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Gynogenetic *Cirrhinus mrigala* produced using irradiated sperm of *Cyprinus carpio* exhibit better cold tolerance

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ABSTRACT

Artificial gynogenesis is an important method for accelerating the selective breeding of varieties and populations. In this study, ultraviolet-irradiated sperm of Common carp (*Cyprinus carpio*) was used to activate the mature eggs of mrigal carp (*Cirrhinus mrigala*, MC), after cold shock at 4–8 °C for 16 min to double the chromosomes, a population of gynogenetic mrigal carp (GMC) was obtained. The fertilization rate, hatching rate and survival rate of GMC exceeded 62.2%, 6.3% and 2.9%, respectively. Comparative analyses of morphological traits, DNA content, chromosome numbers and 5S rDNA sequences showed no significant differences between MC and GMC. However, microsatellite (MFW1, CCA15) analysis revealed that paternal DNA fragments were inserted and a parent DNA fragment was deleted in the GMC. The GMC population survived the natural winter for more than 50 days with a temperature below 10 °C, while all the normal MC individuals perished. In addition, lower critical thermal minimum (CTMin) and lethal temperature (T_{LD50}) values were detected in GMC than that in MC. Our results indicated that the artificial gynogenesis method improved the cold tolerance of mrigal carp. The results of this study are of great significance for fish breeding and application.

1. Introduction

Artificial gynogenesis, an induced developmental process in which the maternal genome is activated by genetically inactivated sperm, is an important method for accelerating the selective breeding of varieties and populations [1–3]. First, artificial gynogenesis has been developed into an effective way to induce all-female stock in some fish species that exhibit sexual growth dimorphism [4,5]. Besides, in some certain fish species including half-smooth tongue sole (*Cynoglossus semilaevis*), large yellow croaker (*Pseudosciaena crocea*), mandarin fish (*Siniperca chuatsi*) and bighead carp (*Hypophthalmichthys nobilis*), artificial gynogenetic offspring are ideal models to investigating all-female sex determination mechanisms [5–8]. In addition, artificial gynogenetic offspring generally exhibit allogynogenetic biological effects with the paternal genetic materials that insert into the genome or remain in the microchromosomes and showed superior traits, including fast growth rates, hypoxia tolerance and strong resistance to diseases, in many farmed fishes such as grass carp (*Ctenopharyngodon idella*) [9], blunt snout bream (*Megalobrama amblycephala*) [10,11], Atlantic salmon (*Salmo salar*) [12], and rainbow trout (*Oncorhynchus mykiss*) [13].

Generally, artificial induction of gynogenesis involves two steps: the first step is to activate the mature eggs with homologous or heterologous inactivated sperm, and the second step is to induce duplication of the chromosomes of the activated eggs, including inhibition of either the release of the secondary polar body or first cleavage [2,14,15]. The methods for artificially inducing chromosome doubling include physical (such as cold shock, heat shock, and hydrostatic pressure) and chemical (colchicine, cytochalasin, SP600125, etc.) treatment methods [11,14,16–18]. However, artificially induced of gynogenesis in fish still faces some challenges, such as egg quality control, and those associated with

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sperm selection and methods of chromosomes doubling. For example, differences in temperature shock sensitivity among fish are related to both genetic background and egg maturity [19].

Indian major carp (*Cirrhinus mrigala*, Hamilton) (MC, 2n = 50) is an economically important bottom-feeding fish in Indian, Bangladesh, and Pakistan [20]. The MC was initially introduced to Guangdong Province, China, for aquaculture in 1982 and was than successfully bred and farmed throughout Guangdong Province in the 1990s [21]. Owing to its hardiness and wide range of trophic and ecological adaptations, MC has mainly served as live bait for carnivorous fishes such as the mandarin fish (*Siniperca chuatsi*) in recent years. However, MC populations were easily perished when the temperature falls below 9 °C, showing a weak ability to survive winter in central and western of China [22]. In addition, although artificial induction of gynogenesis mrigal carp (GMC) has been reported in several previous studies [23–25], the basic biological traits and potential applications of GMC have not been investigated. In this study, a GMC population was successfully obtained by cold treatment to double the chromosomes of the MC eggs which activated by ultraviolet-treated common carp (*Cyprinus carpio*, CC, 2n = 100) sperm. The morphological features, DNA content, chromosome numbers, 5S rDNA sequence, cold tolerance and growth of GMC and MC were compared.

2. Materials and methods

2.1. Experimental fish and gynogenesis

The specimens, including 10 two-year-old male CC individuals (each weighing approximately 500 g) and 10 two-year-old female and 10 male individuals MC (each weighing approximately 2000 g) were provided by Chengyi Aquaculture Co., Ltd. (Guangzhou, China). All fish were bred under natural environmental conditions and maintained in a recirculating aquaculture tank for one month before the experiments.

Spawning was induced by injecting chorionic gonadotropin and domperidone (Ningbo Second Hormone Factory, Zhejiang, China) at doses of 10 μg/kg and 10 mg/kg, respectively. The MC females were given the full dose in one injection, and the MC and CC males were given half the dose. Approximately 5–6 h later, the matured eggs from female MC and semen from male MC and CC were collected by stripping. Then, the eggs and sperm were gently mixed in a Petri dish, maintained for 2–3 min, and transferred into an artificial incubation system with a water temperature of 29–30 °C. Embryos resulting from MC self-crossing and crossing between female MC and male CC were used as control.

