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The formation and study of allogynogenesis *Hemibarbus maculatus Bleeker*

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ABSTRACT

Hemibarbus maculatus Bleeker (HM) is an economically important fish that is highly valued by consumers. Gynogenesis is an important breeding method in aquaculture that has been widely applied to many fish species. In this study, we adopted an improved method of gynogenesis that proceeds as follows: first, inactivated koi carp sperm were used to stimulate eggs of HM, and then the UV-inactivated koi carp (KOC) sperm and HM eggs were mixed and stirred for 20–30 s to activate the eggs. The activated eggs were placed in yellow mud at 0 °C for 20–22 min for cold shock treatment, and then placed in water at 4–6 °C, 8–10 °C, and 15–18 °C in order, each for 1–2 min. After incubation at 22–25 °C, gynogenetic *Hemibarbus maculatus* Bleeker (GHM) were obtained. Morphological measurements and blood cell comparisons showed that there were no significant differences between GHM and HM. At the molecular level, comparative analysis of microsatellites DNA indicated that one fragment of GHM came from the KOC. This study provides the first evidence that DNA fragments from male parents exist in GHM at the molecular level. This discovery has important implications for fish genetic breeding.

1. Introduction

Gynogenesis is a reproductive technology commonly used in artificial breeding. Artificial insemination usually involves the activation of an egg by heterologous sperm initially treated with ultraviolet light and then with cold or heat shock to double the number of chromosomes and thus produce all female offspring [1]. Fish are the most numerous group of vertebrates, and the life histories and reproductive modes of different fish species vary greatly. Therefore, the difficulty of gynogenesis depends on the fish species. In some fish such as crucian carp, the use of traditional gynogenetic methods can make it easier to obtain a large number of gynogenetic offspring. The development cycle of *Hemibarbus maculatus* Bleeker (HM) eggs is long, and gynogenesis is difficult; hence, there are no successful reports of gynogenesis in this species. HM lacks varieties with strong resistance to hypoxia, and thus the development of innovative technology is significant for the breeding of new species of HM. The gynogenetic offspring often display heterospermia, usually appearing in the form of tiny chromosomes, spots, or chromosome groups of heterologous sperm [2].

The microchromosomes of second-generation *Carassius auratus gibelio* were identified by Yi et al [3]. The sperm of *Megalobrama amblycephala* treated with ultraviolet light activated the eggs of female *Carassius auratus gibelio*, providing the evidence at the cellular level that genetic material from heterologous sperm has an impact on the offspring [3]. Peek AS al. found paternal genetic material in feminized albino *Oncorhynchus mykiss* activated by sperm from UV-treated wild-type pigmented *Salvelinus fontinalis* [4]. However, there is little knowledge concerning the role of heterologous sperm in gynogenesis.

Taxonomically, HM belongs to the order Cypriniformes, Cyprinidae, and Gryoideae [5]. It is a common small-to medium-sized freshwater fish [6]. The fish are popular with consumers for their taste and appearance. The fish live in the middle and lower layers of water bodies and have a variety of feeding habits. They primarily eat the larvaes of aquatic insects as well as benthic animals such as snails, clams, small fish and shrimps [7]. However, owing to changes in the natural environment, overfishing, and other reasons, the natural resources of HM have been sharply reduced, and the fish has been difficult to find in the market in recent years. In this study, the eggs of HM activated by UV-treated sperm of *Cyprinus carpio haematopterus* (KOC, 2n = 100) were doubled by low temperature treatment to form gynogenetic *Hemibarbus maculatus* Bleeker (GHM).

In biology, morphological research can help to analyze the function

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and development of the structure of an organism as well as to provide clues as to how various structures formed in evolution, thereby revealing how closely one species is related to another. For fish, the external morphology and segmental characteristics are subjects of morphological research, and morphology is commonly adopted to detect genetic differences. The external morphology of fish generally refers to characteristics such as the body color and body shape, while the segmental characteristics focus on the comparative analysis of countable traits (such as lateral scales) and measurable traits (such as body length/total length). Therefore, morphological study plays an important role in revealing differences between species and is widely used in fish distant hybridization.

