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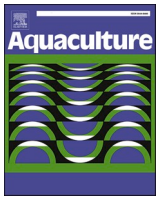

Formation and identification of artificial gynogenetic mandarin fish (*Siniperca chuatsi*) induced by inactivated sperm of largemouth bass (*Micropterus salmoides*)

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ABSTRACT

Gynogenesis is a special method of distant hybridization that has important application value in a variety of areas, such as rapid establishment of pure lines, restoration of endangered species and sexual control. Mandarin fish is an important aquaculture species in China that exhibits sexually dimorphic growth. However, there are few studies on the gynogenesis of mandarin fish at present, and there is no report on gynogenesis induced by cross-family heterologous sperm. In this study, we used the sperm of largemouth bass (*Micropterus salmoides*, LB, Family Centrarchidae, 2n = 46) with UV-inactivated genetic material to stimulate gynogenesis in mandarin fish (*Siniperca chuatsi*, MD, Family Serranidae, 2n = 48) to obtain surviving offspring. To determine whether the offspring obtained by the gynogenetic technique are gynogenetic fish, we analyzed the DNA content, chromosome number, karyotype, countable traits, 5S rDNA and mitochondrial DNA of the offspring. Based on the analysis of the above experimental results, it was found that the offspring was a diploid fish with 48 chromosomes, its karyotype was 6sm + 12st + 30 t, and the 5S rDNA gene included two fragments with sizes of 203 bp and 281 bp. These characteristics were consistent with MD. The DNA content, countable traits, and mitochondrial DNA sequence of the offspring were not significantly different from MD. Afterward, we identified the sex of the gynogenetic mandarin fish (GMD) through sex-specific markers. The results showed that all the mandarin fish obtained in this study are female. The microsatellite pattern showed that GMD contained bands from the male parent. In general, this is the first time that carnivore mandarin fish have obtained full female gynogenesis through gynogenesis. Additionally, this study provides a theoretical reference for the gynogenesis of fish in different subfamilies, and the acquisition of all-female mandarin fish has certain commercial value for production practices.

1. Introduction

Mandarin fish (*Siniperca chuatsi*, MD) is one of the fastest growing fishes in *Siniperca* and is well liked by consumers for its tender, nutritious meat and lack of intermuscular bones (Fang, 1932; Sun et al., 2015). With the growing market demand, MD is gradually becoming an important breeding species in China. However, there are a number of problems in the breeding process. Degradation of germplasm in MD due to excessive inbreeding (Cao et al., 2013). Meanwhile, the growth rate of MD differed by sex. In general, the females of MD grow faster than the males before sexual maturity (Wang et al., 2006). It is an urgent problem to obtain new varieties with better quality to improve the quality of MD and increase its economic benefits.

Distant hybridization refers to the hybridization between two species whose genetic relationship is interspecific or more distant (Zhang et al., 2014). It can combine the genomes of different species together, resulting in significant changes in the phenotype and genotype of the hybrid offspring (Liu, 2022). Gynogenesis is a special method of distant

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hybridization and is also an important technical means to solve the degradation of germplasm resources (Li et al., 2014; Mao et al., 2020). Gynogenesis is divided into natural gynogenetic and artificial gynogenetic processes. Artificially induced gynogenesis involves two key steps: inactivation of the genetic material of the sperm and doubling of the chromosomes of the egg (Chen et al., 2018; Zhang et al., 2014). As an important technique for chromosome manipulation in breeding, artificially induced gynogenesis has important applications in a variety of areas, such as rapid establishment of pure lines (Wang et al., 2004), recovery of endangered species (Grunina et al., 2010) and sexual control (Luo et al., 2011). In recent years, there has been an increasing amount of research on artificial gynogenetics, which has been successfully applied in grass carp (*Ctenopharyngodon idella*) (Luo and Liu, 1991), crucian carp (*Carassius auratus*) (Luo, 1991), silver carp (*Hypophthalmichthys molitrix*) (Zou et al., 2004), carp (*Cyprinus carpio*) (Yosefian, 2005), Lanzhou catfish (*Silurus lanzhouensis*) (Li et al., 2022a), etc. In the study of gynogenesis, some researchers found that heterologous sperm can exchange limited genetic material with the egg while stimulating the development of the egg, which is the "heterosperm effect"(Thorgaard et al., 1985; Yan et al., 2005). This effect often brings some changes to the offspring at the DNA molecular level. Phenotypically, gynogenetic offspring are likely to exhibit some advantageous traits, such as faster growth and greater disease resistance (Liu, 2022; Mao et al., 2020; Zhao et al., 2011). However, there are few studies on the gynogenesis of MD. The sperm currently used to induce female reproduction in MD is mainly intrageneric or even conspecific, such as *Siniperca chuatsi* (Li et al., 2022b) and *Siniperca scherzeri* (Cheng et al., 2019). There are no reports of the use of sperm from interfamilial fish to induce gynogenesis in MD.

