi, 1981). There have been many clonal lines generated by gynogenesis in zebra fish (Streisinger et al., 1981), medaka (Naruse et al., 1985), ayu (Han et al., 1991), common carp (Komen et al., 1991), rainbow trout (Quillet et al., 1991), amago salmon(Kobayashi et al., 1994), tilapia (Mu¨ller-Belecke and Ho¨rstgen-Schwark, 1995; Mu¨ller-Belecke and Ho¨rstgen-Schwark, 2000), Japanese flounder (Yamamoto, 1999) and red sea bream (Kato et al., 2002). Additionally, clones play an important role in immunological, endocrinological, developmental, molecular biological and genetic studies.

Japanese flounder is an important cultured flatfish species that is distributed along the coast of China. It has slow growth rate and low resistance to disease, although artificial reproduction from wild fish several generations, was not suitable for intensive production. Therefore, it is necessary to develop the new breed of the Japanese flounder with a high performance. Producing clonal lines can rapidly fix desirable economical traits. Since gyno-genetic males can be produced by sex-reversal during sex differentiation (Yamamoto, 1999), different clonal lines are also useful to generate recombinant inbred lines in order to bring multiple superior traits together. In order to prepare homozygous strains for cross breeding, this study produced clonal lines using gynogenesis method. The clonal status was verified with microsatellite marker.

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Table 1. Attributes of the 21 microsatellite markers used in this study.

Marker	Core sequence	Primer sequence	Annealing temperature	Number of allele	Size of allele (bp)	Linkage group	GenBank accession number
Poli2TUF	$(CA)_{25}$	F: ACAATAGGATGCAGCTGCCT R: AAGCGCAAATTGTTATTCCG	57	4	94-156	22	AB037978
Poli9TUF	$(CA)_{20}$	F: GATCTGCAGAAACACACACTCA R: GCGAGTTCTTCCTCAAATGC	62	4	137-178	5	AB037980
Poli18TUF	$(CA)_{14}$	F: CACGCACACACAAGCTCC R: CGTGGGGTGAGGTTATGG	57	4	150-172	3	AB037983
Poli23TUF	$(CA)_{27}$	F: CACAGTGTCACAAAGTGGTGG R: GGGTGTTCTGTGTCATGCTG	60	4	97-169	2	AB037985
Poli101TUF	$(CA)_{25}$	F: CTCCAGTCATGCTCCAATGATGAC R: AGGATGTTGTAATGAACATTGTGATGA	60	3	128-157	10	AB086493
Poli107TUF	$(CA)_{27}$	F: TGGAAGAGATGTGCACTTGACTGTC R: AACTGTCACCTCTGAGTGGACCG	60	4	109-144	6	AB037990
Poli123TUF	$(CA)_{35}$	F: TATCTGACCAGAACTGGAGGGTCTG R: GCGTGTGCATTCGATATACATTTTG	64	4	118-156	20	AB037994
Poli130TUF	$(CA)_{24}$	F: GCGGTGAGGACTTTATTTCTGGACT R: GTGGTACTGCAGAAAAGCGACTGTT	60	3	138-172	1	AB037996
Poli139TUF	$(CA)_{12}$	F:GACAGTTAGA GACCATCGGG TTGG R:GCAGCCTGTT TGTTCCATTA AGAGA	60	4	139-173	20	AB459413
Poli141TUF	$(CA)_{32}$	F: TATGCACAGT TTGAATGGGT GAATG R: TATGACTTCC AGTACGACGT GGTGA	52	4	126-178	14	AB086537
Poli9-8TUF	$(CA)_{12}$	F: GAGAGACAGAAGGTCGTCAACGGTA R: ACAAAGACCACGATGCAAAGTGAC	64	3	144-157	15	AB037989
Poli13TUF	$(CA)_{27}$	F:CACCTCCAGGTTCTACAGTCG R:TCCTGCACAGAGGATGAAAA	60	3	150-178	3	AB037982
Poli193TUF	$(CA)_{21}$	F:CTCCCAACTG AAGTGGATTG TGTTT R:GTACACCAAA CCAAGCTCAG CTCAT	60	4	81-166	23	AB459463
Po13	$(CA)_{13}$	F: ATCCCGTAACAGCCAATCAG R: CGTCCAGGACAATCAGGACT	60	3	216-229	15	AB046746
Poli16-79TUF	$(CA)_{21}$	F:GCTGTCTGAC ACCACAGGGT TCTAA R: CCACACTGGT CACACAAGGA AGTAG	60	4	137-175	18	AB459369
Poli12MHFS	$(CA)_{32}$	F:CAGTGCCTAA ACCAGTGT R: TGTGCTACCG TGAATAAT	60	4	189-205	15	AB459319
Poli18-55TUF	$(CA)_{32}$	F:ACTCTCGTGA TGAAGACTGG ACCAT R:ATCATCACTG ACCCTGATGT GTTCA	60	4	61-165	7	AB459376