Microsatellite DNA has unique advantages and a wide range of application. Microsatellite DNA refers to special sequences evenly distributed in the genome of eukaryotes, where one microsatellite site appears on average per 10 kb of DNA [14]. Microsatellite DNA is generally composed of flanking sequences and tandem repeating core sequences; this leads to high levels of polymorphism of the same microsatellite site in the composition of its core sequence bases and the number of tandem repetitions. Owing to the advantages of high polymorphism, easy detection, and good reproducibility, microsatellite DNA has become a fast and effective labeling method [15]. It is also commonly used to examine the genetic composition, parental identity, and germplasm origin of laboratory animals [16, 17]. In this study, a pair of primers designated MFW-M were used to amplify specific microsatellite DNA fragments; the analysis identified GHM, indicating that genetic material from paternal origin affected the characteristics of gynogenetic offspring.

2. Materials and methods

2.1. Experimental fish

KOC were obtained from the State Key Laboratory of Developmental Biology of Freshwater Fish in Hunan Normal University, Changsha, Hunan province; HM was obtained from aquatic products in Liuyang, Hunan, China. In this study, pond culture and artificial feeding methods were adopted to treat fish according to the national wildlife protection regulations and animal experiment management regulations, and the study was approved by the National Science and Technology Bureau. The experiments conducted in this article did not require approval from the wildlife authority. The fish were anesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St Louis, MO, USA) before dissection.

2.2. Traditional and improved methods of gynogenesis

In traditional gynogenesis (the one-step warming method), inactivated heterologous sperm were used to stimulate the ovum to start its development, and then the ovum was placed in 0–4 °C muddy water for 22–24 min of cold shock to double the number of egg chromosome number; the egg was then transferred to 23–26 °C water for development until the hatching of the fish fry.

An improved method of gynogenesis (the multi-step warming method) proceeds by first stimulating the eggs by inactivated heterologous sperm to initiate their development, and then the eggs were placed in 0–4 °C muddy water for 22–24 min of cold shock to double the egg chromosomes. Then, the eggs were placed in 4–6 °C, 8–10 °C, and 15–18 °C water in order for 1–2 min. Finally, they are incubated in a temperature of 22–25 °C water for development until the hatching of the fish fry.

2.3. Origin of experimental broodstock, in vitro reproduction, and progeny rearing

This study was conducted at the State Key Laboratory for Developmental Biology of Freshwater Fishes in Hunan Normal University, Changsha, China. The HM females (n = 30) and KOC males (n = 10) were obtained from Liuyang WuLong Aquatic Products, LiuYang, Hunan, China. All HM and KOC were tested for ploidy by flow cytometry.

All of the progeny groups were produced by artificial spawning. Spawning was induced by injecting chorionic gonadotropin (Ningbo second hormone factory, Ningbo, Zhejiang, China) in doses of 500–600 units/kg body mass, luteinizing hormone releasing hormone (LHRH) A2 in doses of 3–4 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China), and domperidone in doses of 1 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China). The males were given the full dosage in one injection (Human chorionic gonadotrophin (HCG) in doses of 600–800 units/kg body mass, LHRH A2 in doses of 5 μg/kg).

Six hours after males were given the resolving injection, sperm were collected by stripping. Semen was diluted for three to four times with Hank's equilibrium solution. The diluted semen was spread in petri dishes and disinfected under ZW20S UV light (Cnlight, Guangdong, China) (all procedures were carried out in the dark). Next, the eggs of mature female HM were activated by UV-treated KOC semen to conduct cold shock (0–4 °C) for 12 min. Finally, the fertilized eggs were incubated in water at 25 °C. In 2021, about 60,000 embryos were randomly selected to determine the hatchlings rate (the number of hatchlings/eggs × 100%) and the survival rate (the number of normal fry/eggs × 100%). The hatched fish were moved into the pond for further culture.