5S rDNA has a very conserved CDS region and a highly variable NTS region (Liu, 2022). Because of this feature, 5S rDNA, as a species-specific or population-specific molecular marker, is widely used in the evolution of species (Campo et al., 2009; Gong et al., 2021; Liu et al., 2021). Mitochondrial genes are generally considered to be maternally inherited and are widely used to analyze the evolutionary relationship between parents and offspring (Wang et al., 2020; Xia et al., 2018). Microsatellite DNA is a series of core sequences with a length of 2–6 base pairs, so it is also called simple sequence repeats (SSRs) (Hansen et al., 2001). It is often used in genetic breeding analysis of animals (Wang et al., 2022).

There have been no reports of successful crossbreeding of mandarin fish and largemouth bass to obtain surviving offspring. We also tried to cross the two but failed to obtain viable offspring after many attempts. In this study, we induced artificial gynogenesis in mandarin fish (*Siniperca chuatsi,* MD) eggs by using largemouth bass (*Micropterus salmoides*, LB) sperm treated with UV light to obtain offspring (GMD). We determined whether the GMD was a gynogenetic offspring by comparing the genetic characteristics of the GMD with the parents, such as countable traits, ploidy, 5S ribosomal DNA, mitochondrial DNA, microsatellites, etc. Subsequently, we identified the sex of GMD by specific markers. In conclusion, the acquisition of gynogenetic MD has important reference significance for the gynogenesis of MD.

2. Materials and methods

2.1. Ethics statement

The procedures were conducted in accordance with the approved guidelines. Experimental fish were housed in different open pools (2 ha) with a suitable pH (7.0–8.5), water temperature (22–24 °C), dissolved oxygen content (5.0–8.0 mg/L) and adequate forage at the Xuefeng Mountain Fish Breed Base of Wugang, Hunan, China. The fish used as the samples were anesthetized with 100 mg/L MS-222 (Sigma–Aldrich, St. Louis, MO, United States) before dissection.

2.2. The GMD formation process

This experiment was conducted in the Xuefeng Mountain Fish Breed Base of Wugang, Hunan, China. Sexually mature MD and LB were randomly selected as parents during the 2021 fish breeding season (May to July). The females were given the full dose of domperidone 4.0–5.0 mg + LHRH-A2 5.0–6.0 μg in one injection. The males were given the full dose of domperidone 2.0–2.5 mg + LHRH-A2 2.5–3.0 μg in one injection and continuously for one week. After an action period of 16–26 h, mature sperm were obtained by squeezing the abdomen of the LB and treating it with UV light to inactivate the genetic material. The distance between the UV lamp and the shaker was 25 cm, and the irradiation time was 25–30 min. The treated sperm was stored at 4–6 °C away from light. The abdomen of the MD was then squeezed to produce mature eggs, and the UV-treated sperm were mixed with the eggs. Using the wet fertilization method, 1–3 min after fertilization, the eggs were poured into cold water at 4–6 °C and treated with cold shock for 15–30 min. At the end of the cold treatment, all eggs were transferred to the circular ring channel (d = 2.5 m) and hatched in micro-running water at 22–24 °C. The hatched fish were transferred to concrete ponds (length × width × height = 3.5 m × 2.5 m × 2.0 m) for further culture. After the embryos were fertilized, at least 100 embryos were randomly selected at regular intervals, and the development of the embryos was observed and recorded under a stereomicroscope. The time of embryonic development was based on the fact that >60% of the embryos have clearly entered this stage of development (Mi et al., 2009). Approximately 3000 embryos were randomly selected from all embryos to check the fertilization rate (number of embryos/number of eggs at the gastrula stage×100%) and hatching rate (number of fry hatched/number of eggs×100%).

2.3. Measurement of countable traits

We randomly selected 20 (60 in total) three-month-old MD, LB, and GMD fish for morphological examination. We recorded the numbers of scales in the lateral line, upper lateral line and lower lateral line, and the numbers of rays in the dorsal fin, abdominal fin, and anal fin.

2.4. Measurement of DNA content

The DNA contents were used to test the ploidy level of the GMD. The DNA content of the erythrocytes of MD and GMD was measured using a flow cytometer (Cell Counter Analyzer, Partec, Germany), and in each group, 20 fish (three months old) were sampled. The blood samples were treated following the method described in the published paper (Liu et al., 2007). The DNA content of each sample was measured under identical conditions. The DNA content of red crucian carp was used as the control.

2.5. Preparation of chromosome spreads

To determine karyotypes, chromosome preparation was carried out on peripheral blood cells of 10 MD, 10 LB, and 10 GMD fish according to the procedures reported by Liu et al. (2013b).We made minor modifications based the references. The cells were cultivated for 70 h at 25 °C in 5% CO_2, and colchicine was added at a final concentration of 0.07–0.14 μg/mL 3 h before terminating the culture. After the cells were collected by centrifugation, a KCl solution with a concentration of 0.0375 mol/L was used for hypotonicity for 30 min. The chromosome shape and numbers were analyzed under a light microscope. For each fish sample, 100 metaphase spreads (10 spreads per sample) were examined. Chromosomes were classified on the basis of their long-arm to short-arm ratios according to the reported standards (Levan et al., 1964).