Table 1. Continue.

Poli39MHFS	$(CA)_{31}$	F: GGCCTTGTTG TTGTCTGTGA R: ACCGAATGTG AATCTGAAAA	60	4	184-212	15	AB459333
Poli174TUF	$(CA)_{19}$	F:TAGAAACTGG CCTTCATGGT GTCTC R:ATGTCAGAGT TTGAAAGCAG CAACC	60	4	135-168	11	AB459445
Poli182TUF	$(CA)_{28}$	F:CAGTCAACAC GGACTTCATC CTGAG R:TGAACACCTT TGAAAGACAC CTTGA	60	4	133-165	9	AB459453
Po25A	$(GATG)_{10}$	F:AGTCAGGTTTCAGGCCACTG R:CAGAAGTGTTGTCGCAGGAA	60	4	224-243	16	AB046749

MATERIALS AND METHODS

Induction of mitotic gynogenetic diploids

Induction of mitotic gynogenetic diploids was carried out in 2007 at Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences, Qinhuangdao, Hebei Province. Four mature Japanese flounder females were selected from foundation stock. Eggs were stripped from each female and placed into a 100 ml glass beaker. Sperm were collected from red sea bream using the same procedure. Prior to use, eggs and sperm were stored in a refrigerator at a temperature of 4°C. UV irradiation of red sea bream's sperm was conducted according to the method described by Yamamoto (1999). For induction of mitotic gynogenetic diploids, eggs were first fertilized with UV-irradiated red sea bream sperm and cleavage was inhibited with hydrostatic pressure (650 kg/cm^2) for a minute after 60 min fertilization. Subsequently, eggs were then transferred to 17°C seawater for incubation. Four mitotic gynogenetic families were simplified as MIT-A, MIT-B, MIT-C and MIT-D.

Production of clones

Mitotic gynogenetic diploids were cultivated until sexual maturity. For cloning, two females (numbered 2004 and 3165) with well-developed gonads were chosen from families MIT-A and MIT-B, respectively. To develop clonal lines 1 and 2, their eggs were first inseminated with UV-irradiated heterospecific sperm and after 2 min, their second polar body was inhibited by cold shock for 45 min at 0°C

Confirmation of clones

To extract genomic DNA, a fin clip was sampled from each maternal parent and mitotic gynogenetic diploid just after artificial fertilization, as well as from each clone at three months after hatching. Genomic DNA of each sample was obtained using phenol-chloroform extraction (Blin and Stafford, 1976).

A set of 30 microsatellite loci, located in a distal region of linkage groups, in the Japanese flounder linkage maps (Coimbra et al., 2003; Castaño-Sánchez et al., 2010) were used to screen the four maternal parents, two mitotic gynogenetic diploids and their clone progenies. PCR was implemented in a reaction mixture (15 µl) that consisted of 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, 1 U *Taq* polymerase (Takara), and 2 pmol of each primer. The PCR products were isolated by electrophoresis 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).

