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ORIGINAL ARTICLE

Artificial Gynogenesis and Sex Determination in Half-Smooth Tongue Sole (*Cynoglossus semilaevis*)

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Abstract Half-smooth tongue sole (*Cynoglossus semilaevis*) is an important cultured marine fish as well as a promising model fish for the study of sex determination mechanisms. In the present study, a protocol for artificial gynogenesis of half-smooth tongue sole was developed in order to identify the sex determination mechanism and to generate all-female stock. The optimal UV-irradiation dose for genetically inactivating sea perch spermatozoa was determined to be ≥30 mJ/cm^2. The optimal initiation time for cold shock of gynogenetic embryos was determined to be 5 min after fertilization, while the optimal temperature and treatment duration were determined to be 20–25 min at 5°C. Chromosomes from common diploids, gynogenetic haploids, and diploids were analyzed. WW chromosomes were discovered in some of the gynogenetic diploids. The microsatellite marker was applied to analyze gynogenetic diploid fry. Among the 30 gynogenetic diploid fry, 11 fry contained only one allele, while 19 contained two alleles, which had the same genotype as their mother. The female-specific DNA marker was observed in four individuals out of ten gynogenetic diploid fry. Ploidy analysis of 20 putative gynogenetic fry showed them all to be diploid. Thus, a protocol for the induction of artificial gynogenesis has been developed for the first time in half smooth tongue sole, and the sex determination mechanism in the tongue sole was determined to be female heterogametic with the ZW chromosome.

Keywords Half-smooth tongue sole · *Cynoglossus semilaevis* · Artificial gynogenesis · Sex determination mechanism · WW super female

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Introduction

Artificial gynogenesis is an important approach to obtaining all-female stock in aquaculture and elaborating the mechanisms of sex determination in fish. Currently, artificial gynogenesis techniques have been developed in some fish species (Devlin and Nagahama 2002; Piferrer et al. 2004; Luckenbach et al. 2004; Tvedt et al. 2006). The monosex stocks, which have been produced by means of gynogenesis, include the common carp (*Cyprinus carpio*) (Komen et al. 1988), hirame (*Paralichthys olivaceus*) (Yamamoto 1999), European sea bass (*Dicentrarchus labrax* L.) (Peruzzi and Chatain 2003), and Atlantic halibut (Tvedt et al. 2006). Furthermore, sex determination mechanisms were investigated by sex-typing gynogenetic fry in certain fish species (Pongthana et al. 1995; Tabata 1991; Tvedt et al. 2006). However, sex determination is of the male heterogametic (XY), female homogametic(XX) type in these fish species. There are no reports in the literature on artificial gynogenesis and sex determination mechanisms in female heterogametic marine fish (i.e., ZW female and ZZ male).

Molecular marker technique has been demonstrated to be an effective tool for the analysis of genetic diversity, the identification of genetic sex and sex control (Liu and Cordes 2004; Liu et al. 2005; Chen et al. 2007). Few

reports on sex-specific molecular markers are available in teleosts, even though sex-specific molecular markers have been cloned in many mammals. Male-specific DNA markers have been isolated and used for genetic sex identification in salmonids (Devlin et al. 2001; Devlin and Nagahama 2002). Also, male-specific RAPD markers were isolated from African catfish (*Clarias gariepinus*) (Kovacs et al. 2000). Recently, sex-related amplified fragment length polymorphism (AFLP) and microsatellite markers were identified in Nile Tilapia (*Oreochromis niloticus*) (Lee et al. 2003; Ezaz et al. 2004). Two male-specific AFLP markers were also identified in the three-spined stickleback, *Gasterosteus aculeatus L.*(Griffiths et al. 2000). However, these species are of XY-type sex determination. To date, there has not been any report on the application of female-specific DNA marker in the study of sex determination mechanism in fish species with the ZW heterogametic sex determination.

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a newly exploited, commercially important cultured marine fish in China. Females grow two to four times faster than males. The low growth rate of males weakens the quality of the fish, and thus leads to an overall reduction in production. To solve these problems, it is essential to obtain all-female stock with the help of artificial gynogenesis. In addition, the sole is a promising model for studying both sex chromosome evolution and sex determination mechanisms in teleosts. Therefore, artificial gynogenesis and development of all-female stocks in the tongue sole would be of significant benefit for both scientific research and aquaculture. Recently, certain studies on the differentiation of gonads, sex-specific molecular marker screening and chromosome analysis have been performed in the tongue sole (Deng et al. 2007; Chen et al. 2007; Zhou et al. 2005). Seven female-specific AFLP markers have been identified in tongue sole (Chen et al. 2007). In addition, polymorphic microsatellite markers have been isolated from the genomic DNA of the species and used to evaluate the genetic diversity of *Cynoglossus semilaevis* (Liao et al. 2007). The availability of polymorphic microsatellite markers provides an important tool for identifying gynogenetic diploid fry.