2.6. Molecular organization of 5S rDNA

Total genomic DNA was extracted from the peripheral blood cells of 10MD, 10 LB and 10 GMD (the age of each fish is three-month-old) using the Universal Genomic DNA Extraction kit (TaKaRa, Dalian, China). Specific primers (5S-F: 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5S-R: 5′-CAGGTTGGTATGGCCGTAAGC-3′) were used to amplify the 5S rDNA gene and untranslated regions (NTS) from MD, LB and GMD fish. PCRs and sequencing were performed as described by Qin et al. (2010). Sequences were analyzed using BioEdit software v7.0 (Hall et al., 2011).

2.7. Mitochondrial DNA sequence and phylogenetic analysis of GMD

Total genomic DNA from the peripheral blood cells of 10 GMD by routine approaches was used as a template (Zhang et al., 2020). Complete mt DNA sequences were amplified from the whole genome of GMD according to the primers and PCRs reported by Chen et al. (2012). The primers are listed in Table 5 (primers marked with "*" on the upper right of the primer name were newly designed). Sequences were analyzed using BioEdit software v7.0 (Hall et al., 2011). The full-length mitochondrial DNA of mandarin fish (GenBank accession no. JF972568.1) and largemouth bass (GenBank accession no. NC_014686.1) were retrieved from the GenBank database. Mitochondrial structure mapping was performed by an online program (http://mitofish.aori.u-tokyo.ac.jp/annotation/input/).

2.8. Microsatellite DNA

Total genomic DNA was isolated from whole blood collected from the caudal vein of 10 MD, 10 LB and 10 GMD individuals using a standard phenol–chloroform procedure. The DNA concentration and quality were assessed using agarose gel electrophoresis. We amplified the loci of MD, GMD and LB using 33 pairs of microsatellite primers, but only one (JZL108: F:5'-GTGACAGATGAGCGGAGAA-3′ and R: 5′- GATGCTTGAGATACGACTA −3′) conformed our requirements. All primers were from reported articles (Liang et al., 2008; Lutz-Carrillo et al., 2008). Primer sequences are given in Supplementary Table 1 (Table S1). Polymerase chain reaction (PCR) conditions and components followed a previous study (Liu et al., 2010). We used electrophoresis on 8% polyacrylamide gels (PAGE) to separate amplification products, which were sized against a pBR322 DNA/Mspl ladder (Tiangen).

2.9. Sexual identification of GMD

Venous peripheral blood was randomly collected from the tails of 30 GMD individuals and then a Takara Minibest Universal Genomic DNA Extraction Kit v5.0 was used to extract DNA (Zhang et al., 2020). Sex-specific bands from male controls and GMD were amplified according to sex-specific primers (Forward: TTCAGCAATAAGCGAGACAATG, Reverse: CCGATCTGTTGGACCTGTTTAG) reported by Wen et al. (2022). PCRs were performed according to the PCR program in Wen et al., 2022. PCRs were conducted with 0.25 units of Ex Taq DNA Polymerase (Takara), 1 μL of 10× PCR Buffer, 100 μM dNTP mixture, 1 μL of 50 ng/μl genomic DNA, and 0.1 μM of each primer in a total volume of 25 μL. The PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s for 35 cycles. Finally, PCR products were run on 2.0 agarose gels.

3. Results

3.1. Fertilization rate and hatching rate of GMD

We explored the optimal conditions for LB sperm to induce MD for gynogenesis by adjusting the fertilization time (1–3 min) and cold shock time (15–30 min) (Table 1). Under the conditions of fertilization for 2 min and cold shock duration of 20–25 min, the fertilization rate and hatching rate reached 10.12% and 2.06%, respectively, which were the best conditionsamong all experimental gradients, and the hatched fry had almost no deformity. At 2 mins of fertilization, the fertilization rates of the other two cold shock durations were 5.07% and 1.53%, respectively, but the fry did not hatch normally, resulting in a hatching rate lower than 0.05%. In the case of fertilization for 1 min and 3 min, the hatching rate of the three cold shock durations was 0%, and the fertilized eggs basically died before the gastrula stage. The crossing procedure to produce GMD is outlined in Fig. 1.

Table 1
Effects of different fertilization times and cold shock times on gynogenesis in GMD.

Fertilization time	Cold shock time	Number of experimental eggs	Fertilization rate	Hatching rate
1 min	15–20 min	10,000	0%	0%
	20–25 min	10,000	0%	0%
	25–30 min	10,000	0%	0%
2 min	15–20 min	10,000	5.07%	0.05%
	20–25 min	10,000	10.12%	2.06%
	25–30 min	10,000	1.53%	0%
3 min	15–20 min	10,000	0%	0%
	20–25 min	10,000	1.04%	0%
	25–30 min	10,000	0%	0%

3.2. Embryonic development process of GMD

To clarify the developmental process of GMD embryos, we recorded the time from development to hatching of GMD embryos (Fig. 2). The duration of MD from fertilization to hatching was 71 h 40 min (Table 2), which was consistent with previous research results. Moreover, the developmental stages and embryo morphology of GMD are consistent with previous research results (Liu et al., 2013a). Interestingly, the time from fertilization to hatching of GMD was 66 h 20 min, which was 5 h 20 min shorter than that of MD. Among them, the time difference between GMD and MD from the blood circulation stage to the hatching stage was the largest, with a difference of 3 h 26 min. The time difference between the other stages is basically within 30 min.