Spermatozoa were inactivated using ultraviolet (UV) lamp irradiation, utilizing methods described in previous studies with some modifications [11,12]. First, the semen of CC was collected by stripping and diluted with Hank's solution (1:10). Aliquots of the diluted sperm (4.0 mL) were placed in a 15 cm Petri dish, and the thickness of the diluted sperm was approximately 0.23 mm. Second, the Petri dish was placed on a tray with a layer of ice in the UV crosslinker (Scientz 03-II, Scientz Biotechnology Co., Ltd., China) and exposed to UV irradiation for 4–6 min based on the spermatozoa activity. The total UV dosage was in the range of 200–300 mJ/MC^2. Subsequently, the eggs of MC were activated by UV-irradiated sperm of CC. At 2 min postfertilization, the embryos were treated at 4–8 °C for 15–16 min, and then transformed into an artificial incubation system with circulating water for incubation. The experiment was repeated multiple times in 2021 and 2022. The water salinity ranged from 0.9‰ to 2.6‰ in 2021 and 1.5‰–3.2‰ in 2022. The embryos were observed every 2 h with a stereomicroscope. Finally, 10000 embryos were randomly selected to determine the fertilization rate (number of embryos at the gastrula stage/number of embryos × 100%), hatching rate (number of hatched fry/number of embryos × 100%), and survival rate (number of first-feeding fry/number of eggs × 100%). The hatching rate of MC (♀) × CC (♂) were less than 0.1%, the rearing data was not recorded in this study.

2.2. Fish rearing, cold tolerance and the survival rate detection

The MC and GMC larval fish (each group = 500 individuals) were housed in two ponds (30 m × 10 m × 1.2 m) at the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha, China. During the breeding process (from June 2021 to April 2022), the larval fish were exposed to ambient light levels, with a suitable pH (6.0–8.0) and dissolved oxygen content (5.5–7.0 mg/L). Meanwhile, the water temperature was natural and measured (50 cm below the water surface) every month. The two groups of fish were fed with artificial feed routinely two times per day at 9:00 and 18:00 h. The amount of food provided was gradually increased according to the fish's body weight. The two groups of fish were sampled at 2, 6, and 10 months of age. At each time point, 30 individuals from each group of fish were randomly selected for body weight measurement. As the temperature significantly decreased from December 2021 to February 2022, all MC individuals perished. The GMC population was kept rearing, in October 2022, three gonad tissues were randomly selected for histological analysis and using a protocol described in a previous study [26].

The critical thermal minimum (CTMin) and lethal temperature (T_{LD50}) are two widely used indices for evaluating the cold tolerance of fish. CTMin is determined by decreasing the water temperature at a constant rate and then recording the temperature at which the experimental fish loses its ability to maintain equilibrium [27]. T_{LD50} is conventionally the temperature at which 50% of individuals die due to a decrease in water temperature [28]. In June 2022, one month old fish including 200 MC (Body mass = 5.53 ± 0.72 g and body length = 8.42 ± 1.02 cm) and 200 GMC (Body mass = 6.14 ± 0.88 g, Body length = 8.93 ± 1.21 cm) were acclimated in 26 °C for two weeks and then used to perform CTMin and T_{LD50} experiments. Water temperature was controlled by using three constant-temperature chambers (Shanghai Yiheng Science Corporation). The fish were placed into a homemade cage fitting the chamber of the circulators. For CTMin measurement, the water temperature was decreased 0.25 °C/min from the intimal temperature, and the fish (n = 30 in each group) was removed and weighted when equilibrium was lost. For T_{LD50} measurement, the water temperature was decreased 2 °C/h from 26 °C and maintained at 20 °C for 24 h, followed by a decrease of 2 °C/h and maintenance at 14 °C for 24 h. On the fourth day, the water temperature was decreased according to different temperature treatments (10, 9, 8, 7, 6 and 5 °C) and maintained for 2 h, following the procedure in a previous study [22]. To reduce random errors, 10 MC and 10 GMC (n = 20 total/tank) were combined in each treatment. Survival rates were averages of the three replicates for each treatment.

2.3. Measurement of morphological traits

Thirty 10-month-old MC, GMC, and CC were randomly selected for morphological examination. The averages of the measurable traits, i.e., whole length (WL), body length (BL) and width (BW), head length (HL) and width (HW), and caudal peduncle length (CPL) and height (CPH), were taken. Additionally, the average ratios of body length to whole length (BL/WL), body width to body length (BW/BL), head length to body length (HL/BL), head width to head length (HW/HL), and caudal peduncle height to caudal peduncle length (CPH/CPL) were calculated. Countable traits, including the number of lateral line scales, the numbers of scale rows above and below the lateral line scales, and the numbers of dorsal, anal, and pelvic fin rays, were determined.

2.4. Preparation of chromosome spreads and measurement of DNA content

Chromosome preparation was carried out using kidney tissues according to the procedures reported by Wang et al. with minor modifications [29]. Chromosome shape and numbers were analyzed under a light microscope. Twenty metaphase spreads were photographed to

determine the chromosome number. The preparations were examined under an oil lens at a magnification of 330 × .

