クロダイ精子を用いた高水温ショック法によるマダイ雌性発生2倍体の作出

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Gynogenetic Diploid Production in the Red Sea Bream Using UV-irradiated Sperm of Black Sea Bream and Heat Shock

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Gynogenetic diploid red sea bream *Pagrus major* were produced by fertilizing the eggs with black sea bream *Acanthopagrus schlegeli* sperm that had been genetically inactivated with ultraviolet (UV) rays, and then 3 min. after insemination, heat shocking the eggs at 35°C for 2.5 min. to retain the second polar body. This treatment gives a satisfactory survival rate of more than 70% beyond yolk sac resorption. An attempt to produce gynogenetic diploids by suppression of the first cleavage was unsuccessful.

There are at least 14 fixed differences at enzyme loci between red and black sea bream that can be detected by electrophoresis. No evidence was found of male contribution in two families of gynogenetic diploids, indicatiing a 100% success rate for induced gynogenesis.

In this study, no differences in the growth rates between control and gynogenetic offsprings was observed up to the age of 6 months. However, significant differences in the growth rate between the two families were found either in control or gynogenetic groups. These differences may be caused by maternal effects as well as genetic factors.

Red sea bream *Pagrus major* is one of the important species for commercial coastal fisheries in Japan. Although, various studies has been performed on specific aspects relating to red sea bream culture, few studies have been directed towards genetic improvements which will be determining factors in the future of red sea bream culture.

One of the genetic techniques which may assist in the improvement of fish breeding is gynogenesis (all maternal inheritance).[1,2] Gynogenesis has numerous potential applications, such as the production of inbred lines, all female populations and gene mapping.[3,4]

Gynogenesis is usually induced in fish by fertilizing eggs with sperm which has been irradiated with UV-ray.[5] To confirm that paternal genes are not transmitted, it is essential in induced gynogenesis to use foreign sperm in which female and male species have distinguishable alleles at one or more loci and the presence of any male contribution can be detected by electrophoresis.[3]

After fertilization with inactivated sperm, diploid gynogenetic individuals can be produced by treatments that either suppress the first cleavage or the second meiotic division. The first case results in 100% homozygosity, and the second case produces portions of heterozygosity, depending on the segregation of the second division (y).[4]

In freshwater fish, cold shock, heat shock and hydrostatic pressure have been used to induce gynogenetic diploid and polyploidy.[6,7,8] However, data on the optimum condition for inducing gynogenetic diploids in red sea bream by heat shock have not been reported.

In this paper we summarize the results of our experiments on the production of gynogenetic diploid red sea bream using UV-irradiated sperm of black sea bream and heat shock. Electroporetic banding pattern of red and black sea bream hybrids and gynogenetic individuals are shown, as well as growth rate and heterozygotes in induced gynogenetic offsprings.

Materials and Methods

Induction of Gynogenetic Diploids

All the procedures used in the collection of broodfish and eggs, sperm irradiation, fertilization and incubation of fertilized eggs before and after treatments were the same as described by Sugama *et al.*[9] In this study black sea bream sperm was

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inactivated by 3,000 erg/mm^2 of ultraviolet (UV) rays. Various heat shock temperatures (32.5, 35.0, 37.5 and 40.0°C) and durations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 min.) were examined. Fertilized eggs were incubated at 18°C and the shocks were applied 3 min. after insemination, to coincide with the second meiotic division and the extrusion of the second polar body in red sea bream eggs.[9] The experiments were repeated twice using different female broodfish. Shock treatments were also applied at the approximate time of the first cleavage. The first cleavage takes place about 55 to 60 min after fertilization under present conditions, as determined by microscopic observation. In the present experiments, the shocks were applied 1 min intervals from 50 to 60 min after fertilization at temperatures and durations mentioned above.

In one experimental series, a group of eggs was collected by hand stripping from a single female, and a sample of 800–1,000 eggs was used in each treatment. Three controls were made, *i.e.*, eggs fertilized with non-irradiated sperm of red and black sea bream (hybrids) and eggs fertilized with UV-irradiated sperm of black sea bream without heat shock (UV-C) for control of egg and sperm quality.