2.4. Observation of chromosome

Chromosome counts were performed using 20 GHM, 20 KOC, and 20 HM kidney tissues at the age of 6 months to determine the ploidy of GHM, KOC, and HM. Chromosomes were prepared according to the method of Wang et al. [8]. After 2–3 days of cultivation at 20 °C, the samples were injected with one to three times the concanavalin dose of 6–15 μg/g body weight for 12–24 h. Two to three hours before dissection, each sample was injected with colchicine at a dose of 4–6 μg/g body weight. Kidney tissue was ground with 0.8% NaCl and then fixed with 0.075 mol/L KCl at 37 °C for 40–60 min and 3: 1 methanol-acetic acid three times. Cells were dropped onto cold and wet slides and stained with 4% Giemsa for 40–60 min. The shape and number of chromosomes were observed under a microscope. Sixty metaphase chromosomes were analyzed for each fish (20 metaphase chromosomes per sample). Observations were performed under a 100 × magnification oil mirror.

2.5. Morphological traits

The traits of 20 HM, 20 KOC, and 20 GHM were counted and measured. Countable features were lateral scales, above lateral scales, below lateral scales, dorsal fin, ventral fin, and anal fin. The measurable traits were whole length (WL), body length (BL), body width (BW), head length (HL), head width (HW), caudal peduncle length (CPL), and caudal peduncle width (CPW). Subsequently, the ratios of BL/WL, BW/BL, HL/BL, HW/HL, CPW/CPL, and HW/BW were calculated. The covariance analysis of morphological traits (all of the above traits) of KOC, HM, and GHM was performed using SPSS 22.0 software [9].

2.6. Microsatellite DNA

Genomic DNA (gDNA) was isolated from 20 caudal fin tissues from GHM, KOC, and HM using a DNA extraction kit (Sangong, Shanghai, China) according to the manufacturer's instructions. The primers (MFW-M F: 5′-GTCCAGACTHMATCAGGAG-3′ and R: 5′-GAGGTGA-CACTGAGTCAHM-3′) were synthesized to amplify the gDNA sequence extracted from each fish. The PCR reaction was conducted in a 30 μL volume comprising 1 μL gDNA (about 20 ng), 15 μL 2 × Taq PCR MasterMix (Tiangen, Beijing, China), 13 μL ddH_2O, and 0.5 μL of each primer. The temperature curve during amplification comprised initial

denaturation at extension step at 72 °C for 7 min. PCR products were separated by polyacrylamide gel electrophoresis (PAGE).

The DNA fragments were purified by gel extraction using a kit (UNIQ-10 rotating column DNA gel extraction kit for PAGE, Sangon) and ligated onto a pMD18-T vector. The plasmid was transformed into *E. coli DH5α* and purified. Then, the DNA fragments inserted in the pMD18-T vector were sequenced using an automatic DNA sequencing instrument (ABI PRISM 3730, Applied Biosystems, Carlsbad, California).

2.7. Measurement of appearance and nuclear volume of red blood cells

Red blood cells were extracted from the tail veins of GHM, KOC, and HM with a needle that had been immersed in ACD (Citrate glucose), and then diluted to the appropriate concentration with normal saline. Subsequently, 10 μL of blood was added to the slide, and then a coverslip was used to make an even smear. The slide was allowed to dry naturally to form a blood film, and then stained with Swiss dye for 2–3 min. The slide was diluted with phosphoric acid buffer and then stained for 5–8 min. The back of the slide was washed with running water. After natural drying, the slides were observed under a 100 × oil immersion microscope. The red blood cells of each fish were observed by visual micrometer under oil immersion, and the red cell volume of each fish was calculated using the formula of (4/3) πab^2, where a and b were separately the long and short half axes of an ellipsoid [10]. The covariance analysis of erythrocyte karyotypes of KOC, HM, and GHM was performed by SPSS software (Version 19.0).