The DNA contents of erythrocytes from CC, MC, and GMC (each group n = 30 individuals) were measured using a flow cytometer (Cell Counter Analyser, Partec, Germany). Blood samples (1–2 ml) were collected from the caudal vein of each individual using a syringe containing ~100 units of sodium heparin. The blood samples were processed following the method and investigated under the same conditions as described in a previous study [30]. The MC DNA contents were used as controls. The χ^2-test (with a Yates correction) was used to test for deviation from the expected values.

2.5. 5S rDNA cloning and sequencing

Caudal fin samples were collected from CC, MC and GMC individuals (each group n = 10), and then total genomic DNA was extracted with a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. The DNA concentration and quality were assessed using agarose gel electrophoresis. Specific primers (5S-F: 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5S-R: 5′-CAGGTTGGTATGGCCGTAAGC-3′) from Cyprinidaes were used to amplify the 5S rDNA gene and untranslated regions (NTS) of the three groups of fish [29]. PCRs and sequencing were performed as previous study described [29]. The obtained origin sequences were analyzed by using BioEdit software v7.0.

2.6. Microsatellite DNA cloning and analysis

Two specific microsatellite primers for CC, namely MFW1 (F: 5′-GTCCAGACTGTCATCAGGAG-3′, R: 5′- GAGGTGTACACTGAGTCACGC-3) and CCA15 (F: 5′-CAGCCGCTGGATCCCAACTG-3′, R: 5′-TGCAGATGCGTAG.

CAATGTAAACC-3′), were used for locus analysis based on published studies [31,32]. PCR conditions and components followed those in a previous study [30]. Electrophoresis on 8% polyacrylamide gels (PAGE) was used to separate amplification products, which were sized against a 25–250 bp DNA ladder (Sangon Biotech, Shanghai). Finally, silver staining was performed to visualize microsatellite DNA according to a previous study [33].

2.7. Data analysis

SPSS Statistics 19.0 (IBM Corp., NY, USA) was used to analyze the date that were presented as mean ± standard deviation (SD), and analysis of variance (ANOVA) was used to determine significant difference between MC and GMC. $p < 0.05$ represents a significant difference between the two groups.

3. Results

3.1. Formation of GMC

In this study, GMC population was obtained in both 2021 and 2022. The production of GMC was outlined in Fig. 1. At 2 min postfertilization, the embryos were treated at 4–8 °C for 16 min to prevent the release of the second polar body of the embryos for chromosome doubling. The embryos of MC (self-crossing) and GMC hatched approximately 15–22 h post-fertilization. The salinity of the hatchery water was 2.4‰ in 2022 and 1.9‰ in 2021. The fertilization rate, hatching rate and survival rate of MC and GMC were presented in Table 1. Interestingly, the fertilization rate, hatching rate, and survival rate of MC was observed higher in 2021 than 2022, in accordance with the water temperature in 2021 was higher than that in 2022. The fertilization rate, hatching rate of GMC in 2022 was higher than that in 2021, but the survival rate was lower in 2022 than that in 2021. Three ovary tissues from 15-month-old GMC fish (body weight = 754.67 ± 34.89 g) were randomly collected for histological analysis, and the results showed that the ovarian tissues of was normal and developed into typical ovaries with numerous primary oocytes (at stage I) (Fig. 1).

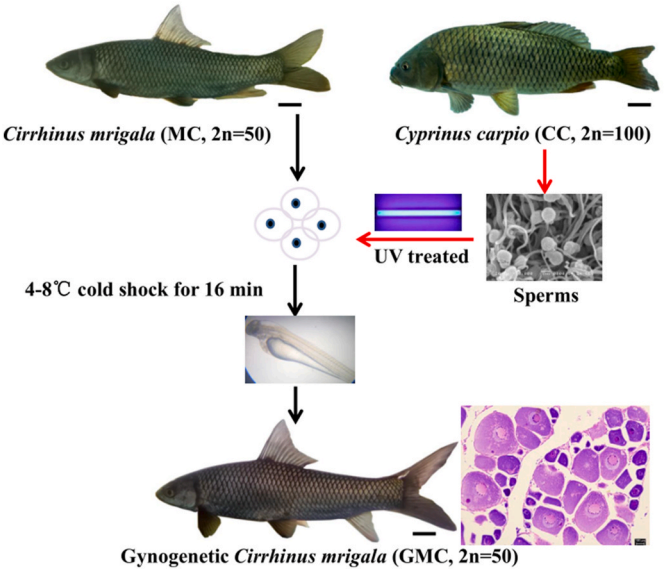

Fig. 1. Production processes of GMC.
UV-inactivated sperm of CC were used to induce GMC, and histological analysis showed that the ovary tissue of 10-month-old fish was normally developed. I: oocyte at stage I. Bar = 1 MC.

Table 1
Fertilization rate, hatching rate, survival rate, and water temperature of MC and GMC.