The hatching rate of normal larvae was the primary criterion for estimating the success of induced diploidization, with all percentages expressed in relation to the initial egg number.

In the mass production of gynogenetic diploids, the treatments were applied at the time found to be optimal in the previous experiments. In this case 1 male black sea bream, 1 male and 2 female red sea bream were used. Three controls were made as mentioned above. The number of eggs collected from both females are presented in Table 1. After collection of eggs and sperm all parental fish were killed for electrophoresis.

Three days after hatching approximately equal numbers of fry derived from each female were randomly collected and transferred from hatching nets to 2,000 *l* capacity of rearing tanks. Two families of normal controls as well as 2 families of gynogenetic diploid groups were mixed and then reared uniformly in the same tank and net cage ($3 \times 3 \times 2$ m). The two groups were reared separately under the same size of tank and net cage. The rearing conditions of temperature, salinity and feeding shcedule was the same for both groups with water temperatures and salinities ranging from 18.6–24.3°C and 29.8–33.4‰, respectively. The larval rearing techniques of Fukuhara[10] were followed. Larvae were fed on cultured zooplankton, *Brachionus plicatilis*, *Tigriopus japonicus* and nauplii of *Artemia salina*. Chopped meat of anchovies and commercial fish feed was added to the rearing tanks from 35 days after hatching.

Four hundred each of two months old fingerlings from both groups were teranferred and reared in net cages. The fish were fed at 7% of body weight per day with commercial feed pellet. The total fish weight in each group was measured at 15-day intervals and feeding levels and feed pellet size were readjusted for each group.

The offsprings derived from each female broodfish could be identified due to the presence of different alleles at *Est* locus in the two broodfish. Two-month old fingerlings and 6 months old fish were sampled to confirm the diploidy of gynogenetic individuals and to compare the growth rate between normal control and gynogenetic diploid groups as well as growth rate of fish derived from different females broodfish.

Estimation of Induction Efficiency

Starch gel electrophoresis was used to identify isozyme genotypes of parents and offsprings. A complete absence of paternal gene expression was the second criterion in estimating the success rate of induced gynogenesis. Muscle and liver tissues were assayed. For this purpose, the juveniles were reared for at least 2 months until they achieved adequate size (3 to 5 cm in body length) for easy removal of the liver. Electrophoresis techniques and allele designation followed the procedures outlined in Taniguchi and Okada.[11]

Results and Discussion

Determination of Optimum Shock Temperature and Duration

The variation of hatching rate and incidence of haploid syndrome for each treatment are shown in Fig. 1. These results were derived from female 1 only, as the survival pattern of female 2 was similar.

Red sea bream eggs fertilized with normal sperm of black sea bream (hybrid) showed normal viability and hatching rate was comparable to that of diploid controls of red sea bream.

The hatching rate of normal control and hybrids were 97.1% and 96.6% respectively, indicating good quality of eggs and sperm. Eggs fertilized with UV-irradiated sperm of black sea bream resulted in 100% hatching of abnormal larvae. Abnormal larvae showed short caudal