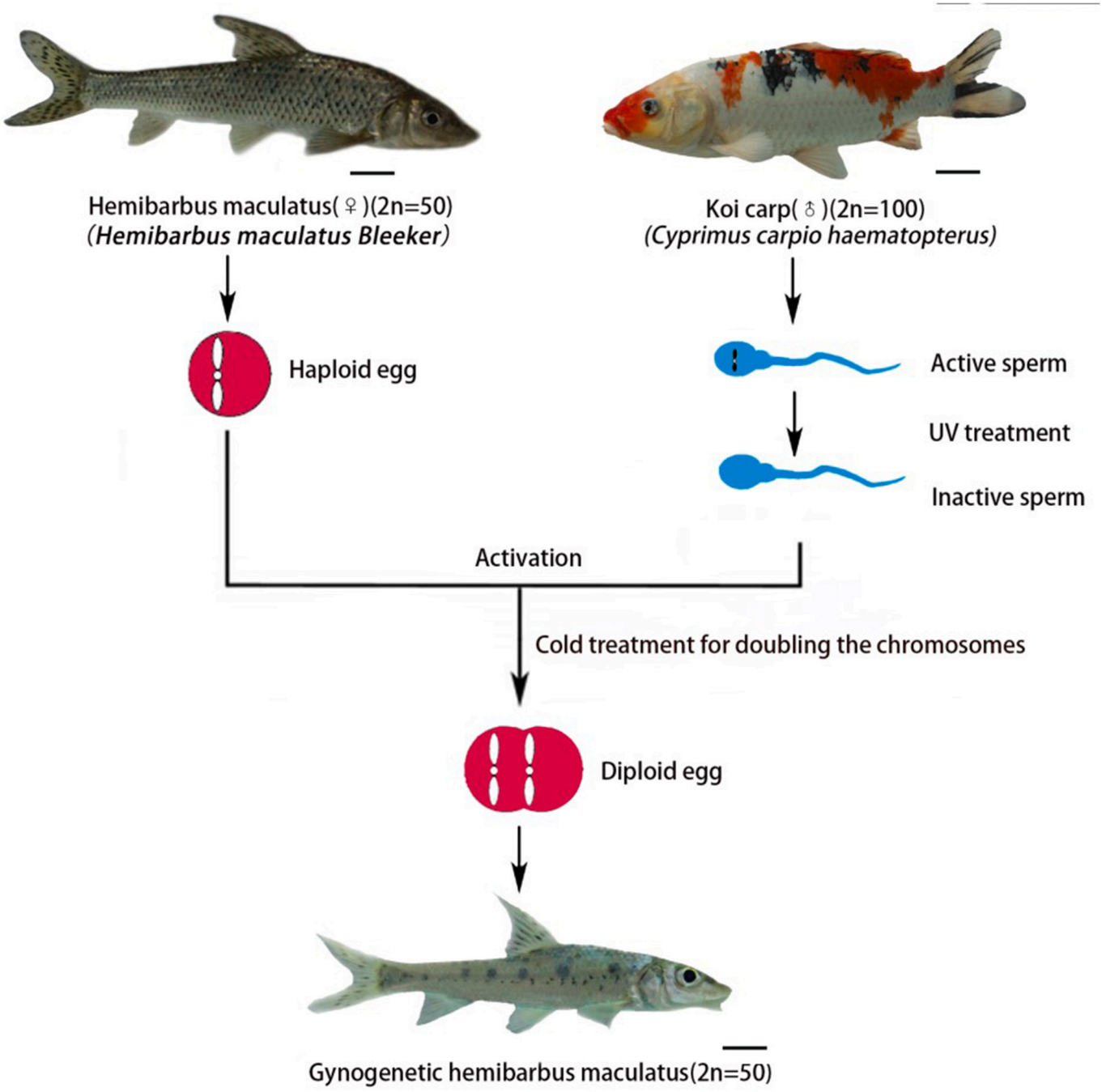

Fig. 1. Gynogenesis of *Hemibarbus maculatus Bleeker*, Bar = 2 cm.