	MC		GMC	
	2021	2022	2021	2022
Fertilization rate (%)	94.72 ± 3.03	88.53 ± 2.56	62.2 ± 5.54	70.7 ± 3.42
Hatching rate (%)	89.33 ± 2.94	83.63 ± 1.87	6.39 ± 0.65	7.61 ± 0.34[b]
Survival rate (%)	81.66 ± 2.84	72.85 ± 2.04[a]	4.26 ± 0.46	2.97 ± 0.25[b]
Water temperature	29–31 °C	23–25 °C	29–31 °C	24–26 °C

[a] and [b] represent significantly different between 2021 and 2022 in MC or GMC group fish ($p < 0.05$).

3.2. Morphological traits

The phenotype of GMC resembled that of wild-type MC. The measurable and countable traits of CC, MC and GMC are presented in

Table 2
Comparison of the morphological traits of CC, MC and GMC.

Phenotypes	Fish type		
	CC	MC	GMC
BL/WL	0.82 ± 0.05	0.84 ± 0.04	0.83 ± 0.04
BW/BL	0.35 ± 0.02	0.26 ± 0.01	0.28 ± 0.03
HL/BL	0.24 ± 0.04	0.21 ± 0.02	0.20 ± 0.03
HW/HL	0.82 ± 0.05	0.83 ± 0.03	0.86 ± 0.05
CPH/CPL	0.85 ± 0.14	0.76 ± 0.01	0.74 ± 0.03
HW/BW	0.59 ± 0.03	0.64 ± 0.06	0.63 ± 0.05
No. lateral scales	35–42	40–45	41–42
No. upper lateral scales	6–7	6–7	7
No. lower lateral scales	5–6	5–6	6
No. dorsal fins	III + 17-18	III + 12-14	III + 12-13
No. abdominal fins	7–8	8–9	8–9
No. anal fins	III + 6-7	III + 6-7	III + 6-7

Table 2. The measurable traits of GMC, such as BW/BL, HL/BL, HW/HL, CPH/CPL, and HW/BW, were more similar to those of MC than to those of CC. Some countable traits of GMC, i.e., the numbers of lateral scales, dorsal fins and anal fins, were similar to those of MC, whereas other showed significant differences from those in CC. However, the numbers of lateral scales and abdominal fins were similar among the three groups of fish. Furthermore, the countable traits were stable in the individuals of GMC.

3.3. DNA content and chromosome numbers

DNA contents were detected by flow cytometry, and the average relative fluorescence intensities of CC, MC and GMC in the DNA histogram were 104.09, 63.85 and 62.28, respectively (Fig. 2A, C and E). The DNA content of GMC was similar to that of MC ($p > 0.05$). Chromosome preparations were performed from kidney tissue, in which 100 metaphase phases of each sample were counted. In total, 82% of metaphases showed 50 chromosomes in GMC (Table 3). The largest submetacentric