3.3. The countable traits of GMD

The countable traits of MD, GMD and LB are shown in Table 3. The results showed that the number of abdominal fins of MD, GMD and LB is the same, all being I + 5. In addition, the number of dorsal and anal fins of the three species did not differ significantly. Notably, the number of lateral line scales, upper lateral line scales and lower lateral line scales do not differ much between MD and GMD but are significantly more numerous than in LB.

3.4. DNA content of GMD

To clarify the DNA content of GMD, we measured the DNA content of MD and GMD with red crucian carp as a control (Fig. 3). The DNA content results of MD and GMD are shown in Supplementary Table 1. The determined DNA content of GMD was not much different from that of MD, and the ratio of the two was 1:1, which means that GMD was diploid like MD.

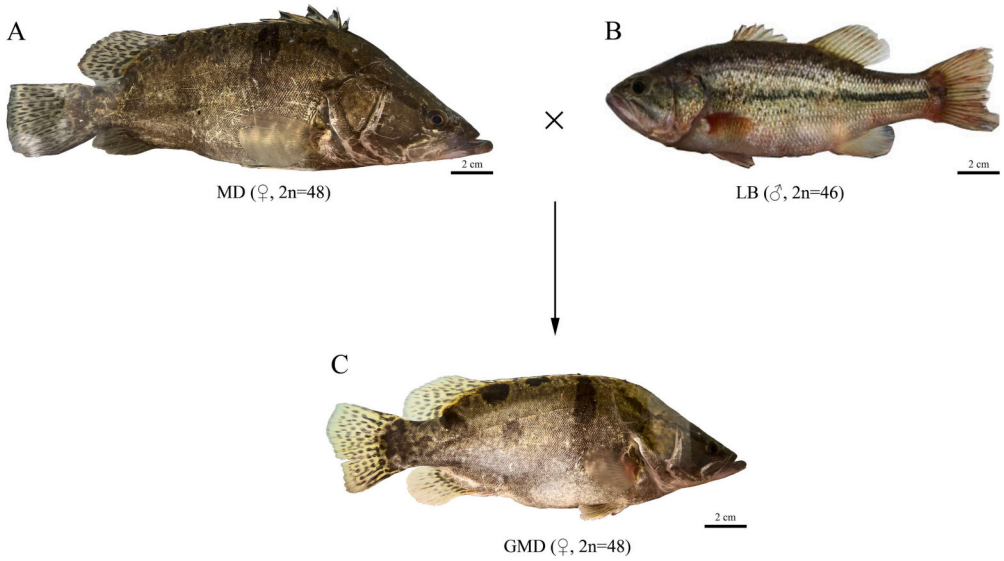

Fig. 1. Formation procedure and appearance of MD, LB and GMD fish. (A) The appearance of MD. (B) The appearance of LB. (C) The appearance of GMD.