3. Results

3.1. Formation of GHM and comparison of two methods of gynogenesis

In March 2021, we used the traditional gynogenesis method and the improved gynogenesis method to induce HM, and both methods produced GHM. The hatchability and survival rates of fish obtained by traditional gynogenesis were 5% and 3%, respectively, while the hatchability and survival rates of fish obtained by the improved gynogenesis method were 15% and 8%, respectively (Fig. 1).

3.2. Comparison of morphological characteristics

The morphological traits of KOC, HM, and GHM were shown in Table 1. GHM was similar to the maternal HM for many traits (Fig. 1 , Table 1 and Table 2). In this study, the body colors and shape of GHM individuals differed from those of KOC. The ratio of body length to whole length (BL/WL) of GHM was slightly higher than those of KOC and HM. The ratio of head width to head length (HW/HL) and the ratio of body width to body length (BW/BL) of GHM were between those of KOC and HM.

3.3. Chromosome numbers and karyotypes

Table 3 presented the distribution of chromosomes in KOC, HM, and GHM. Among the metaphase photographs, 95% showed 50 chromosomes with a karyotype of 16 m + 14sm + 16st+4t. Among KOC photographs, 90% showed that the KOC had 100 chromosomes with 34sm + 22 m + 22st+22t. Among GHM photographs, 85% showed that the GHM had 50 chromosomes with 16sm + 14sm + 16st+4t. In addition, 90% of the chromosome metaphase images supported GHM as being a diploid (Fig. 2).

3.4. Measurement of the nuclear volume of erythrocytes

The results of the measurements of mean erythrocyte nuclear volume for KOC, HM, and GHM were shown in Fig. 3. The mean erythrocyte nuclear volume ratio of GHM to HM was not significantly different from a ratio of 1:1 ($P > 0.05$), suggesting that the GHM were diploids. There was no significant difference between the mean erythrocyte nuclear volume ratio of GHM to KOC and a ratio of 1:1, indicating that the GHM were diploids. There was no prominent difference between the mean erythrocyte nuclear volume ratio of the HM to KOC and a ratio of 1:1, indicating that the HM were diploids.

3.5. DNA fragment from the paternal parent was identified in GHM

A pair of microsatellite DNA primers designated MFW-M were used to amplify the DNA fragments from GHM and their parents (HM and KOC) in this study. Three kinds of distinct and bright bands were amplified in GHM using the primers of MFW-M. In contrast, only four bands were presented in GHM. To detect microsatellite DNA patterns among KOC, HM, and GHM individuals, we amplified fragments with the MFW-M primer. In GHM, we found homologous fragments approximately between 220 bp and 320 bp in length. In GHM, each individual contained both fragments, indicating that GHM inherited them from KOC and HM (Fig. 4).

Table 1
Comparison of measurable traits among HM, KOC and GHM.

Fish type	BL/WL	HL/BL	HW/HL	CPW/CPL	HW/BW	BW/BL
KOC	0.8 ± 0.05	0.32 ± 0.05	0.80 ± 0.05	0.74 ± 0.05	0.82 ± 0.05	0.31 ± 0.05
HM	0.8 ± 0.01	0.29 ± 0.01	0.62 ± 0.01	0.48 ± 0.01	0.84 ± 0.01	0.21 ± 0.01
GHM	0.79 ± 0.01	0.28 ± 0.01	0.65 ± 0.01	0.80 ± 0.01	0.78 ± 0.01	0.23 ± 0.01

Table 2
Comparison of countable traits among HM, KOC and GHM.

Fish type	No. of lateral scales	No. of upper lateral scales	No. of lower lateral scales	No. of abdominal fins	No. of anal fins	No. of dorsal fins
KOC	36	6	5	10	9	21
HM	49	7	3	9	8	9
GHM	47	7	5	9	8	22

Table 3
Mean erythrocyte nuclear volume measurements for KOC, HM, and GHM.