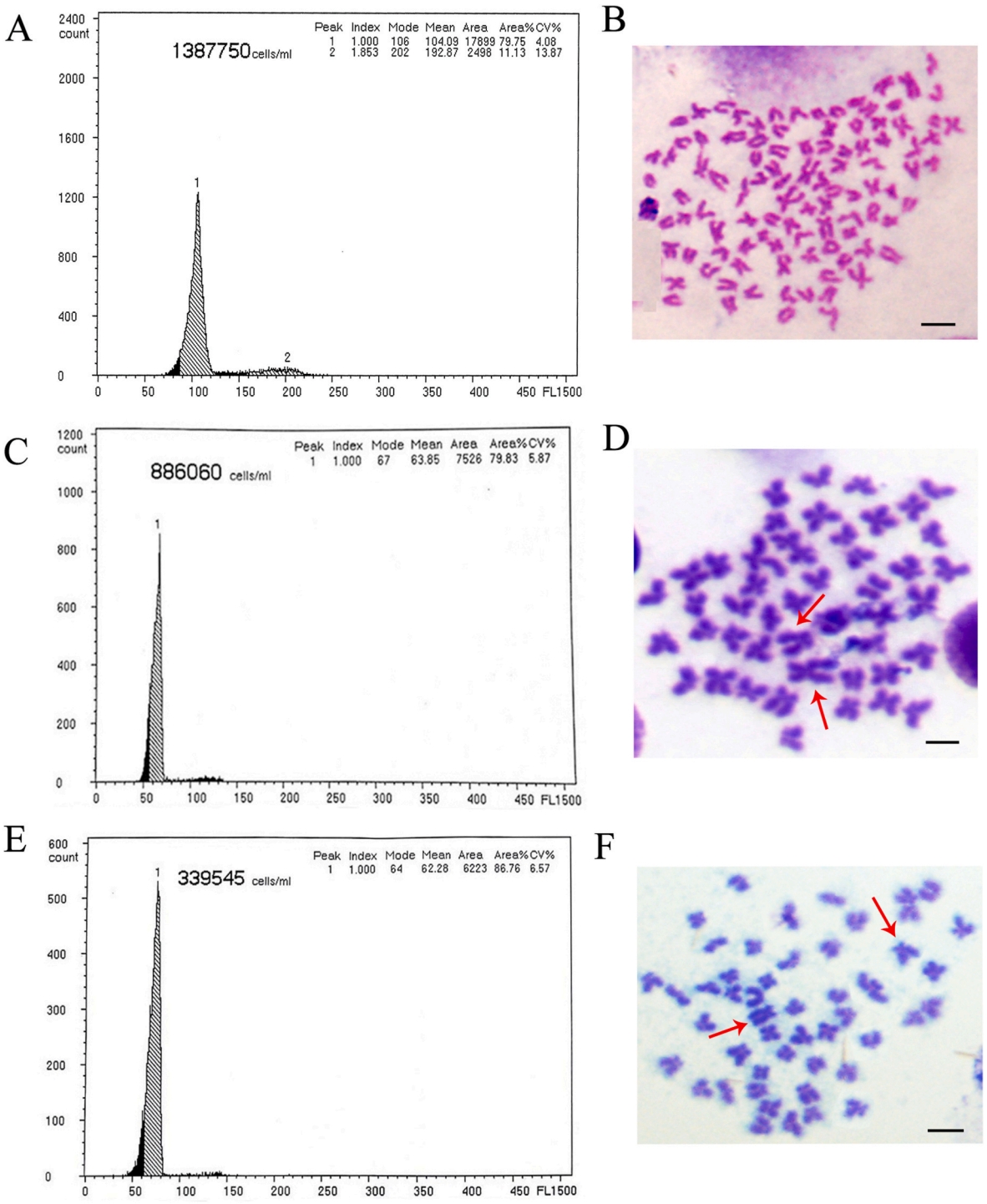

Fig. 2. Cytometric histogram of DNA fluorescence and chromosome spreads at metaphase in CC, MC and GMC. (A) The mean DNA content of CC (peak 1: 104.09); (B) the 100 chromosomes of CC; (C) the mean DNA content of MC (63.85); (D) the 48 chromosomes of MC; (E) the mean DNA content of GMC (62.28); (F) the 50 chromosomes of MC. The red arrow shows the large pair of submetacentric chromosomes. Bar = 3 μm.

Table 3
Chromosome numbers in CC, MC and GMC.

Fish lines	No. of metaphase photographs	Distribution of chromosome numbers			
		<50	50	<100	100
CC	100			16	84
MC	100	13	87		
GMC	100	18	82		

chromosome was present in the metaphase chromosome set of GMC (Fig. 3D), which was a marker chromosome for MC (Fig. 3H). The distribution of chromosome number indicated that GMC was diploid.

3.4. Molecular organization of 5S rDNA and microsatellite DNA analysis

Using the 5S rDNA primer pair, two fragments in CC (~200 bp and ~400 bp) and two fragments in both MC and GMC (~200 bp and ~300 bp) were amplified. To determine the sequences of each 5S rDNA gene, a total of 60 clones were sequenced from CC, MC, and GMC. The PCR products of CC comprised two fragments of 205 bp and 404 bp; those of both MC and GMC comprised two PCR fragments of 193 bp and 294 bp. In this study, each 5S rDNA molecule contained two fragmented coding regions (5′ −99 bp and 3′ −21 bp) and a separated NTS sequence. In CC, only one fragment, the 202 bp 5S rDNA, contains a 120 bp coding region and 82 bp NTS unit. The 404 bp fragment was simply two repeats of 202 bp. In MC and GMC, the 193 bp fragment (each fish contains two types) contains a 120 bp coding region and a 73 bp NTS unit (Fig. 3A). The 294 bp fragment (each fish contains two types) was separated by two NTS sequences (73 + 73 bp), which contain a 120 bp coding region and a 28 bp coding region (Fig. 3B). The sequence similarity of the two types of 5S rDNA between MC and GMC was exceeded 96.8%.

Two pairs of microsatellite DNA primers, MFW1 and CCA15, were used to amplify the DNA fragments from CC, MC and GMC. For MFW1, a special band was amplified in GMC and parental CC but not in maternal MC. Meanwhile, a new band was amplified only in GMC (Fig. 4A). For CCA15, a specific band was amplified in the parents CC and MC but not in GMC (Fig. 4B). These results demonstrated the incorporation of the genetic material of the paternal parent, as well as insertions and deletions in genomic DNA sequence of GMC.

3.5. Cold tolerance and growth of GMC

To better understand the relationship between the growth and cold tolerance of MC and GMC, the body weight and temperature were recorded. The water temperature was gradually increased from June to August and remained high until October, at which point it significantly decreased from October (~33.0 °C) to December (~12.0 °C). On 27 December, snow caused the water and air temperatures to drop below 7 °C. The low air (ranging from 0.5 to 10 °C) and water (ranging from 4.5 to 9.5 °C) temperatures lasted until 24, February 2022, after which both significantly increased to 10 °C from February to April 2022 (Fig. 5A). The growth of GMC was higher than that of MC, with the body weight of GMC and MC were 137.37 ± 13.52 g and 171.28 ± 15.33 g in 6-month-old ($p < 0.05$). During the breeding process from December 2021 to February 2022, all MC individuals perished, while the GMC individuals survived the natural winter, despite exposure to low water temperatures of 5.0–9.5 °C. The results suggested that the cold tolerance of GMC was improved. The body weight of MC and GMC are compared in Fig. 5B and Table S1.