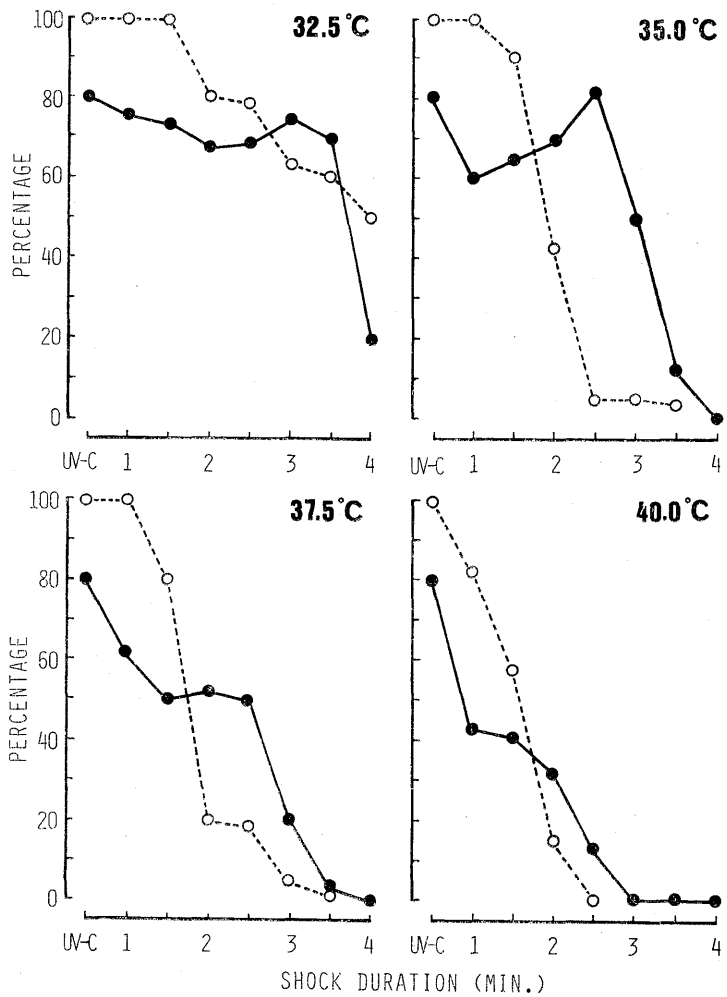

Fig. 1. The survival rates (solid circles) and the incidences of haploid syndrome (open circles) in hatched larvae of red sea bream eggs fertilized with UV-irradiated sperm of black sea bream and heat shocked at various temperatures and durations, begining 3 min. after insemination. UV-C is the eggs fertilized with UV-irradiated sperm without heat shock treatment.

vertebrae, curved tails and died within 3 hours after hatching. These abnormalities are known generally as the haploid syndrome. The non-viability of these individuals may indicate that the paternal DNA in the UV-irradiated sperm were indeed genetically inactivated.

In treated eggs, hatching rate and rate of normal larvae varied with the treatments. Heat shock (35–40°C) for longer than 4.0 min. caused 100% mortality. A high hatching rate (81.2%) and high rate of normal larvae (78.3%) were obtained in the shock treatment 35.0°C for 2.5 min applied 3 min. after insemination (Fig. 1).

Hatching rate and rate of normal larvae were very poor in the treatment applied at the approximate time of the first cleavage (data not shown). A hatching rate of more than 10% were found in the treatments applied at 35°C for 2.5 min beginning 50 and 55 min after fertilization, but 99.7 and 100% of hatched larvae were haploid and died several hours after hatching. Several combinations between shock temperatures and durations were attempted to suppress the first cleavage, but the survival rate of normal larvae was still very poor. This suggests that heat shock treatment may not be suitable for suppression of the first cleavage in red sea bream.

Mass Production of Gynogenetic Diploids

Two female broodfish were used in the mass pro-

Table 1. Survival rate of normal control (C-2N), red and black sea bream hybrids (R×B) and Gynogenetic diploids (G-2N) at 1 day and 3 days after hatching

Group	Female	Number of eggs	Survival at	
			1 day (%)	3 days (%)
C-2N	I	6,120	98.6	98.1
	II	6,840	97.9	96.3
R×B	I	4,320	96.5	95.0
	II	—	—	—
G-2N	I	7,560	80.2	74.2
	II	9,720	78.3	70.6