In the present study, a protocol for the induction of diploid gynogenetic tongue sole fry with heterogeneous sperm was developed. Diploid gynogenetic fry were obtained in which some of the gynogenetic diploids consisted of two W chromosomes. Female-specific AFLP markers and microsatellite markers were used to identify the gynogenetic diploid fry.

Materials and Methods

Broodstock and Gamete Collection Six hundred parental half-smooth tongue sole were collected from two commercial hatcheries (Laizhou Mingbo Aquatic at Laizhou City and Marine 863 High-tech Base at Haiyang City, China), and one third were females with an average weight of 1.5–2.5 kg, while the average weight of males was 200–500 g. They began to spawn under natural conditions or artificial induction after sexual maturity by reproductive manipulation. Artificial induction of spawning was carried out as described (Yang JF and Chen SL, unpublished), and unfertilized eggs were collected by pressing the abdomen and maintained in dry beakers. Cryopreserved sperm of sea perch (*Lateolabrax maculates*), olive flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*) and summer flounder (*Paralichthys dentatus*) were used for gynogenesis induction in preliminary experiments, the results showed that sea perch sperm was the most effective. Thus, in the present study, sea perch frozen sperm were selected for developing the gynogenesis technique for half-smooth tongue sole. Sea perch sperm were cryopreserved and thawed as described (Ji et al. 2004; Chen 2007).

Feasibility Analysis of Artificial Gynogenesis Using Heterogeneous Frozen Sperm Frozen-thawed sea perch sperm with motility of 60–80% were used. The sperm were divided into two groups, one group was irradiated in a Hoefer UV crosslinker (Amersham biosciences) for genetic inactivation, and the other not irradiated but used as normal sperm. One hundred microliters of thawed milt was diluted in 1 mL modified plaice Ringer solution (MPRS) (Ji et al. 2004; Chen 2007), and then spread in a 9-cm Petri dish for irradiation. The doses of UV irradiation were 50–90 mJ/cm^2 in order to achieve sperm motility at a level of 30% after irradiation.

The unfertilized eggs were separated into two groups, 1 and 2. The eggs in group 1 were fertilized with sea perch sperm. After fertilization, the eggs in group 1 were also divided into two groups, A and B. Group A was the hybridized-group. Group B received a 20 min-cold shock at 5 min after fertilization, and this group was the hybridized-shocked group. The unfertilized eggs in group 2 were artificially fertilized with irradiated sea perch sperm, and then separated into two groups: C and D. Group C was the irradiated-hybridized group. Group D received a 20-min cold shock at 5 min after fertilization, and this group was the irradiated-shocked group.

After the respective treatments, the four groups were incubated in seawater at a temperature of 22–23°C. The survival rates of embryos and fry were determined at 12 h postfertilization (gastrula stage), 24 hpf (somite stage), 36 hpf (hatching stage), and 48 hpf (1 day after hatching).

Ploidy Determination in Tongue Sole Embryo and Fry Chromosome ploidy of embryos and fry of tongue sole was determined using flow cytometry (Partec, Germany). In brief, 200 μL phosphate-buffered saline (PBS) (Ph 7.4) was

added to 1.5-mL Eppendorf tubes containing one gastrula embryo or fry for washing. After the addition of 800 μL one-step 4,6-diamidino-2-phenylindole (DAPI) staining solution, the gastrula embryo was ground with a glass grinding stick to homogenize the tissues. Then the homogenates were filtered through a 50-μm pore nylon filter into a plastic tube. After staining for 5 min at room temperature, nuclear suspensions were subjected to flow cytometry for measurement of the relative nuclear DNA content. In each sample, 6,000–20,000 nuclei were analyzed. The distribution of fluorescence intensities after flow cytometric analyses are usually given as the channel number. For ploidy screening, the scale was calibrated with a reference (a mature female half-smooth tongue-sole blood sample). The flow cytometer was adjusted in order that the dominant peak of the reference was set at channel 25. This setting was kept constant and the other samples were characterized by the relative positions of their dominant peaks.

Screening for the Optimal UV-Irradiation Dose of Sea Perch Sperm Thawed semen was diluted at a ratio of 1:9 in MPRS. Then, 1 mL of diluted semen was spread in a glass Petri dish (9 cm diameter) and irradiated under UV light. Eight different UV doses (0, 2, 5, 10, 15, 20, 30, and 50 mJ cm^{-2}) were tested for their efficacy to yield gynogenetic haploids upon activation of eggs with UV-irradiated sperm. After irradiation, 0.3 mL of irradiated semen was divided into three equal volumes and added to three replicate batches of eggs, mixed, and then immediately activated with 10 mL of seawater at 23°C. When the fertilized eggs developed to the gastrula stage, the ploidy level of 20 embryos from each replicate was measured as described above using flow cytometry. Twelve hours after fertilization, the fertilization rate was calculated by examining 100–150 embryos at the gastrula stage. The experiment was replicated three times.