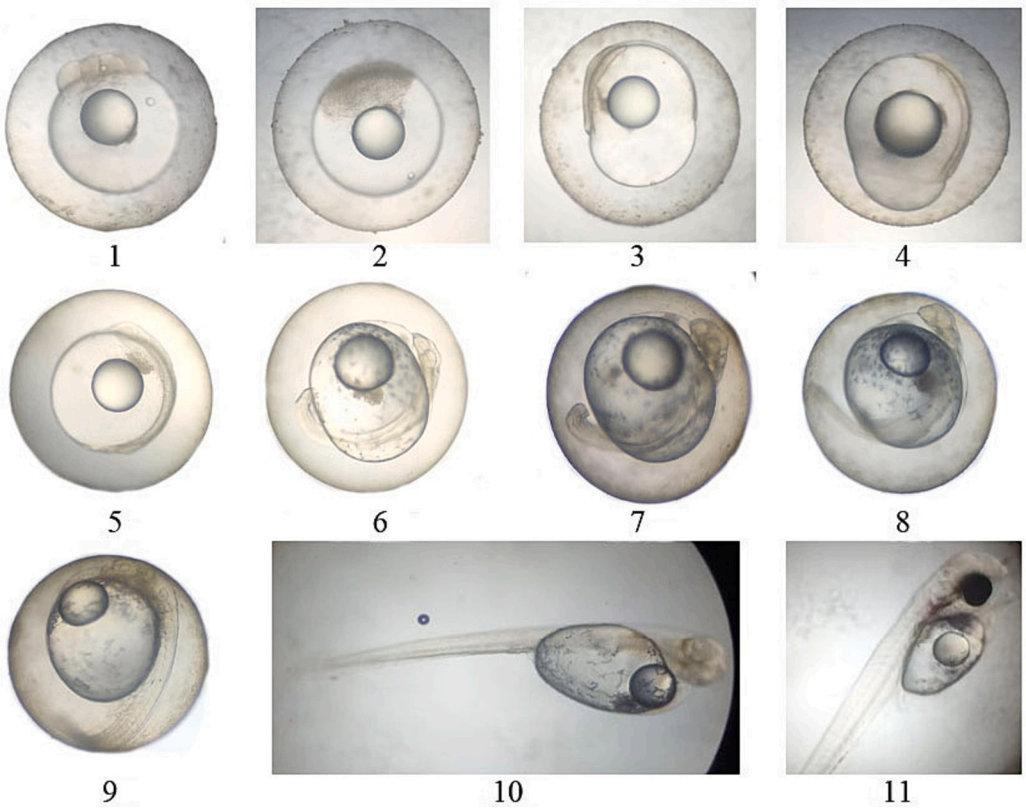

Fig. 2. Embryo development of GMD. 1. Multicellular stage; 2. Blastula stage; 3. Gastrula stage; 4. Neurula stage; 5. Myotome formation stage; 6. Tail bud stage; 7. Muscle effect stage; 8. Heart beat stage; 9. Blood circulation stage; 10. Hatching stage; 11. 24 h after hatching.

3.5. Chromosome numbers and karyotypes

To examine whether the chromosome number and karyotype of GMD are consistent with those of MD, we randomly selected 100 metaphase spreads of each of the three, counted the chromosome numbers of the three and determined their karyotypes (Table 4 and Fig. 4). The results showed that 89% of MD cells had 48 chromosomes in the nucleus with a karyotype formula of 6sm + 12st + 30 t (Fig. 4A-B); 91% of LB cells had 46 chromosomes in the nucleus with a karyotype formula of 2 m + 2st + 42 t (Fig. 4C-D); and 87% of GMD cells had 48 chromosomes in the nucleus with a karyotype formula of 6sm + 12st + 30 t (Fig. 4E-F). The above results showed that the chromosome numbers and karyotypes of GMD and MD were identical.

3.6. Molecular organization of 5S rDNA

As one of the genetic molecular markers, we amplified 5S rDNA gene sequences in the whole genomes of MD, LB and GMD. Agarose gel

Table 2
Comparison of the length of time after fertilization at different developmental stages of GMD and MD.

Developmental stages	After fertilization Length of time	
	GMD	MD
Multicellular stage	2 h 10 min	2 h 30 min
Blastula stage	5 h 10 min	5 h 35 min
Gastrula stage	15 h 30 min	16 h 10 min
Neurula stage	18 h 35 min	19 h 35 min
Myotome formation stage	19 h 00 min	20 h 06 min
Tail bud stage	22 h 20 min	23 h 28 min
Muscle effect stage	38 h 04 min	39 h 24 min
Heart beat stage	40 h 04 min	41 h 39 min
Blood circulation stage	43 h 30 min	45 h 24 min
Hatching stage	66 h 20 min	71 h 40 min

Table 3
Comparison of the countable traits in MD, GMD and LB.

Fish type	lateral line scales	Upper lateral line scales	Lower lateral line scales	Dorsal fins	Abdominal fins	Anal fins
MD	112–116	31–32	53–55	XI+12–14	I + 5	III + 9–11
GMD	111–114	30–32	52–55	XI+12–14	I + 5	III + 9–10
LB	63–66	7–8	17–20	X-XI+13–14	I + 5	III + 10–11

electrophoresis showed that all three had two bands (Fig. 5), and the positions of the two bands of GMD and MD were consistent. To determine the sequence of each 5S rDNA gene, we randomly selected three samples to clone the 5S rDNA of each fish species and sequenced 10 clones from each band in each sample (180 clones in total). The sequence results showed that the sizes of the two fragments from MD were 203 bp and 281 bp, the sizes of the two fragments from LB were 313 bp and 712 bp, and the lengths of the two fragments from GMD were the same as those of MD. In MD, there were two monomeric 5S rDNA classes (class I: 203 bp; class II: 281 bp), a result consistent with previous studies (Wang et al., 2022). In LB, two monomeric 5S rDNA classes (class III: 313 bp; class IV: 712 bp) were characterized by three NTS types (class III: 184 bp, class IV: 373 bp and 187 bp). In GMD, there are two monomeric 5S rDNA classes (class V: 203 bp; class VI: 281 bp), characterized by two NTS types (class V: 83 bp, class VI: 161 bp) (Fig. 6, Fig. 7). By comparing the sequence similarity, we found that the 5S rDNA sequence similarity of GMD and MD was 100%.

3.7. Mitochondrial DNA sequence and phylogenetic analysis of GMD

To ascertain the homology of the mitochondrial DNA sequence of GMD with MD and LB, we amplified the mitochondrial DNA sequence of GMD. The gene distribution is shown in Fig. 8. The full length of the GMD mitochondrial DNA obtained by sequencing and splicing was 16,497 bp. The full-length mitochondrial DNA of MD (GenBank accession no. JF972568.1) and LB (GenBank accession no. NC_014686.1) was 16,496 bp and 16,486 bp, respectively. Compared with the two, the full-

Table 4
Chromosome numbers in MD, GMD and LB.

