International Scholars Journals

Advances in Aquaculture and Fisheries Management ISSN: 9424-2933 Vol. 7 (1), pp. 001-007, January, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Creation and confirmation of heterozygous clones in Japanese flop, Paralichthys olivaceus by microsatellite marker

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Accepted 12 November, 2018

Mitotic gynogenetic diploids Japanese flounder *Paralichthys olivaceus* were produced by activating eggs with ultra violet (UV) irradiated sperm of red sea bream (*Pagrus major*), followed by hydrostatic pressure treatment to block the first mitotic division. By crossing two mitotic gynogenetic diploid females with two males, two heterozygous clones of Japanese flounder were produced. Microsatellite marker was used to confirm the genetic status of maternal parents and their progenies. 20 polymorphic microsatellite markers were chosen, covering 16 out of the total 24 linkage groups. The four maternal parents used in this experiment were completely heterozygous, while four mitotic gynogenetic diploids were homozygous for each marker. The genotypes of heterozygous clone progenies were identical and the combination of parental alleles, demonstrates the successful development of cloning.

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

INTRODUCTION

Gynogenesis by chromosome manipulation has increasingly been applied to improve the economic traits of cultured fishes and to produce various useful breeds and strains in aquaculture (Thorgaard, 1983). Mitotic gyno-genesis is an effective technique to produce all-homozygous diploids fish in just one generation (Arai, 2001; Komen and Thorgaard, 2007). All-homozygous diploids can be produced by inhibition of the first mitotic cell divisions after the eggs were stimulated by genetically inactivated sperm, which will be used to generate clonal lines by a second round of gynogenesis (Purdom et al., 1985; Arai, 2001; Komen and Thorgaard, 2007). There were many clonal lines that have been induced by the 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, 2000), Japanese flounder (Yamamoto, 1999) and red sea bream (Kato et al., 2002). The mitotic gynogenetic diploids can also be used to generate heterozygous clones by crossbreeding between two homozygous diploids, because 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 homozygous diploids, via mitotic gynogenesis. It seems probable that heterozygous clones with superior reproductive traits can be developed, furthermore, they will have a high power in detecting and mapping novel QTL relating to embryo - and early larval mortality (Komen and Thorgaard, 2007).

Japanese flounder is an important cultured flatfish species that is distributed along the coast of China, Korea and Japan. Its slow growth rate and low resistance to

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disease, although artificial reproduction from several generations of wild fish, was not suitable for intensive production. Therefore, it is necessary to develop the new breed of Japanese flounder with faster growth and strong resistance to disease. The Japanese flounder has already been the target of several studies on the induction of meiotic and mitotic gynogenesis (Yamamoto, 1999). The aim of the present work was to develop a reliable technique in order to produce heterozygous clones to be subsequently used for genetic improvement. The clonal status was verified with microsatellite marker.

MATERIALS AND METHODS

Induction of mitotic gynogenetic diploids

The experiment was carried out in 2007 at Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences, Qinhuangdao, Hebei province. Eggs and sperm were obtained from 2 females (F1, F2) and 2 males (M1, M2) at the age of four to five years; foundation stocks were used to produce the normal diploid full-sib family ND-A and ND-B. Four mature females of Japanese flounder were selected from foundation stock. Eggs were stripped from each female and put into a 100 mL glass beaker, and enough sperms were collected from red sea bream using the same procedure. Prior to use, all the eggs and sperms were stored in a refrigerator at a temperature of 4°C. The ultra violet (UV) irradiation of red sea bream's sperm was conducted according to the method by Yamamoto (1999). For induction of mitotic gynogenetic diploids, eggs were fertilized with UV-irradiated red sea bream sperm and the cleavage was inhibited with hydrostatic pressure (650 kg/cm^2) for 6 min duration after 60 min fertilization. Subsequently, eggs were transferred to 17°C seawater for incubation. Four mitotic gynogenetic families were designated as MIT-A, MIT-B, MIT-C and MIT-D.