Screening for the Optimal Initiation Time for Cold Shock Cold shock treatment was used for chromosome diploidization by retention of the second polar body. The gynogenetic eggs were immerged in seawater at a temperature of 3–6°C at different time points after fertilization (2, 3, 4, 5, 6, 7, 8, 9 min) for 20–30 min. After cold shock, the eggs were transferred into 23°C seawater for incubation. The gynogenetic diploid rate was determined with flow cytometry when gynogenetic fry hatched. The experiment was replicated three times.

Screening for the Optimal Cold Shock Temperature and Duration The cold shock temperature and duration for chromosome diploidization were examined. Eggs fertilized with UV-irradiated sperm were grouped into six temperature gradients: 2°C, 3°C, 4°C, 5°C, 6°C, and 8°C. Cold shock was carried out at each gradient for 15, 20, 25, 30, 35, and 60 min. Thus, there were 36 groups in total. After cold shock, the eggs were transferred into 23°C seawater for incubation. The gynogenetic diploid rate was determined when gynogenetic fry hatched. The experiment was replicated three times.

Chromosome Preparation of Tongue Sole Chromosome preparation of tongue sole embryos and fry was performed as follows. In brief, the embryos at tailbud stage were treated in 0.02% colchicines at room temperature for 2 h, and then transferred into 0.075 mol/L KCl solution and held for 30 min. Fry of 3–6 cm were incubated in seawater containing 0.02% colchicines at room temperature for 6 h. The fin of fry was sheared, transferred into 0.0375 mol/L KCl solution and incubated for 30 min. The embryos and fin were fixed in fresh Carnoy's fluid (methanol: glacial acetic acid= 3:1) three times, and each fixation lasted for 20 min. The fin was taken out and put into 50% glacial acetic acid, then lacerated with cuspidal forceps to dissociate the cells. After being thermally dripped, it was stained in 10% Giemsa for 20–30 min, and finally observed and photographed under ×1,000 light microscopy (Nikon Eclipse 80i).

Gynogenetic diploid embryos of half-smooth tongue sole were induced by UV-irradiated sea perch sperm and treated with cold shock for chromosome diploidization. Haploid embryos were induced by UV-irradiated sea perch sperm without cold shock. Normal diploid embryos were collected from natural sole spawning ponds.

Extraction of Genomic DNA For DNA extraction, the fry or fins fixed in anhydrous ethanol were placed in 1.5-mL centrifuge tubes. The minced tissues were homogenized in 400 μL Tris-NaCI-EDTA-SDS (TNES) lysis buffer (10 mM Tris–HCl, pH 7.5; 400 μM NaCl; 100 mM EDTA; 0.6% SDS, and freshly added 10 μL proteinase K, 10 mg/mL), then the homogenate was lysed at 55°C. After that, 140 μL saturated NaCl solution was added, and the mixture was centrifuged at 12,000 rpm for 30 min at 4°C. The DNA was precipitated by two volumes of ethanol, which had been refrigerated at −20°C. Then the DNA pellets were washed twice with 70% ethanol, dried and dissolved in 50 μL TE buffer (10 mM Tris–HCl, pH 8.0, 10 mM EDTA).

Identification of Gynogenetic Fry with Microsatellite Markers Diploid gynogenetic fry of half-smooth tongue sole were confirmed by microsatellite analysis. Microsatellite markers of half-smooth tongue sole and sea perch were isolated and characterized as described (Liao et al. 2007; Shao et al. 2008). Microsatellite analysis of the gynogenetic fry was performed using microsatellite markers from the sea perch and tongue sole. Meanwhile, microsatellite analysis

was performed in eight individual adult sea perch randomly sampled from wild captives using tongue sole markers.

Genetic Sex Identification of Gynogenetic Fry Genome DNA extraction from 5-day-old gynogenetic fry and normal diploid fry of half-smooth tongue sole was performed as described above. Genetic sex identification of gynogenetic and normal tongue sole fry was performed using female-specific AFLP marker as described (Chen et al. 2007). A pair of female-specific polymerase chain reaction (PCR) primers (CseF382N1: ATTCACTGACCCCTGAGAGC, CseF382C1: AACAACTCACACACGACAAATG) was used for PCR amplification. With this pair of primers, female individuals produced a female-specific DNA band of 350 bp, but male individuals did not.

Data Analysis Analysis of variance (ANOVA) was used to determine significant differences between groups using SPSS software. When differences were significant, Turkey was used for comparison. Values were taken to be significant when $p<0.05$.