Fish type	Number of metaphase photographs	Distribution of chromosome number				
		<46	46	47	48	>48
MD	100	2	3	1	89	5
GMD	100	1	2	2	87	8
LB	100	5	91	1	1	2

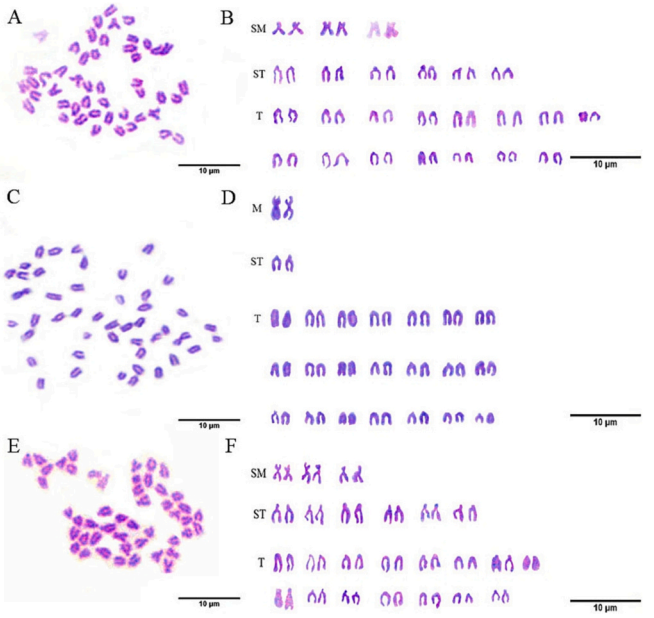

Fig. 4. Chromosomal traits of MD, LB and GMD fish. (A-B) The chromosomal numbers and karyotype of MD. (C-D) The chromosomal numbers and karyotype of LB. (*E*-F) The chromosomal numbers and karyotype of GMD.

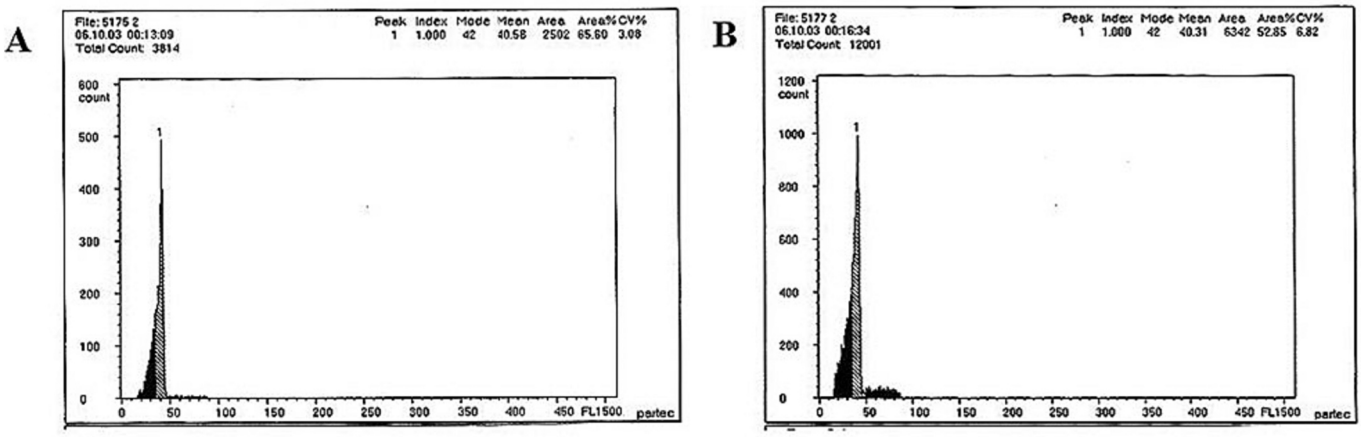

Fig. 3. Cytometric histograms of DNA fluorescence for MD and GMD. (A): MD, (B): GMD.

Table 5
Mitochondrial full-length amplification primer sequence of GMD.

Primers	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)
P-mt1	TTCTTCGCCTTCCACTTCCTCT	AGGCGGATAGCAGGTGTAAGGT
P-mt2	AGGACTTGGCGGTGCTTTAGAT	CGTTGTGCCATTCATACAGGTC
P-mt3	CACCTTACACCTGCTATCCG	ATGTTACGACTTGCCTCCC
P-mt4	AGACGAGAAGACCCTATGGAGC	TAGATGGGCGATTGAGGAGTAG
P-mt5	AAGCCTCGCCTGTTTACC	CTTCTTTTCGGTCCTTTCGT
P-mt6	TACTTTCTTACCCAAGCCACCG	GAGGCAAGAAGGAGAAGGAAGG
P-mt7	GCAGAACTAAGCCAACCAG	CAGTAGGGATTGCGATGA
P-mt8	TTCCTTCTCCTTCTTGCCTCTT	CGTGGTCGTGGAAATGAAGG
P-mt9	TAATGGCACATCCCTCAC	AGGGCTAATAGTCGGTTGT
P-mt10	AGCCTCCCTTGAATCCTTT	GCTCAGAGGAAGGTGGTTAG
P-mt11	CATCTGAGCACCATTTCGG	TGGGTTCGTTCGTAGTTGG
P-mt12	CCTCTGACTCCCAAAAGCAC	ATAGGGCGACTGGGGTAAAG
P-mt13	CGTATTCACAACCCTCACAC	AAGTGGTGGGTGGTTAGTTG
P-mt14–1*	CACCCACCACTTCTCCAA	GAGAGCCAAAGTTTCATCAG
P-mt14–2*	GAAGCAACCGCAACCAAC	GAGGAAGTGGAAGGCGAAG

Table 6
Identity of the four mitochondrial protein-coding genes for MD, LB, and GMD (GMD1–3).