Based on the results above, CTMin and T_{LD50} were used to evaluate the acute cold tolerance of GMC and MC. The CTMin value of GMC was 11.65 ± 0.25 °C, significantly lower than that of MC (13.08 ± 0.35 °C) ($p < 0.05$), indicated that the GMC population was better able to tolerate low temperatures than the MC population (Fig. 6A). For T_{LD50} analysis, the survival rate of both GMC and MC at 10 °C was 100%. There was no change in the GMC survival rate at 9 °C, which gradually decreased to 86.67% at 8 °C, showing no difference compared with that of MC (83.33%) ($p > 0.05$). Then, the survival rate significantly decreased for both GMC and MC as the temperature decreased from 8 °C to 6 °C. At 5 °C, all MC had perished, while GMC fish lost their balance and then perished, and showed an average survival rate of 10.0%. The lethal temperature for GMC ($T_{LD50} = 6.7$ °C) was 0.5 °C lower than that for MC ($T_{LD50} = 7.2$ °C) (Fig. 6B). Together, the results indicated that GMC fish were able to survive at lower temperature environment.

4. Discussion

The temperature and salinity of hatchery water are two major factors that influence the fertilization, hatching and survival rates of many fish in artificial environments [34,35]. In this study, the GMC population was obtained by cold treatment to double the chromosomes of the mrigal carp eggs (Fig. 1). A higher fertilization rate, hatching rate and survival rate were observed in 2021 than in 2022, consistent with the water temperature in 2021 was higher than that in 2022 (Table 1), suggesting that a water temperature >26 °C was better for MC spawning and hatching. Although the fertilization rate and hatching rate of GMC was higher in 2022 than that in 2021, the survival rate was decreased and <4.6%. In several previously studies, heterogeneous sperm (common carp or crucian carp) used for the induction of gynogenetic fish generally showed a higher survival rate, such as grass carp (>12.3%), Japanese crucian carp (*Carassius cuvieri*) (>15.7%), bighead carp (*Hypophthalmichthys nobilis*) (>7.36%) and blunt snout bream (~30%) [14,36,37]. Meanwhile, the method of heat shock to induce chromosome doubling, also showed a higher hatching rate and survival rate than cold shock in mrigal carp [24,25]. Therefore, the lower survival rate in our study indicated that water temperature and salinity (salinity of the hatchery water was 2.4‰ in 2022 and 1.9‰ in 2021) may have major effects on the early development of MC larvae, especially since MC shows lower cold resistance [22,38]. Besides, the hatching rate and survival rate of MC eggs may also be influenced by UV irradiation conditions, heterologous sperm and egg quality, cold-shock duration time and activation time [11,14,17,25].

Microsatellites, tandemly repeated sequence motifs that are ubiquitously distributed throughout the eukaryotic genome, are widely used as a genetic marker due to their polymorphic information was easily detected by polymerase chain reaction (PCR) [17,39]. By microsatellite analysis, several previous studies have observed the incorporation of paternal genetic material in some gynogenetic fish including common carp, grass carp, blunt snout bream, silver crucian carp (*Carassius auratus gibelio* Bloch) and rainbow trout (*Salmo gairdneri*) [17,32,40–42]. Similarly, our results also revealed that DNA fragments in GMC were inherited from the paternal parent CC. In addition, a new fragment was appeared and the parent fragment was deleted in GMC (Fig. 4). These results indicated that the sperms from CC inactivated and finally degraded but may left the microchromosomes in MC eggs [30]. And recombination events were occurred which results in DNA from CC (fragments or base loci) was randomly inserted (as well as deletion and mutation) into the nuclear DNA of MC eggs [43–45]. Identification of these DNA variations and exploration of their function in gynogenetic fish are needed in the future.

Water temperature, is widely recognized as a crucial environmental factor that influences the physiology and behavior of aquatic organisms. Although fish can cope with daily or seasonal temperature fluctuations in their aquatic environments, when suffer temperature decreases that exceed their thermal tolerance capability may result in mortality. In this study, we observed that the GMC population can successfully overwintered and adapt to cold water, while the MC fish perished (Fig. 5). The CTMin and T_{LD50} value of GMC was also detected lower than that of MC (Fig. 6), suggesting that artificial gynogenesis improved the cold tolerance of *Cirrhinus mrigala*. The possible reasons for artificial gynogenesis improving the cold tolerance of GMC may include the following:

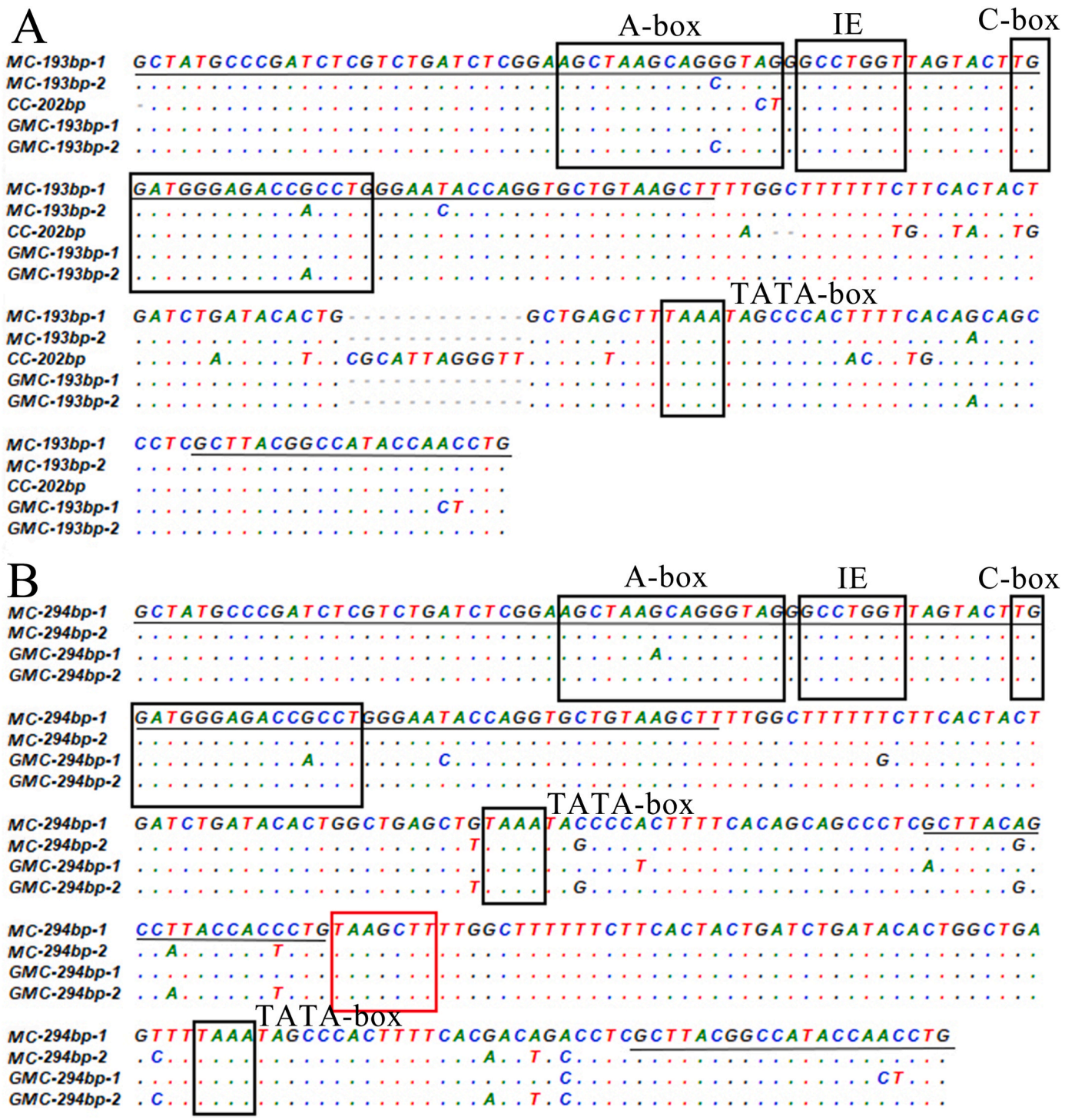

Fig. 3. Nucleotide sequence alignment of sequenced 5S rDNA fragments in MC, CC and GMC. (A) nucleotide sequence alignment of 5S rDNA fragments in CC (202 bp), MC and GMC (193 bp). (B) Nucleotide sequence alignment of 5S rDNA fragments (294 bp) in MC and GMC. The black straight line and red box represent the coding region. The regulatory sequences [A-box (AGCTAAG(A)CAGG(C)GTA(C)G), intermediate element (GCCTGGT), C-box (TGGATGGGAGACCG(A)CCTG) and TATA-box (TAAA)] are shown in black boxes. The hyphens represent insertions/deletions.

1) cold shock, as a selective stress (causing poor-quality eggs to die), improved the development of MC eggs; 2) the increased frequencies of some homologous recessive genes caused a proportion of GMC fish to die; and 3) the DNA fragments of common carp, well known for its robustness to cold tolerance and ability to survive several months of exposure to low temperatures of 0–4 °C, were randomly integrated and inserted into the nuclear DNA of MC eggs and resulting in "hybrid" effect [17,27,32,42]. Besides, cold acclimation, namely the water temperature gradually decreased from October to January, can enable the physiological activities of fish to adapt to a lower temperature and improve their survival rate [41,42]. Interestingly, we also observed the growth of GMC fish was higher than that of MC individuals (Table S1), but slowed as the water temperature decreased. The decline or termination of most vital activities was also observed in many fish when exposed to lower water temperatures. For example, the metabolic rate decreased and energy homeostasis disrupted in zebrafish (*Danio rerio*), orange-spotted