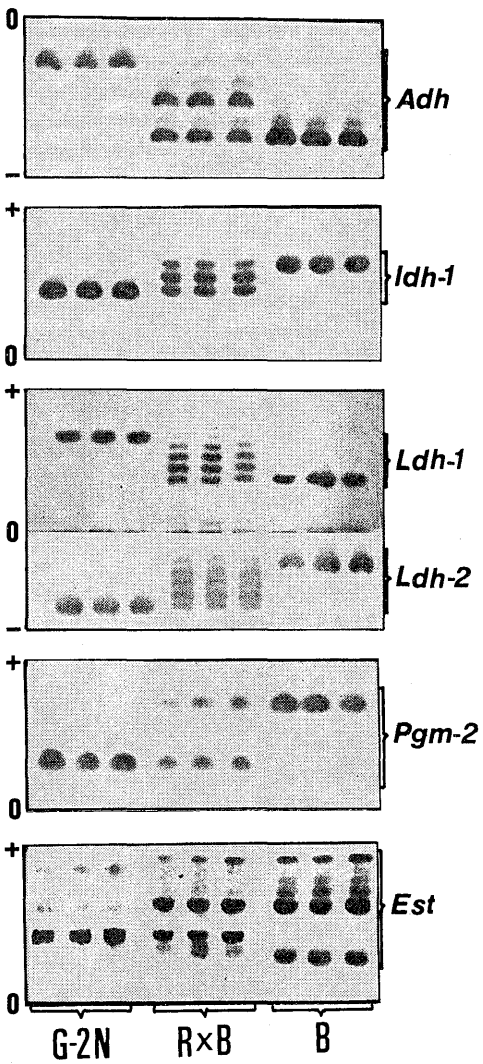

Fig. 2. Zymograms of *Adh*, *Idh-1*, *Ldh-1,2*, *Pgm-2* and *Est* loci, illustrating the banding patterns of red sea beram or gynogenetic diploid red sea bream (G-2N), red and black sea bream hybrids (R×B) and black sea bream (B).

duction of gynogenetic diploid offspring. A treatment was applied, 3 min. after insemination, of 35.0°C for 2.5 min. The number of eggs used from each female and the survival rates of normal hatched larvae at one and three days after hatching are presented in Table 1. The hatching rate of normal larvae did not greatly differ between females. The survival rate up to initial feeding (3 days after hatching) was more than 70% in the larvae from both females. All hatched larvae from eggs fertilized with UV-irradiated sperm (UV-C) without heat shocking were abnormal and died within 3 hours after hatching. Red and black sea bream hybrids showed normal viability and could grow to adult. The surviving fish from this experiment were raised and sampled at particular months to confirm the diploidy of gynogenetic individuals and recorded data on the growth rate will be explained below.

Efficiency of Gynogentic Diploid Induction

Electrophoretic analysis of parental species of red and black sea bream confirmed that, there are at least 14 fixed differences at enzyme loci between them *i.e.*, *Aat-2*, *Adh*, *Est*, *α Gpd-1*, *Gpi-2*, *Idh-1*, *Ldh-1,2*, *Mdh-2*, *Pgm-1,2*, *Sp-1,2* and *Sdh* loci. Allelic contributions form both parental species were observed in every hydrid at each of the loci showing parental allelic differences, confirming that these fish were not spontanious androgenetic or gynogenetic offsprings (Fig. 2). However, paternal alleles did not appear in any of the gynogenetic offsprings. Only the alleles which were present in the female were found in the gynogenetic offsprings, indicating a 100% success rate of induced gynogenesis. During UV-treatment, some sperm may receive insufficient irradiation,[3] and such cases may produce hyperdiploid and triploid hybrids. If donor sperm is used from different species, where different alleles are present in the two species, triploid hybryds could be distinguished from gynogenetic or hy-

brid individuals by differencies in relative intensity of maternal and paternal alleles as observed in triploid hybrid of brown trout × brook trout,[12] or chum salmon × coho salmon.[13]

In this study, no evidence was found of any male contribution in gynogenetic offspring, and this implies that the UV-treatment was effective in inactivation of the sperm DNA.

Distribution of Genotype in Control and Gynogenetic Diploid Offspring

The genotypes of parents, and segregation of offspring in the normal control and gynogenetic diploid, are given in Table 2. Control mating showed that polymorphism of each of the 3 loci (*Adh*, *Est* and *Pgm-1*) is controlled by codominant allele segregating in a Mendelian manner. The observed number of each genotype in offspring agreed with the numbers expected from the combination of parent genotypes.

Gynogenetic offspring possessed only the alleles of their mother (Table 2). The presence of two homozygous classes and heterozygotes of the maternal genotype confirmed that the second meiotic division of the eggs was retained. Tests for significant differences between the two homozygous classes in both families did not show any significant deviation from a 1:1 ratio, indicating that none of the homozygous classes had reduced viability.