Results

Feasibility of Half-Smooth Tongue Sole Gynogenesis Induction by Sea Perch Sperm The survival of hybrids between sea perch sperm and tongue sole eggs was monitored along with the effects of sperm irradiation and cold shock treatment on the survival rate of gynogenetic embryos and fry (Fig. 1). UV irradiation of sperm had a subtle effect on developing rate of half-smooth tongue sole embryos (12 hpf), and the decrease of embryo developing rate is not obvious. The hybridized group and irradiated-hybridized group both exhibited a high survival rate 24 h after fertilization, but both the hybridized group and hybridized-shocked group mostly died before the hatching stage (36 hpf). It was also observed that hybridized embryos, whether shocked or not, always developed aberrantly with heavy malformation. The irradiated-hybridized group could survive to the hatching stage (36 hpf), but the fry were not obtained or died soon after they were hatched (48 hpf). Only when the eggs were fertilized with irradiated sperm and then were cold-shocked did 2.5% of the embryos develop into fry and survive after 48 hpf, which is to say, only gynogenetic diploid fry could be hatched and survive.

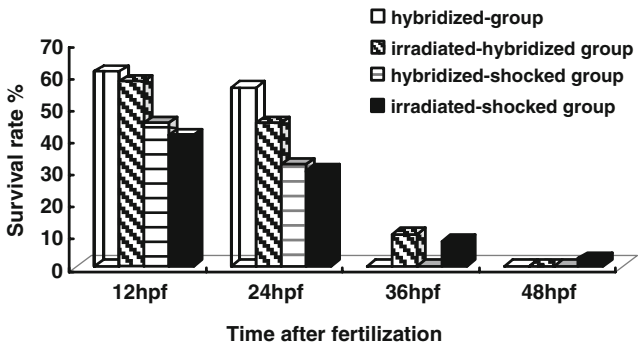

Fig. 1 Effect of sperm irradiation and cold shock treatment on the survival rate of gynogenetic embryos and fry

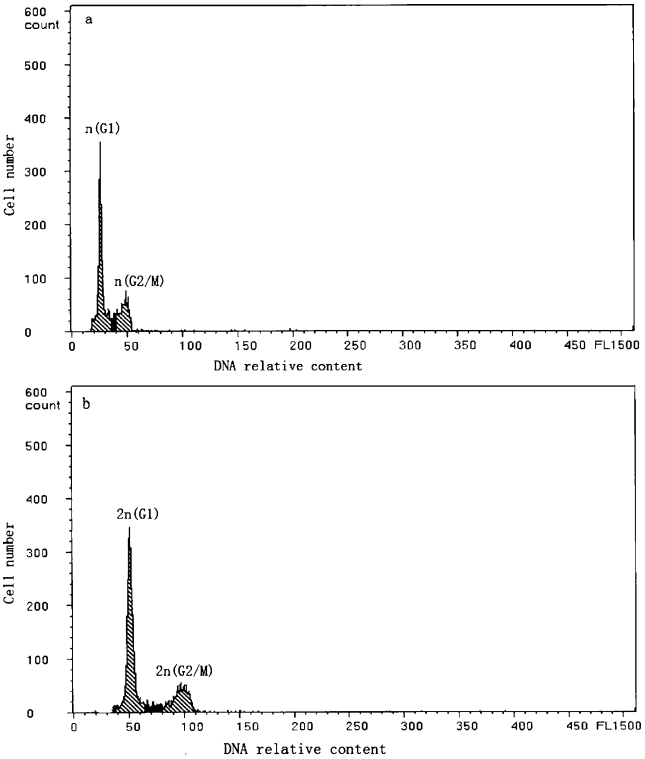

Fig. 2 Histogram of the DNA relative content of haploid (**a**) and diploid (**b**) embryos

Screening for the UV-Irradiation Dose of Sea Perch Sperm The ploidy analyzer can identify the chromosomal ploidy state of tongue sole embryo (Fig. 2). A histogram of the DNA relative content of one haploid embryo is shown in Fig. 2a. The dominant peak at channel 25 indicates that most of cells are in the G1 phase. There is a minor peak at channel 50 representing cells in the G2/M phase. A histogram of the DNA relative content of one diploid embryo is shown in Fig. 2b. There is a dominant peak of cells in the G1 phase around channel 50 and a minor peak of cells in the G2/M phase around channel 100. The dominant peak reflects the ploidy level of the sample. The UV-irradiation dose for sperm inactivation had a significant effect on the ploidy level of embryos ($P<0.05$) (Table 1). When the UV dose was lower than 5 mJ/cm^2, 100% embryos were diploid. When the UV dose was ≥30 mJ/cm^2, 100% embryos were haploid. However, there was not any

Table 1 Effect of ultraviolet dosage on the fertilization rate of sperm and the ploidy level of fertilized eggs