Fish type	Major axis (μm)	Minor axis (μm)	Volume (mm^3)	Volume ratio	
				Observed	Expected
KOC	5.15 ± 0.52	3.25 ± 0.23	29.25 ± 8.22		
HM	4.78 ± 0.37	2.66 ± 0.22	17.78 ± 3.11		
GHM	4.74 ± 0.34	2.63 ± 0.24	17.19 ± 3.12	GHM/HM = 0.97[a]	
				GHM/KOC = 0.59[a]	

Note: [a]The difference between the observed value and the expected value is not significant ($P > 0.05$).

4. Discussion

The results of this study indicated that the oosperm of HM were sensitive to the changes of temperature during hatching. A sharp change in temperature could cause a large number of deaths. In our experiments, after cold shock treatment, the activated eggs were directly transferred into water with a temperature of 22–25 °C. Without gradient heating treatment, the survival rate of the offspring of gynogenesis was only about 1–2%. However, gradient heating of the activated eggs after cold shock treatment could reduce the damage to the fertilized eggs and thereby significantly improved the hatchability of the fertilized eggs and the survival rate of the offspring.

The morphological analysis found no significant differences in countable or measurable traits between GHM and HM. Through the analysis of chromosome number and karyotype, we found that the GHM was a diploid with 50 chromosomes, which was consistent with the female parent (HM). The results showed that the gynogenetic fish retained the maternal characters in external morphology. The microsatellite DNA analysis indicated that there were fragments of heterologous sperm from the paternal KOC in the genome of GHM, indicating the hybridization effect in GHM. The hybridization effect caused by the insertion of small fragments had been previously reported in the study of gynogenetic fish. For example, in the process of stimulating *Carassius auratus gibelio* with inactivated *Megalobrama amblycephala* sperm, microchromosomes appeared in the gynogenetic *Carassius auratus gibelio* [11]. In the process of natural gynogenesis of KOC and HM, a set of heterologous chromosomes was found in the genome of GHM. In the process of artificial gynogenesis of *Hypophthalmichthys molitrix* with *Cyprinus carpio* sperm, *Cyprinus carpio* DNA was found in the genome of gynogenetic *Hypophthalmichthys molitrix* [12]. It has been reported in the literature that the offspring of gynogenetic fish might display desirable biological characteristics such as disease resistance, hypoxia tolerance, and growth rate [13,14]. According to the breeding experiment, the GHM had greater hypoxia tolerance; this might be related to the heterologous sperm fragments inserted into the GHM genome. The reasons for this might be as follows: first, in the process of gynogenesis, the eggs were activated by sperm treated with ultraviolet light, and thus only high-quality eggs could develop further. Therefore, the surviving offspring might be stronger. Second, in the process of gynogenesis, the