RESULTS AND DISCUSSION

Using gynogenesis for Japanese flounder in China, we induced 152 mitotic gynogenetic diploids, from which, two clonal lines were produced, containing 286 and 174 survivors, respectively. Through screening by 30 microsatellite makers in four maternal parents, we found that 21 markers performed polymorphism and can be therefore used to identify genetic status of two mitotic gynogenetic diploids and their clone progenies. These 21 markers were found to be distributed in 16 linkage groups of the total 24, indicating the genome wide sampling in the study. Of the 21 markers, four were located on the 15th linkage group, two markers were located on the 3rd and 20th linkage group, and one marker was located on each of the other 13 linkage groups. Table 1 shows core and primer sequences, annealing temperatures, sizes of alleles, number of alleles observed in the present study, the located linkage groups and access numbers in GenBank of 21 polymorphic microsatellite markers. Further detecting results demonstrated that 21 polymorphic markers for all clone progenies were homozygous and identical with that of corresponding mitotic gynogenetic diploid. The allele sizes (bp) of the 21 markers are listed in Table 2. With Japanese flounder in China, we successfully produced homozygous clones by the combination of inducing mitotic gynogenetic diploids and meiotic gynogenetic diploids over two generations. Genetic identity within a cloned population was confirmed by multiple microsatellite makers. As good material for fish breeding and commercial fish culture (Yamamoto, 1999), some heterozygous clones were also generated by crossing between different clones. For wide

Table 2. Genotyping results of the 21 microsatellite markers in maternal parents and their progenies of Japanese flounder.

Marker	Mother-2004	2004	Clone 1	Mother-3165	3165	Clone 2
Poli2TUF	124/132	124/124	124/124	136/160	160/160	160/160
Poli9TUF	137/142	142/142	142/142	145/150	145/145	145/145
Poli18TUF	134/156	156/156	156/156	139/158	139/139	139/139
Poli23TUF	90/130	90/90	90/90	95/111	95/95	95/95
Poli101TUF	140/150	140/140	140/140	119/131	131/131	131/131
Poli107TUF	130/156	130/130	130/130	144/159	159/159	159/159
Poli123TUF	112/125	125/125	125/125	100/116	116/116	116/116
Poli130TUF	130/138	130/130	130/130	136/141	141/141	141/141
Poli139TUF	159/162	159/159	159/159	140/157	157/157	157/157
Poli141TUF	156/165	156/156	156/156	120/132	120/120	120/120
Poli9-8TUF	145/149	145/145	145/145	154/158	158/158	158/158
Poli13TUF	157/178	157/157	157/157	162/182	182/182	182/182
Poli193TUF	128/138	138/138	138/138	119/130	130/130	130/130
Po13	212/216	216/216	216/216	222/226	226/226	226/226
Poli16-79TUF	118/139	118/118	118/118	133/148	133/133	133/133
Poli12MHFS	192/206	192/192	192/192	196/212	212/212	212/212
Poli18-55TUF	133/150	133/133	133/133	115/138	115/115	115/115
Poli39MHFS	188/194	188/188	188/188	209/213	213/213	213/213
Poli174TUF	134/164	134/134	134/134	131/161	161/161	161/161
Poli182TUF	112/150	112/112	112/112	118/152	152/152	152/152
Po25A	220/226	220/220	220/220	228/232	232/232	232/232

application of homozygous clones to commercial breeding, however, some key technologies are still required to work on, such as improvement of survival rate of mitotic gynogenetic diploids and homozygous clones from genetic and farming environmental aspects. In addition to its value of generating superior breeds of Japanese flounder in aquaculture, homozygous diploids and clonal lines derived from them, should also provide useful reagents for genomic and genetic studies of Japanese flounders.

Microsatellite markers have been used to ascertain the genetic status of mitotic gynogenetic diploids in African catfish (Galbusera et al., 2000), European sea bass (Bertotto et al., 2005), channel catfish (Waldbieser et al., 2010), as well as homozygous clones of Nile tilapia (Ezaz et al., 2004). The fact that the loci away from centromere exhibit high marker-centromere, recombination proportion has been reported in zebrafish (Kauffman et al., 1995), channel catfish (Liu et al., 1992), loach (Morishima et al., 2001), Japanese eel (Nomura et al. 2006), pink salmon (Matsuoka et al., 2004), barfin flounder (Lahrech et al., 2007), large yellow croaker (Li et al., 2007) and half-smooth tongue sole (Ji et al., 2009). In this study, we identified 21 polymorphic microsatellite markers on the distal regions of 16 linkage groups, as polymorphic and of high marker-centromere recombination proportion in parental populations. This indicates that the genetic test is a reliable one for testing clones.

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