Dosage (mJ/cm^2)	Fertilization rate	Ratio of haploid embryos	Ratio of diploid embryos
0	79.0±6.2%[a]	0.0±0.0%[a]	100.0±0.0%[a]
2	78.6±4.5%[a]	0.0±0.0%[a]	100.0±0.0%[a]
5	77.2±7.8%[a]	0.0±0.0%[a]	100.0±0.0%[a]
10	77.5±10.3%[a]	10.0±8.6%[b]	90.0±8.6%[b]
15	77.2±7.6%[a]	42.1±10.5%[c]	57.8±10.5%[c]
20	72.7±9.6%[a]	88.8±14.3%[d]	11.1±14.3%[d]
30	73.2±4.5%[a]	100.0±0.0%[e]	0.0±0.0%[e]
50	72.4±9.0%[a]	100.0±0.0%[e]	0.0±0.0%[e]

The data with different letters in superscript are significantly different.

significant difference in the fertilization rate among the various UV-irradiation dose groups ($p>0.05$).

Determination of the Initiation Time for Cold Shock The relative induction rate of gynogenetic diploids is shown in Fig. 3. The effects of the time of initiation of cold shock on the gynogenetic diploid induction rate of half-smooth tongue sole are shown in Fig. 3A. Gynogenetic diploids could be induced if gynogenetic eggs received cold shock treatment 3–6 min after fertilization. However, the gynogenetic diploid induction rate at 5 min after fertilization was significantly higher than the other time points ($P<0.05$).

Screening of the Cold Shock Temperature for Chromosome Diploidization Effects of cold shock temperature on the gynogenetic diploid induction rate of the sole is shown in Fig. 3B. Gynogenetic diploids could be produced by cold shock induction in the range of 3–7°C, and the induction rate was the highest at 5°C ($P<0.05$).

Screening of the Optimum Cold Shock Duration for Chromosome Diploidization Effects of cold shock duration on the sole gynogenesis were examined. The gynogenetic diploid induction rates for different periods of cold shock duration are shown in Fig. 3C. Gynogenetic diploid fry could be produced by cold shock treatment when the period of cold shock duration was in the range of 15–30 min. However, the induction rate in the 20 and 25 min groups was significantly higher than the others ($P<0.05$).

Chromosome Analysis of Gynogenetic Half-Smooth Tongue Sole The chromosome number of the sea perch is $2n=48$ and the nuclear type is 48 t, while the chromosome number of the sole is $2n=42$, and the nuclear type is 42 t (Fig. 4A). There is an obvious difference in chromosome number between sea perch and half-smooth tongue sole, so chromosome analysis can be applied to substantiate that these gynogenetic fry have developed from gynogenesis. It was observed that the chromosome number of the gynogenetic haploid was 21 (Fig. 4B), and the chromosome number of gynogenetic diploid was 42 (Fig. 4C), which was identical with normal half-smooth tongue sole. In addition, two huge WW chromosomes were observed in some of the gynogenetic embryos (Fig. 4D), implying that these individuals were WW super-female individuals induced by gynogenesis. The availability of the WW super-female individuals in gynogenetic diploids demonstrated that the tongue sole is female heterogametic.

Microsatellite Analysis of Gynogenetic Fry Microsatellite analysis in eight individual sea perch using tongue sole microsatellite markers demonstrated that there were not any