Production of heterozygous clones

Mitotic gynogenetic diploids were cultivated until sexual maturity. Two females (numbered as 2075 and 8033) and two males (numbered as 9791 and 1381) with well-developed gonad were randomly chosen from mitotic gynogenetic families. Two heterozygous clones were designated as H1 (2075×9791) and H2 (8033×1381). After fertilization, eggs were incubated at the hatchery. Each heterozygous clone was transferred to a separate rearing tank (300 × 100 × 100 cm) with flow-through seawater after day 30. The water temperature of the tanks varied from 15.4 to 22.3°C. The fish were fed *Brachionus plicatilis* from day 0 to 25, *Artemia salina* from day 15 to 60 and dry pellets after day 60.

Confirmation of heterozygous clones

As material for extracting genomic DNA, a fin clip was sampled from each parental fish and mitotic gynogenetic diploids just after artificial fertilization, while from each heterozygous clone at 3 months after hatching. Thirty individuals from each of two full-sib families and 30 individuals taken from each of two heterozygous clones were collected. Genomic DNA of each sample was isolated using phenol-chloroform method (Blin and Stafford, 1976).

A set of 24 microsatellite markers located at relatively distal region of linkage groups in the Japanese flounder linkage maps (Coimbra et al., 2003; Castaño-Sánchez et al., 2010) were used to screen in four maternal parents, four mitotic gynogenetic diploids and their heterozygous 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, 10mM 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 pairs. The PCR products were separated using 8% 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).

Genetic analysis

The Mendelian inheritance at each marker was examined in both control families ND-A and ND-B. The Mendelian segregation pattern was tested using the chi-square (χ^2) test (α=0.05). The polymorphic microsatellite markers were chosen to examine the homozygosity of the mitotic gynogenetic diploids and genetic status of the heterozygous clones. The number of alleles and allele sizes of these markers were examined in 60 individuals from the Beidaihe wild population.

RESULTS

Using gynogenesis for Japanese flounder in China, 152 survival mitotic gynogenetic diploids were produced. We chose the 24 microsatellite markers located at relatively distal region of linkage groups to examine the complete homozygosity of the progeny from the four mitotic gynogenetic lines (MIT-A, B, C and D). Through screening by 24 microsatellite markers in four maternal parents, we found that 20 markers performed polymorphism and can be therefore used to identify genetic status of four mitotic gynogenetic diploids.

The number of alleles and allele sizes of 20 microsatellite markers in the 60 individuals from a wild population are listed in Table 1. Furthermore, Table 1 also shows core sequences, primer sequences, annealing temperatures observed in the present study, the located linkage groups and access numbers in GenBank of 20 polymorphic microsatellite markers. Mendelian segre-garion of alleles at these 20 markers was confirmed in two full-sib families (ND-A and B) (Table 2). No null alleles were detected in the two families.

Using eggs from the complete homozygotes: #2075, #8033 female and #9791, #1381 male, two heterozygous clones containing 207 and 174 survivors, respectively were produced. Four mitotic gynogenetic diploids were completely homozygous at all 20 markers. Thirty progenies per heterozygous clone were randomly selected from all survivors and found that their genotype were identical and the combination of corresponding parental alleles at all polymorphic 20 markers, indicating successful generation of heterozygous clonal lines. The allele sizes (bp) of 20 markers are listed in Table 3.

DISCUSSION

In this study, we identified genetic status of the double haploids and heterozygous clones by multiple micro-

Table 1. Marker, core sequences, primer sequences, annealing temperatures, number of allele, allele sizes, linkage group and accession number in GenBank of 24 microsatellite markers in the *Paralichthys olivaceus*.