The frequency of heterozygotes among loci is expected to vary according to the frequency of second division segregation (y).[3] The proportion of heterozygotes in the offspring derived from female heterozygous at *Adh*, *Est* and *Pgm-1* loci were 0.24, 0.82 and 0.57 respectively (Table 2). Compared to an earlier study on induced gynogenetic diploid of red sea bream by cold shock treatment,[9] there are no differences in the proportion of heterozygotes at *Adh* and *Est* loci, indicating that both heat and cold shock treatments provided the same amount of heterozygotes in gynogenetic diploid offspring.

The high proportion of heterozygosites for *Est* and *Pgm-1* loci after gynogenesis involving second meiotic division, demonstrates that this is not effective for producing a homozygous line in red sea bream; treatments suppressing the first cleavage may be promising as observed in zebra fish[1]; rainbow trout[6] and ayu.[8]

Growth Rate

Three days after hatching, approximatly 3,000 fry from each family were randomly collected from hatching nets and transferred to rearing tanks. The rearing methods as explained in materials and methods.

Two months old fingerlings of each group were counted, and the surviving fish in the control and gynogenetic groups were 576 and 585 fish respectively. Four hundred fish from each group were randomly selected and then transferred to net-cages. The remaining fish were used for electrophoresis and size measurement. At six months age, 200 fish from each group were kept for further rearing to assess gonad development, whereas the

Table 2. Distribution of genotype at the *Adh*, *Est* and *Pgm*-1 loci in control mating (C-2N) and Gynogenetic diploid (G-2N) offspring from homozygous and heterozygous females of red sea bream

Locus	Group	Family	Genotype of parents		Genotype of offspring	y*
			Female	Male		
Adh	C-2N	I	*CC*	*AA*	*AC*: 160	
		II	*BC*	*AA*	*AB*: 68, *AC*: 76	
	G-2N	I	*CC*	—	*CC*: 164	
		II	*BC*	—	*BB*: 51, *BC*: 31, *CC*: 48	0.24
Est	C-2N	I	*EE*	*EE*	*EE*: 160	
		II	*DG*	*EE*	*DE*: 70, *EG*: 74	
	G-2N	I	*EE*	—	*EE*: 164	
		II	*DG*	—	*DD*: 12, *DG*: 107, *GG*: 11	0.82
Pgm-1	C-2N	I	*AB*	*AA*	*AA*: 121, *AB*: 39	
		II	*AA*	*AA*	*AA*: 144	
	G-2N	I	*AB*	—	*AA*: 38, *AB*: 93, *BB*: 33	0.57
		II	*AA*	—	*AA*: 130	

* y values are the second division segregation rate estimated by counting the proportion of heterozygous individuals in G-2N offspring (s) derived from heterozygous female.

Table 3. Average fork length (FL) and body weight (BW) of normal control (C-2N) and Gynogenetic diploid (G-2N) red sea bream at 2 and 6 months of age

Age (month)	Family	C-2N		N	G-2N		N
		FL±SD (Cm)	BW±SD (g)		FL±SD (Cm)	BW±SD (g)	
2	I	3.66±0.36	1.09±0.29	98	3.57±0.82	1.03±0.75	112
	II	3.34±0.41	0.74±0.28	78	3.15±0.69	0.69±0.43	73
6	I	10.94±0.56	31.87±4.96	62	10.67±1.15	28.77±8.57	52
	II	10.25±0.59	25.50±5.15	66	9.84±0.84	22.75±6.04	57

N: number of fish examined

remaining fish were sampled for electrophoresis and size measurement.

The offspring derived from each female, although mixed, were readily identified because genotype differences were present in the two female broodfish at *Est* locus (Table 2).

The sizes and numbers of fish examined for 2- and 6-month old fish are shown in Table 3. The survival rates did not greatly differ between control and gynogenetic groups and there are no significant differences ($P>0.05$) in fork length (FL) and body weight (BW) between control and gynogenetic groups in either family. However, FL and BW between families within the control and gynogenetic groups were significantly different ($P<0.01$).