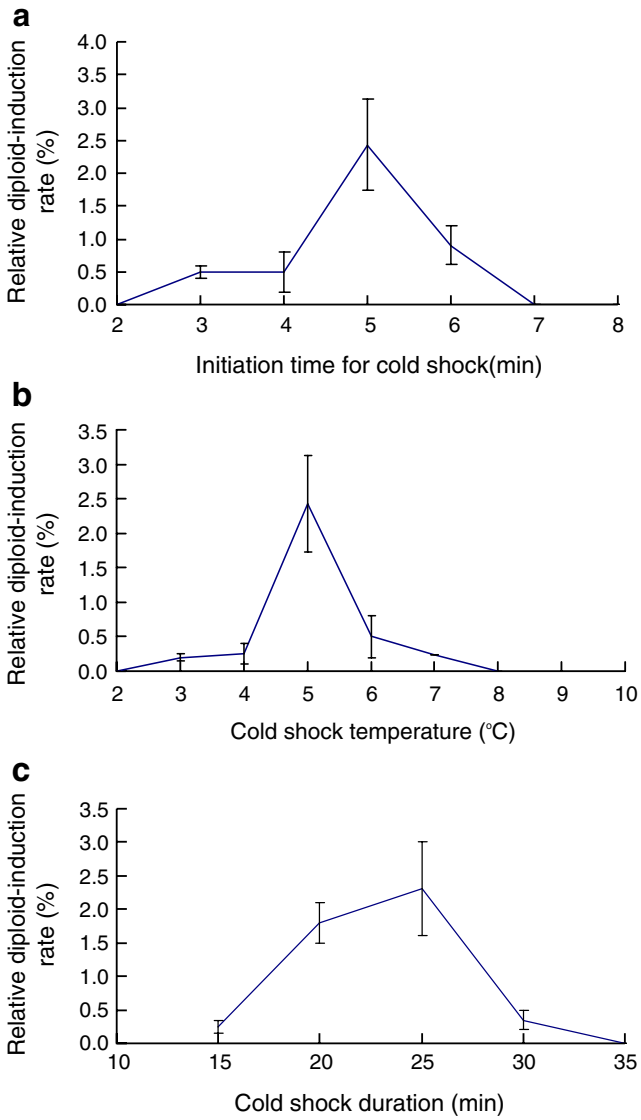

Fig. 3 Effects of the initiation time of cold shock (**A**), cold shock temperature (**B**), and duration (**C**) on the gynogenesis induction rate in half-smooth tongue sole

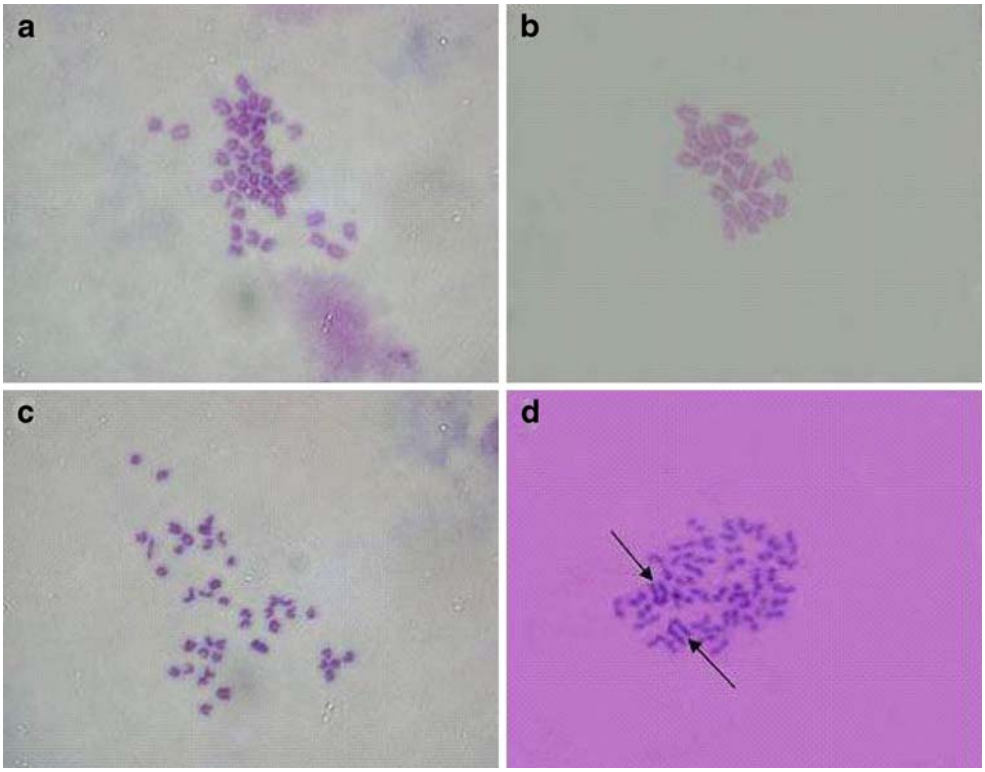

Fig. 4 Chromosome analysis of normal embryos and gynogenetic embryos. (*A*) Normal male tongue sole; (*B*) Gynogenetic haploids; (*C*) Gynogenetic diploids, showing a ZZ individual; (D) gynogenetic diploids, showing a WW individual, two huge WW chromosomes were observed (*arrowhead*)

amplification products (data not shown). The 30 gynogenetic fry were used for analysis using microsatellite markers of sea perch and tongue sole. No amplification products were detected in the 30 gynogenetic individuals and the maternal parent using eight sea perch microsatellite markers (*Lama04, 07, 10, 18, 21, 23, 24* and *28*) (data not shown). The SSR marker Cyse17 from the tongue sole analysis showed that 11 of the 30 gynogenetic fry have one allele of the same size as one of the two alleles in the maternal parent, and 19 of the 30 ones have two alleles with the same size as that of the two alleles in the maternal parent (Fig. 5).