Marker	Core sequences	Primer sequence (5′-3′)	Annealing temperature (°C)	Number of alleles	Allele sizes (bp)	Linkage group	GenBank accession no.
Poli1TUF	$(CA)_{39}$	F: GATCTCTGGCTGAGTCAGCG R: TGAGTGTGAAGCCAAAGGC	52	4	59-152	12	AB037977
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

Table 1. Continue

Poli18-55TUF	$(CA)_{32}$	F:ACTCTCGTGA TGAAGACTGG ACCAT R:ATCATCACTG ACCCTGATGT GTTCA	60	4	61-165	7	AB459376
Poli24MHFS	$(GT)_{21}$	F:CCACCTTATT TTTCCTGCTC TGTA R:TCTCTGTCTT ATCACCTTTC ATCC	60	4	100-147	3	AB459326
Poli104MHFS	$(CA)_{37}$	F:GCCGCTCGCT GTTTCTCTC R:ACTGCTCCTC TTTGTGTCG	60	4	215-248	11	AB459342
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

satellite markers. Actually, microsatellite maker has been used to indentify homozygosity of mitotic gynogenetic diploids in African catfish (Galbusera et al., 2000), European sea bass (Bertotto et al., 2005) and channel catfish (Waldbieser et al., 2010), and that of homozygous clones in Nile tilapia (Ezaz et al., 2004) as well. But for ascertaining the clonal status of heterozygous clone using microsatellite marker, similar studies on other fish species have not been reported.

The locus away from centromere with 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). Twenty microsatellite markers are found on sixteen of the total twenty-four linkage groups, therefore, representing an approximately genome-wide sampling. Of the 20 markers, *Poli9-8TUF*, *Po13* and *Poli12MHFS* are located on the fifteenth linkage group, *Poli18TUF* and *Poli13TUF* are located on the third linkage group, *Poli123TUF* and *Poli139TUF* are located on the twentieth linkage group, and one marker is located on each of the other thirteen linkage groups. These markers were identified to be polymorphic and of high marker-centromere recombination proportion in parent population, indicating that the result of genetic confirmation using microsatellite marker is reliable for heterozygous clones.

For Japanese flounder in China, we success-fully produced heterozygous clones by crossing between mitotic gynogenetic diploids. As good material for fish breeding and commercial fish culture, heterozygous clones of Japanese flounder in Japan were also generated by crossing between different clones (Hara et al., 1993; Yamamoto, 1999). Mitotic gynogenetic diploids produced by gynogenesis theoretically should be all-female. But in the present study, a minor proportion (4 of 152) of homozygous gynogenetic diploid males occurred. The mechanism responsible for the rare occurrence of such homozygous male progeny is unknown at present. We put forward hypotheses that environmental factors might play a role in spontaneous inducing female-to-male sex reversal. Heterozygous clones will open up new exciting areas for selective breeding. For wide application of heterozygous clones to commercial breeding, however, some key technologies are still required to be solved, such as improvement of survival rate of mitotic gynogenetic diploids and heterozygous clones from genetic and farming environmental aspects.

ACKNOWLEDGEMENTS

The authors would like to thank Ligeng Yang, Lingling Xue and Xiufeng Jiang for their help during the experiments. This research was financially supported by the earmarked fund for

Table 2. Genotypic segregation and P value of χ^2 test in two control families ND-A and ND-B of normally fertilized diploids at 20 microsatellite markers.