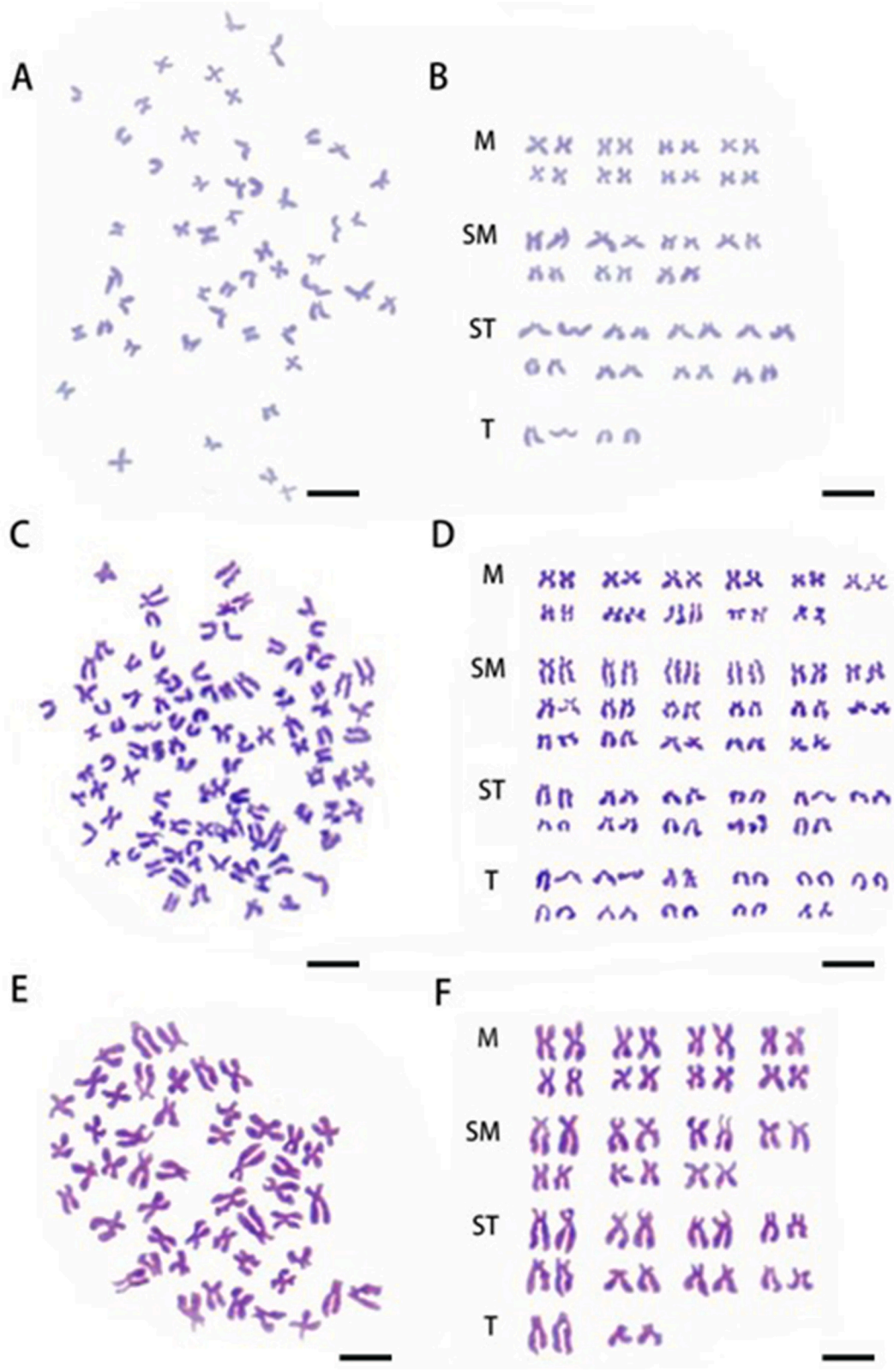

Fig. 2. Chromosome spreads at metaphase and corresponding karyotypes of GHM, KOC, and HM
(A) The 50 chromosomes of GHM (B) The karyotype of GHM was 16 m + 14sm + 16th+4t. (C) The 100 chromosomes of KOC (D) The karyotype of KOC was 22 m + 34sm + 22st+22t. (E) The 50 chromosomes of HM. (F) The karyotype of HM was 16 m + 14sm + 16th+4t. Bar = 5 μm.

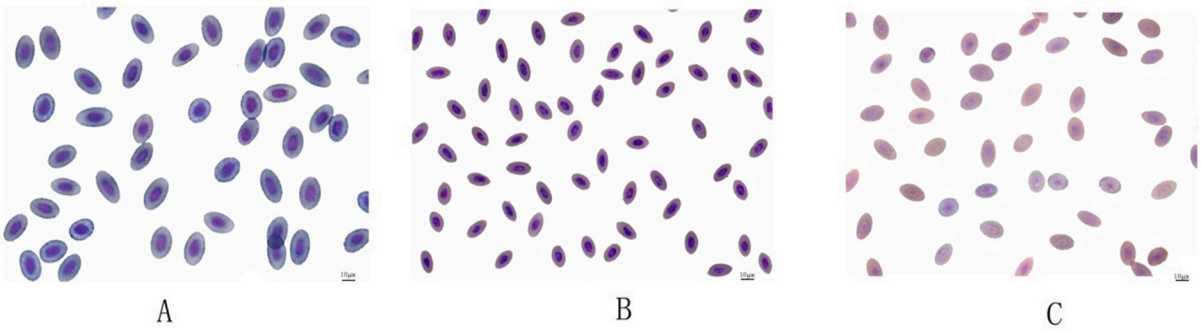

Fig. 3. Erythrocytes of KOC, GHM and HM in peripheral blood
(A)Mature erythrocytes of KOC, 100 × ,(B)Mature erythrocytes of HM, 100 × , (C) Mature erythrocytes of GHM, 100 × , Bar = 3 μm.