Genetic Sex and Ploidy Level Identification of Half-Smooth Tongue Sole Gynogenetic Fry The genetic sex of half-smooth tongue sole gynogenetic fry was identified using the female-specific marker. Among ten 4-day-old heterogeneous gynogenetic fry, four fry contain the 350-bp female-specific DNA band. Among the 10 normal fry, four fry also contain the 350-bp female-specific DNA band (Fig. 6). This indicates approximately 40% of the heterogeneous gynogenetic fry were genetically female individuals. Ploidy analysis of 20 putative gynogenetic fry showed them all to be gynogenetic diploid, and no triploids were observed.

Application of the Half-smooth Tongue Sole Gynogenesis Technique According to the above-described established procedures for half-smooth tongue sole gynogenesis, experiments of gynogenesis induced by heterogeneous sperm have been performed more than 20 times, with ten experiments achieving success, and over 6,600 half-smooth tongue sole gynogenetic fry were produced. The results of artificial gynogenetic induction in half-smooth tongue sole are summarized in Table 2. The gynogenetic diploid induction rate was approximately 0.1–2.8%.

Discussion

Gynogenesis was considered to be a powerful approach of producing all-female stock, studying sex determination mechanisms, and developing pure lineages in fish. Artificial gynogenesis has been reported in many freshwater fish and a few marine fish species, and gynogenetic fry have been produced (Devlin and Nagahama 2002; Castro et al. 2003; Tvedt et al. 2006). Nevertheless, all fish species in which gynogenesis has been induced to date have the XY sex determination mechanism, namely, males are XY heterogametic

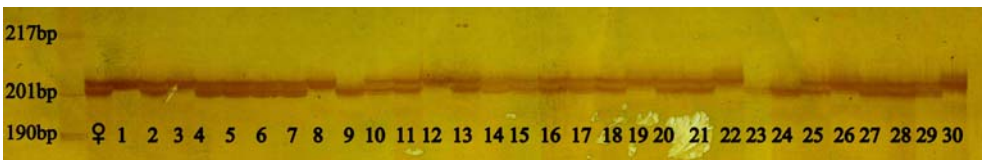

Fig. 5 Microsatellite analysis of half-smooth tongue sole gynogenetic fry. ♀: Female parent; 1–30: gynogenetic fry at 30 days old

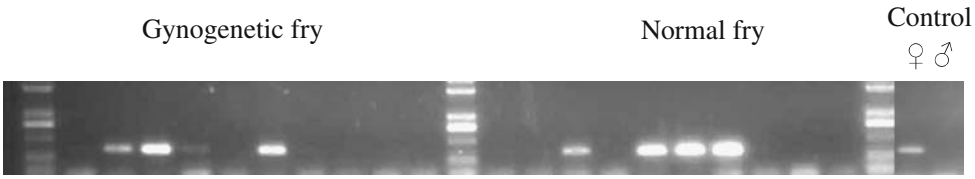

Fig. 6 Genetic sex identification of half-smooth tongue sole gynogenetic fry

and females are XX homogametic. Females produce only one kind of eggs (X-eggs), but males produce two kinds of sperm (X-sperm and Y-sperm). However, research on the artificial gynogenesis in fish with ZW sex determination has rarely been reported. To date, no report is available on gynogenesis in the half-smooth tongue sole.

In most gynogenesis studies, fresh homogeneous sperm were used to activate eggs to develop. Gynogenetic diploids have not proven easy to distinguish from normal diploids when homogeneous sperm were used if the sperm were not completely inactivated by UV light. In the present study, cryopreserved sperm from various marine fish such as turbot, olive flounder, summer flounder, oriental red-globefish, sea perch, and starry flounder were examined for effectiveness in inducing gynogenesis in tongue sole (data not shown), and ultimately sea perch sperm was found to be the most effective. Gynogenetic diploid fry were successfully obtained by inducing half-smooth tongue sole eggs with frozen sea perch sperm. Since the sea perch has 48 chromosomes (Chen et al. 2003) and the half-smooth tongue sole has 42, it is straightforward to identify gynogenetic fry through chromosome analysis. Heterogeneous sperm, especially those with a different chromosome number, do not truly fertilize eggs for producing hybrids, but rather, they just trigger the eggs. Therefore, gynogenetic fry induced by heterogeneous sperm are readily identified with high precision. In addition, the possibility that normally fertilized fry were produced by incomplete irradiation of homologous sperm was eliminated. Therefore, using heterogeneous sperm with a different chromosome number for gynogenetic induction has become a new method to study gynogenesis in fish (Váradi et al. 1999; Luckenbach et al. 2004). Furthermore, the sea perch sperm applied in this research was cryopreserved for more than 1 year, so the successful application of heterogeneous frozen sperm in half-smooth tongue sole gynogenesis not only widens the application range of frozen sperm, but also creates a new approach to sex control in fish. To date, there has been no other report on obtaining gynogenetic diploid fry induced with long-term cryopreserved fish sperm. However, it is difficult to determine the appropriate UV dose for inactivating sperm because the hybridized larvae fertilized with sea perch sperm are also abnormal and difficult to differentiate from haploid larvae. In the present study, a flow cytometer was used to determine the ploidy of gynogenetic embryos and fry in order to optimize the UV dose to inactivate sea perch sperm, which led to a precise discrimination of haploids and diploids. Flow cytometry is a useful application in gynogenesis induction and sex control in fish.