Marker	Family	Parents		Progeny				Total	P value
		Female	Male	ac	ad	bc	bd		
Poli2TUF	ND-A	123/127 (ab)	94/129 (cd)	12 (7.5)	8(7.5)	5(7.5)	5 (7.5)	30	0.221
	ND-B	122/130 (ab)	104/156 (cd)	6 (7.5)	8(7.5)	9(7.5)	7 (7.5)	30	0.881
Poli9TUF	ND-A	158/170 (ab)	137/147 (cd)	9 (7.5)	5(7.5)	8(7.5)	8 (7.5)	30	0.753
	ND-B	156/166 (ab)	162/178 (cd)	4 (7.5)	9(7.5)	9(7.5)	8 (7.5)	30	0.881
Poli18TUF	ND-A	155/158 (ab)	150/164 (cd)	9 (7.5)	7(7.5)	6(7.5)	8 (7.5)	30	0.881
	ND-B	155/157 (ab)	153/172 (cd)	6 (7.5)	8(7.5)	7(7.5)	9 (7.5)	30	0.881
Poli23TUF	ND-A	126/144 (ab)	122/141 (cd)	4 (7.5)	10 (7.5)	10 (7.5)	6 (7.5)	30	0.308
	ND-B	97/142 (ab)	122/169 (cd)	8 (7.5)	10 (7.5)	6(7.5)	6 (7.5)	30	0.690
Poli101TUF	ND-A	145/157 (ab)	154/154 (cc)	16 (15)		14 (15)		30	0.715
	ND-B	154/154 (aa)	128/144 (cd)	13 (15)	17 (15)			30	0.465
Poli107TUF	ND-A	112/144 (ab)	136/140 (cd)	8 (7.5)	9(7.5)	5(7.5)	8 (7.5)	30	0.753
	ND-B	113/115 (ab)	109/109 (cc)	14 (15)		16 (15)		30	0.715
Poli123TUF	ND-A	118/156 (ab)	124/130 (cd)	4 (7.5)	9(7.5)	7(7.5)	10 (7.5)	30	0.424
	ND-B	142/158 (ab)	119/131 (cd)	8 (7.5)	8(7.5)	9(7.5)	5 (7.5)	30	0.753
Poli130TUF	ND-A	143/143 (aa)	138/172 (cd)	15 (15)	15 (15)			30	1.000
	ND-B	147/151 (ab)	161/161 (cc)	13 (15)		17 (15)		30	0.465
Poli139TUF	ND-A	141/158 (ab)	160/173 (cd)	6 (7.5)	9(7.5)	5(7.5)	10 (7.5)	30	0.519
	ND-B	146/167 (ab)	139/169 (cd)	9 (7.5)	7(7.5)	6(7.5)	8 (7.5)	30	0.881
Poli141TUF	ND-A	149/178 (ab)	126/144 (cd)	7 (7.5)	9(7.5)	6(7.5)	8 (7.5)	30	0.881
	ND-B	140/150 (ab)	152/154 (cd)	9 (7.5)	7(7.5)	8(7.5)	6 (7.5)	30	0.881
Poli9-8TUF	ND-A	146/151 (ab)	157/157 (cc)	12 (15)		18 (15)		30	0.273
	ND-B	144/144 (aa)	149/149 (cc)	30 (30)				30	1.000
Poli13TUF	ND-A	150/150 (aa)	178/178 (cc)	30 (30)				30	1.000
	ND-B	150/150 (aa)	164/173 (cd)	13 (15)	17 (15)			30	0.465
Poli193TUF	ND-A	81/138 (ab)	84/152 (cd)	6 (7.5)	5(7.5)	13 (7.5)	6 (7.5)	30	0.141
	ND-B	132/160 (ab)	128/166 (cd)	4 (7.5)	4(7.5)	11 (7.5)	11 (7.5)	30	0.088
Po13	ND-A	220/220 (aa)	219/229 (cd)	16 (15)	14 (15)			30	0.715
	ND-B	218/218 (aa)	216/216 (cc)	30 (30)				30	1.000
Poli16-79TUF	ND-A	150/152 (ab)	137/137 (cc)	18 (15)		12 (15)		30	0.273
	ND-B	152/173 (ab)	143/175 (cd)	6 (7.5)	8(7.5)	10 (7.5)	6 (7.5)	30	0.690
Poli18-55TUF	ND-A	61/114 (ab)	100/126 (cd)	11 (7.5)	6(7.5)	5(7.5)	8 (7.5)	30	0.424
	ND-B	61/117 (ab)	68/165 (cd)	10 (7.5)	7(7.5)	6(7.5)	7 (7.5)	30	0.753