	CO1		ND4		ND5		Cytb	
	MD	LB	MD	LB	MD	LB	MD	LB
GMD-1	99.8%	83.9%	99.2%	79.6%	99.7%	77.8%	99.6%	81.3%
GMD-2	99.4%	83.7%	99.2%	79.5%	99.7%	77.8%	99.5%	80.9%
GMD-3	99.6%	83.9%	99.2%	79.6%	99.6%	77.9%	99.5%	81.0%

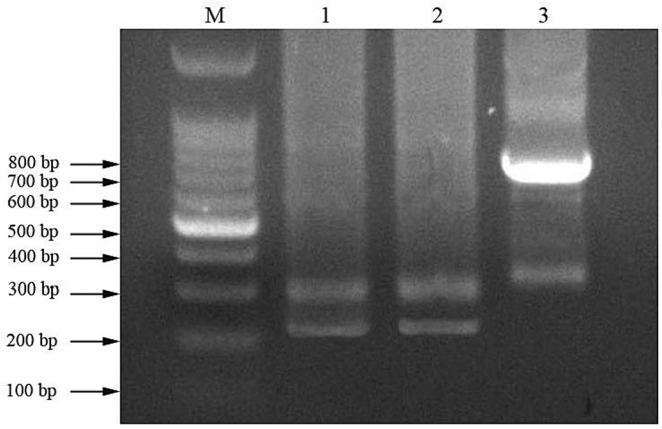

Fig. 5. PCR amplification map of 5S rDNA among MD, GMD and LB lines. Lanes 1–3: DNA bands amplified from MD, GMD and LB.

length mitochondrial DNA of GMD was closer to that of MD. All three had two rRNAs, 22 tRNAs and 13 protein-coding genes, and the GMD was 99.5% homologous to the MD and 83.0% homologous to the MD, a result consistent with matrilineal inheritance. Next, we further compared the four protein-coding gene sequences of CO1, NADH4, NADH5 and Cytb among the three. The results showed that all four protein-coding gene sequences of GMD were >99% similar to those of MD (Table 6), and sequence comparison revealed individual base differences, insertions or deletions (Fig. 9).

3.8. Microsatellite DNA

To detect microsatellite DNA patterns in MD, LB and GMD individuals, we amplified fragments with JZL108 primers (Fig. 10). In MD, we found seven obvious homologous fragments, with sizes of approximately 527 bp, 450 bp, 350 bp, 309 bp, 270 bp, and 147 bp. In GMD, we found homologous fragments of the same size as MD, although there was a difference in the colour of the band between the two. Importantly, there was a band consistent with LB at approximately 280 bp and 320 bp in GMD but not in MD. This result indicated that GMD was basically consistent with MD, but there were fragments of LB inserted.

3.9. Sexual identification of GMD

The above research results basically indicated that GMD was a gynogenetic offspring. To further determine whether the GMD was female or male, we used sex-specific markers of MD to identify GMD. The agarose gel results showed that the male control had a bright band at approximately 1000 bp, while the GMD did not (Fig. 11). This result means that the randomly selected GMDs were all female.

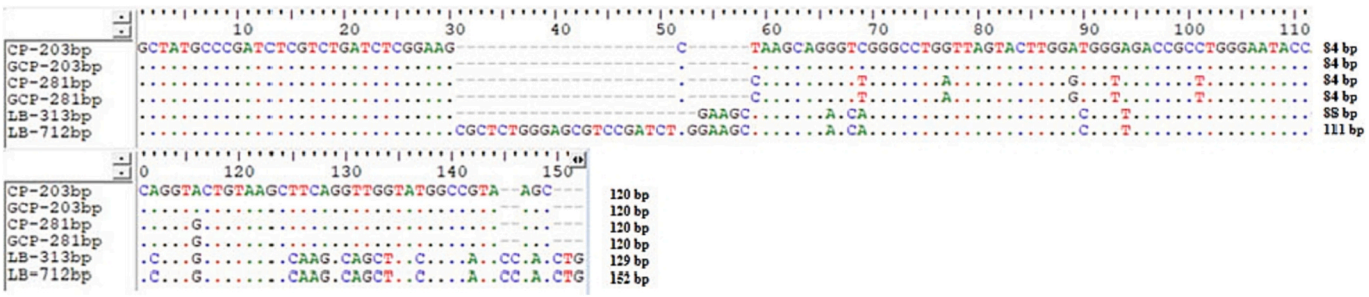

Fig. 6. Comparison of complete 5S coding regions from MD, GMD and LB fish. Dots indicate identical nucleotides.