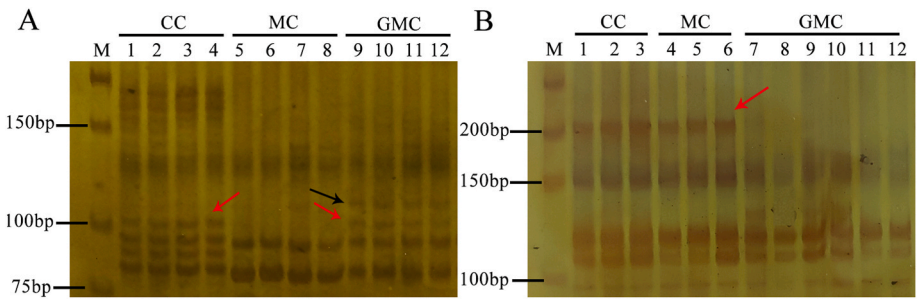

Fig. 4. Microsatellite patterns of MFW1 and CCA15 in CC, MC and GMC fish.
(A) Electropherogram of microsatellite DNA patterns produced by the primer pair MFW1 in CC, MC and GMC. The red arrow indicates the DNA bands derived from CC. The black arrow indicates that the different DNA bands that occurred in GMC. (B) Electropherogram of microsatellite DNA patterns produced by the primer pair CCA17 in CC, MC and GMC. The red arrow indicates the DNA bands shared in CC and MC. M: DNA marker (25–500 bp, Sangon Biotech) 1–12: represent individuals of CC, MC and GMC.

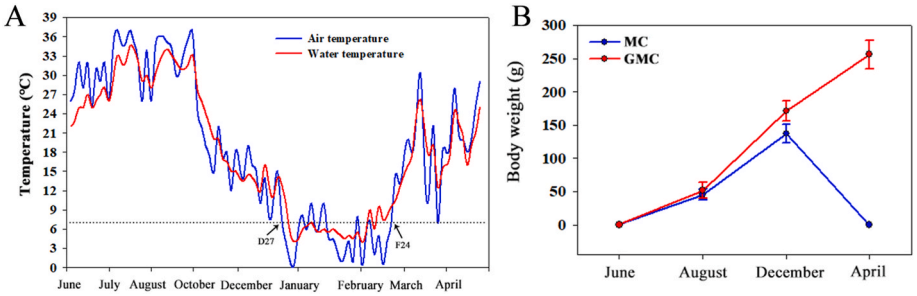

Fig. 5. Temperature variation and growth of MC and GMC during the rearing process. (A) Changes in air (blue line) and water (red line) temperatures from June 2021 to April 2022. (B), Changes in the body weight of MC and GMC from June 2021 to April 2022. The dotted line indicates the temperatures below 10 °C. D27: 27, December 2021; F22, 22, February 2022.

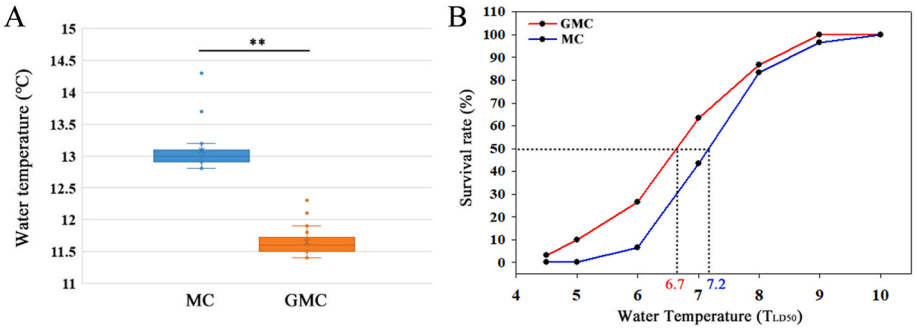

Fig. 6. Cold tolerance of GMC and MC.
(A) Critical thermal minimum (CTMin) of GMC and MC. (B) Lethal temperature (T_{LD50}) of GMC and MC. ** represents a significant difference between the MC and GMC groups.

grouper (*Epinephelus coioides*) and coho salmon (*Oncorhynchus kisutch*) when exposed to low temperature [38,46,47]. In a future study, the regulatory mechanism underlying the adaptation of GMC populations to low water temperature, including genomic variations, methylation, dynamic metabolism and transcription, will be investigated.

In summary, we successfully obtained GMC fish with cold treatment to double the chromosomes after the MC eggs were activated by UV-irradiated common carp sperm. The gonads of GMC showed normal development. No significant differences were found between MC and GMC in terms of appearance, DNA content, chromosome numbers and 5S rDNA gene sequence. The incorporation of heterologous genetic material from male parent was proven in gynogenetic GMC by microsatellite analysis. Interestingly, the cold tolerance (CTMin and T_{LD50}) and growth of GMC were significantly improved compared with those of maternal MC. The results of this study are of great significance for fish breeding and application.

Author contributions

Shaojun Liu and Wuhui Li conceived and designed the research, Zexun Zhou, Qilong Liu, Hongqing Li, Ping Wu and Jie Hu, performed the experiments and sampling, Jisen Su, Shi Wang, Zhongyuan Shen, Lei Zeng analyzed the data, Wuhui Li wrote the manuscript, Min Tao, Chun Zhang, Qinbo Qin revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

All experiments were approved by the Animal Care Committee of Hunan Normal University and followed the stated guidelines of the Administration of Affairs Concerning Animal Experimentation of China. The MC, GMC, and CC individuals used in this research were managed according to the guidelines of the Animal Ethics Committee of the Life Science Institute. In addition, all the experimental fish were anesthetized with 100 mg/L MS-222 (Sigma–Aldrich, St. Louis, MO, United States) before dissection.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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