Table 2 Summary of artificial gynogenetic results in half-smooth tongue sole induced with sea perch sperm during 2006–2007

Exp. No.	Relative diploid-induction rate of gynogenesis (%)	Approximate No. of Gynogenetic diploid fry obtained
1	0.9	300
2	1.3	400
3	2.8	500
4	0.6	200
5	2.5	2,000
6	2.5	1,000
7	1	1,000
8	0.8	120
9	0.1	1,000
10	0.5	100

In the present study, the induction rate of gynogenetic diploids is low partly because the fertilization rate of heterogeneous sperm was usually lower than that of homologous sperm. In the present study, the diploid induction rate of gynogenetic tongue sole was only 0.1–2.8%, which was below the induction rate in other marine fish. For example, Yamamoto (1999) obtained the diploid gynogenetic rate of about 34% in flounder when induced by homologous sperm. Piferrer et al. (2004) used homologous sperm to induce turbot gynogenesis, and a 25-min cold shock in −1 to 0°C seawater was carried out 6.5 min after the eggs were fertilized with UV-irradiated sperm, and 10% gynogenetic diploid fry were obtained. However, similar to our results, a low gynogenetic diploid induction rate was observed in Atlantic Halibut (Tvedt et al. 2006). In addition, Luckenbach et al. (2004) discovered that the gynogenetic diploid rate of *Paralichthys lethostigma* induced by homologous sperm was 2.1%, while the rate reached 1.6–11.4% when heterogeneous sperm from *Mugil cephalus* were used for gynogenetic induction.

In the present study, two huge WW chromosomes were observed in gynogenetic half-smooth tongue sole, indicating that these WW individuals had developed from W chromosome duplication through gynogenesis. In addition, analysis of the female-specific marker showed approximately 40% of the gynogenetic fry contained the female-specific DNA marker, which strongly suggested that there are two types of eggs in half-smooth tongue sole. Therefore, there are also two types of gynogenetic fry. All of the above demonstrate

that the half-smooth tongue sole has the ZW sex determination mechanism and the female is heterogametic with Z and W chromosomes. The WW "super-female" genotypes discovered in gynogenetic diploid soles could be used to generate all-female populations if crossed to normal homoganetic males (the ZZ-type).

Microsatellite DNA marker technology is a powerful approach for the monitoring of genetic diversity and genetic linkage map construction (Liu and Cordes 2004). Microsatellite markers have also greatly facilitated the molecular identification of the gynogenetic offspring of fish, because of their PCR-based, codominant Mendel Ian inheritance pattern and highly polymorphic characteristics (Castro et al. 2003; Chen et al. 2005; Li et al. 2008). In the present study, gynogenetic tongue sole fry were confirmed using microsatellite analysis. As the sea perch is distantly related to the tongue sole, hybridization between the two species was lethal. Furthermore, microsatellite of tongue sole used for cross-species amplification in sea perch demonstrated that no target products were amplified and vice versa. Microsatellite analysis demonstrated that the gynogenetic offspring obtained in the study inherited exclusively maternal alleles, and thus it is unambiguously demonstrated that gynogenesis in the tongue sole was induced with 100% success. The result presented in Fig. 5 also showed that locus *Cyse17* has a high recombination rate with a frequency greater than 0.63 (19/30), and similar results were observed at other microsatellite loci (Liao X et al., unpublished data). The loci with high recombination rates may be present on telomeres (Li et al. 2008). High recombination rate levels have also been reported in other fish, such as the zebra fish (*Danio rerio*) (Kauffman et al. 1995), catfish (*Ictalurus punctatus*) (Liu et al. 1992), and the large yellow croaker (*Pseudosciaena crocea*) (Li et al. 2008).

In summary, an artificial gynogenesis technique in half-smooth tongue sole was developed and is here reported for the first time. Sea perch sperm was found to be effective in inducing gynogenesis in the tongue sole. Genetic inactivation conditions for sea perch sperm were developed. The optimum initiation time, temperature, and treatment duration of cold shock were determined. In addition, chromosome analysis, microsatellite analysis, and female-specific marker were applied to confirm gynogenesis in the gynogenetic diploid fry. The discovery of WW "super-female" individuals in gynogenetic diploid fry provides important evidence for a ZW sex determination mechanism in the tongue sole. These findings lay the foundation for sex control and the breeding of all-female stock in the half-smooth tongue sole.

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