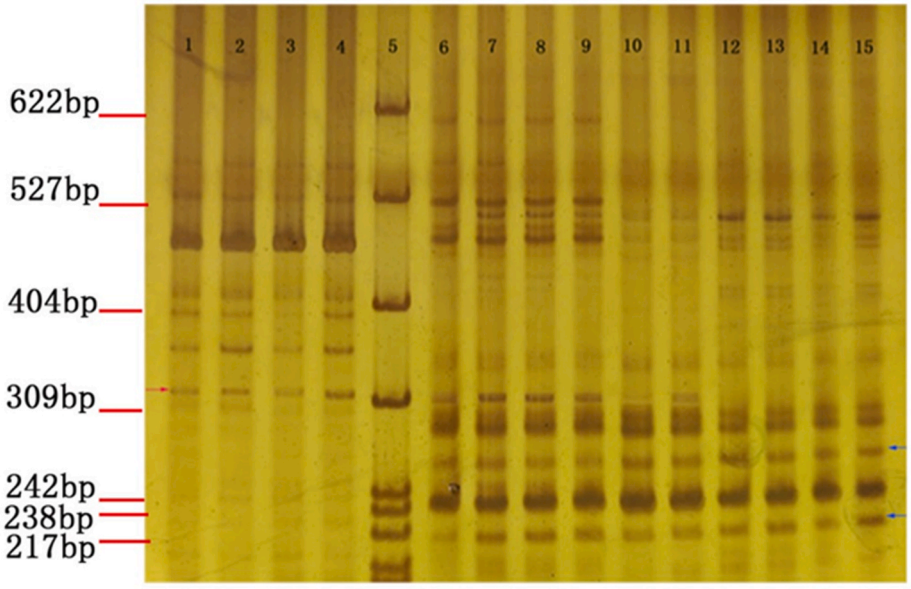

Fig. 4. Microsatellite patterns of KOC (Nos. 1-4), GHM (Nos. 6-11), and HM (Nos. 12-15)
Electropherogram of microsatellite DNA patterns produced by the primer pair MFW-M in KOC, GHM, and HM. Lanes 1–4 represent KOC; Lanes 6–11 represent GHM, and Lanes 12–15 represent HM. The blue arrow indicates the DNA bands derived from HM. The red arrow indicates the DNA bands derived from KOC. M represents the pBR322 DNA/Msp Marker.

eggs activated by sperm treated with ultraviolet light experience cold shock or heat shock that could seriously damage the development of the eggs, indicating that the surviving offspring of gynogenesis experienced a screening process. Third, the increase in the frequency of some harmful homologous recessive genes leads to the death of female reproductive offspring. Therefore, the surviving female offspring might include improved traits. Fourth, if some paternal genetic material was inserted into the genome or existed in the form of microchromosomes, this might produce a “hybridization” effect.

In this study, an improved method was developed to obtain a large number of fish with desirable traits, a high survival rate, and genetic stability. It is expected to obtain a variety of desirable traits through secondary gynogenesis fish strains, and then develop quality varieties with high hypoxic tolerance and disease resistance.

Author contributions

Y-DW: conceptualization, investigation, writing original draft, and funding acquisition; A-ML and CG: conceptualization and investigation; H-FT: data curation; Q-BQ and K-KL: investigation; MT and CZ: software; SW, MW and R-RZ: methodology; S-JL: conceptualization, writing–review and editing, supervision, project administration, and funding acquisition. All the authors read and approved the final manuscript.

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Declaration of competing interests

The authors declare no competing interests.

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