Suzuki *et al.*[14] reported a lower survival rate and growth rate in gynogenetic diploid loach than in controls. They found that these differences were mainly caused by a high level of abnormal fish in gynogenetic diploids. In the present study, the absence of significant differences between normal and gynogenetic groups was consistent with the normal morphology shown by the gynogenetic fish.

The offsprings derived from female I had an average weight significantly higher than those derived from female II both in normal control and gynogenetic groups (Table 3). These differences may be caused by maternal effects as well as genetic factors, as both families in each group were reared uniformly in the same tank and net-cage in order to eliminate enviromental factors. These finding agreed with Taniguchi *et al.*[15], who found growth rate differences between half-sibs family of red sea bream. In addition, the two females had genotype differences at *Est* locus (Table 2). Torrissen[16] obtained association between the genotype of trypsin like isozyme (TRP) and growth rate in Altantic salmon, and he reported that individuals with genotype TRP-2 (92/92) grew faster than other genotypes. A similar study in red sea bream, in which juveniles reared from fertilized eggs to 45 days of age showed significant differences in growth rate between esterase (*Est*) genotype, and *Est-HH* genotype act as a marker for fast growth rate.[17] In the present study, it is possible that red sea bream with *Est-EE* genotype grew faster than *Est-DG* genotype, due to differences in the structure of this enzyme probably causing different rates of digestion, which may reflect the growth rate of the fish. This data is not sufficient to prove this possibility bacause only two families were compared. Further study on the production of gynogenetic diploids using different genotypes of the *Est* locus are of great interest for clarification of the associasion between genotype and growth rate.

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References

1) G. Streisinger, C. Walker, N. Dower, D. Knauber, and F. Singer: *Nature*, **291**, 293–296 (1981).
2) A. Nagy, V. Csanyi, J. Bakos, and M. Bercsenyl: *Aquacultura Hungarica*, **4**, 7–16 (1984).
3) G. H. Thordgaard, F. W. Allendorf, and K. L. Knudsen: *Genetics*, **103**, 771–783 (1983).
4) F. W. Allendorf, J. E. Seeb, K. L. Knudsen, and G. H. Thordgaard: *J. Hered.*, **77**, 307–312 (1986).
5) H. Onozato and E. Yamaha: *Nippon Suisan Gakkaishi*, **49**, 693–699 (1983).
6) D. Chourrout: *Aquaculture*, **36**, 111–126 (1984).
7) N. Taniguchi, A. Kijima, T. Tamura, K. Takegami, and I. Yamasaki: *Aquaculture*, **57**, 321–328 (1986).
8) N. Taniguchi, S. Seki, and J. Fukai: *Nippon Suisan Gakkaishi*, **54**, 1483–1491 (1988).
9) K. Sugama, N. Taniguchi, and H. Nabeshima: in

"Proceeding The Second Asian Fisheries Forum" (ed. by R. Hirano and I. Hanyu), Asian Fisheries Society, Manila, Philippines, 1990, pp. 543–548.
10) O. Fukuhara: *Nippon Suisan Gakkaishi*, **51**, 731–743 (1985).
11) N. Taniguchi and Y. Okada: *Nippon Suisan Gakkaishi*, **46**, 437–443 (1980).
12) P. D. Scheerer, G. H. Thordgaard, and J. E. Seeb: *Trans. Am. Fish. Soc.*, **116**, 92–97 (1987).
13) J. E. Seeb, G. H. Thordgaard, and F. M. Utter: *Aquaculture*, **72**, 31–48 (1988).
14) R. Suzuki, T. Oshiro and T. Nakanishi: *Aquaculture*, **48**, 45–55 (1985).
15) N. Taniguchi, K. Azuma, and S. Umeda: *Nippon Suisan Gakkaishi*, **54**, 553–557 (1988).
16) K. R. Torrinsen: *Aquaculture*, **62**, 1–10 (1987).
17) N. Taniguchi, R. Hamada, and H. Fujiwara: *Nippon Suisan Gakkaishi*, **47**, 731–734 (1981).

Nippon Suisan Gakkaishi : Formerly *Bull. Japan. Soc. Sci. Fish.*