Table 2. Contd

Poli39MHFS	ND-A	212/212 (aa)	184/188 (cd)	15 (15)	15 (15)			30	1.000
	ND-B	190/212 (ab)	187/209 (cd)	8 (7.5)	7 (7.5)	11 (7.5)	4 (7.5)	30	0.343
Poli174TUF	ND-A	139/154 (ab)	149/151 (cd)	8 (7.5)	5 (7.5)	9 (7.5)	8 (7.5)	30	0.753
	ND-B	135/156 (ab)	154/168 (cd)	12(7.5)	6 (7.5)	5 (7.5)	7 (7.5)	30	0.276
Poli182TUF	ND-A	137/137 (ab)	152/165 (cc)	13 (15)		17 (15)		30	0.465
	ND-B	133/155 (ab)	154/163 (cd)	12(7.5)	8 (7.5)	5 (7.5)	5 (7.5)	30	0.221
Po25A	ND-A	226/237 (ab)	227/243 (cd)	11(7.5)	10 (7.5)	4 (7.5)	5 (7.5)	30	0.177
	ND-B	224/232 (ab)	228/228 (cc)	16 (15)		14 (15)		30	0.715

Table 3. Genotyping results of 20 microsatellite markers in maternal parents and their progenies of Japanese flounder.

Marker	Mother-2075	Mother-9791	2075	9791	H1	Mother-8033	Mother-1381	8033	1381	H2
Poli2TUF	114/129	129/160	129/129	129/129	129/129	100/126	126/156	126/126	126/126	126/126
Poli9TUF	152/157	157/174	157/157	157/157	157/157	145/152	152/186	152/152	52/152	152/152
Poli18TUF	136/152	139/160	152/152	139/139	139/152	136/159	150/172	136/136	50/150	136/150
Poli23TUF	145/159	95/145	145/145	145/145	145/145	130/144	90/102	144/144	102/102	102/144
Poli101TUF	134/155	144/155	155/155	155/155	155/155	119/153	135/153	153/153	153/153	153/153
Poli107TUF	112/116	108/116	116/116	116/116	116/116	144/151	112/130	151/151	112/112	112/151
Poli123TUF	110/156	95/156	156/156	156/156	156/156	128/159	114/159	159/159	159/159	159/159
Poli130TUF	128/134	126/134	134/134	134/134	134/134	131/138	131/144	131/131	131/131	131/131
Poli139TUF	134/159	140/159	159/159	159/159	159/159	155/162	145/155	155/155	155/155	155/155
Poli141TUF	129/150	150/165	150/150	150/150	150/150	170/196	181/202	196/196	202/202	196/202
Poli9-8TUF	142/147	147/154	147/147	147/147	147/147	152/156	144/148	152/152	144/144	144/152
Poli13TUF	152/157	162/177	152/152	177/177	152/177	136/154	180/191	154/154	180/180	154/180
Poli193TUF	103/127	119/127	127/127	127/127	127/127	125/138	125/143	125/125	125/125	125/125
Po13	212/219	219/226	219/219	219/219	219/219	221/234	216/230	221/221	216/216	216/221
Poli16-79TUF	145/156	114/120	145/145	114/114	114/145	139/148	148/165	148/148	148/148	148/148
Poli18-55TUF	95/115	115/133	115/115	115/115	115/115	86/112	112/145	112/112	112/112	112/112
Poli39MHFS	204/211	211/217	211/211	211/211	211/211	196/214	188/194	214/214	188/188	188/214
Poli174TUF	134/140	155/164	134/134	155/155	134/155	137/142	142/166	142/142	142/142	142/142
Poli182TUF	156/178	163/178	178/178	178/178	178/178	114/120	152/175	120/120	175/175	120/175
Po25A	206/225	225/229	225/225	225/225	225/225	202/228	228/232	228/228	228/228	228/228

Modern Agro-industry Technology Research System (nycytx-50-G02) and Special Fund for Agro-scientific Research in the Public Interest (200903046) in China.

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