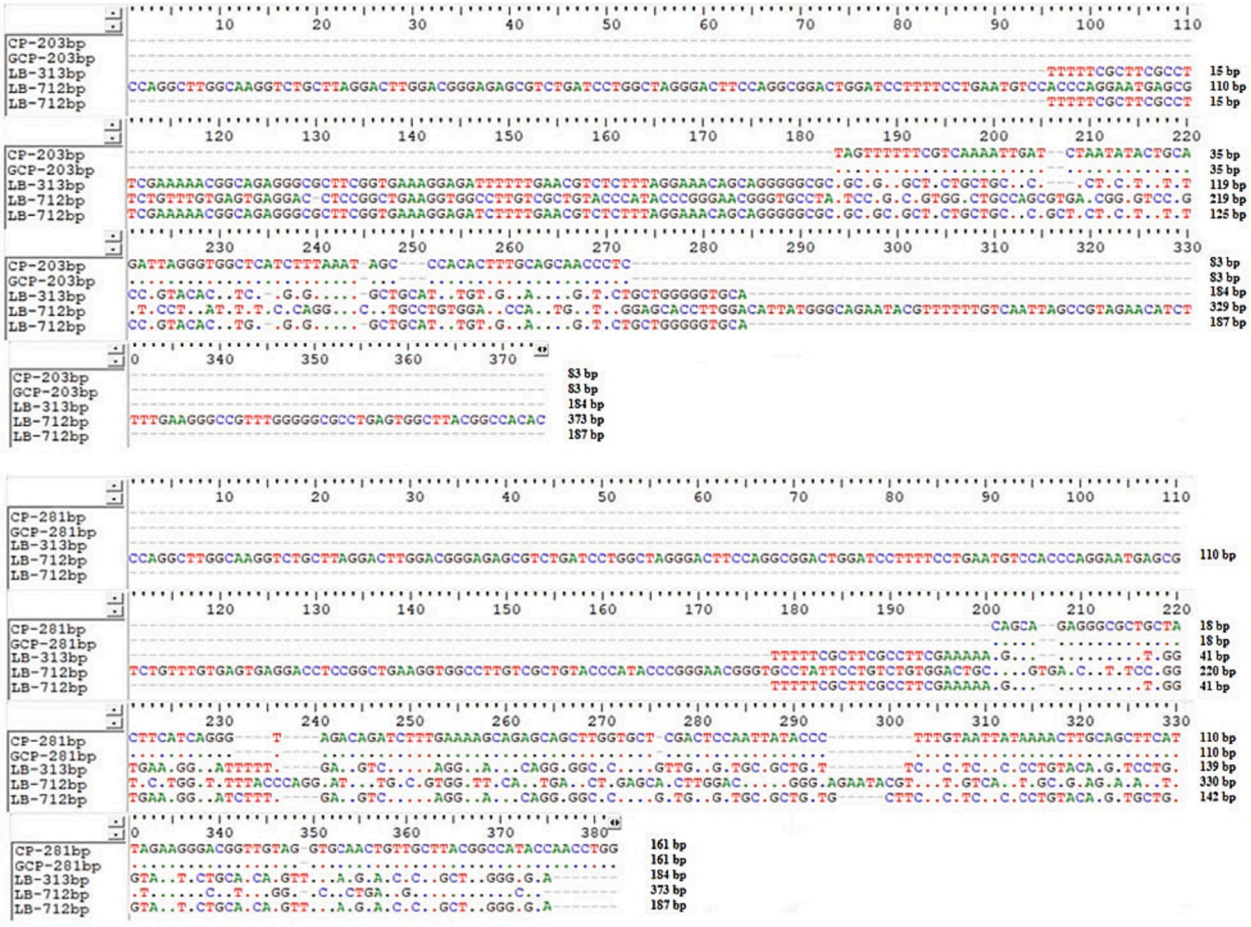

Fig. 7. Comparison of the NTS sequences from MD, GMD and LB fish. Dots indicate identical nucleotides.

4. Discussion

In this study, we used LB sperm to induce MD gynogenesis and explored the optimal fertilization time and cold shock time for gynogenesis of MD. The surviving offspring were successfully obtained under the best conditions, and the development process and development time of the embryos were recorded. Through methods such as DNA content, chromosomes, countable traits, 5S rDNA, and mitochondrial DNA, it was comprehensively proven that the offspring were gynogenetic MD. The microsatellite pattern showed that GMD contained a band from the male parent. In addition, we identified the sex of the gynogenetic offspring, thus confirming that the gynogenetic MD is an all-female population.

Fertilization time and cold shock time are key factors for the successful induction of gynogenesis. In our study, embryo survival decreased with prolonged fertilization time and cold shock time. This may be due to the late treatment of the cold shock, and the second polar body was discharged after the egg was fertilized, resulting in unsuccessful chromosome doubling. At the same time, long-term cold shock treatment stimulates eggs too much, resulting in increased mortality (Gong et al., 2019; Wang et al., 2006). In addition, when the fertilization time was 1 min, the embryo fertilization rate and hatching rate were both 0%. According to previous reports, this may be due to the short fertilization time; some embryos were not fully fertilized, and the remaining embryos died after undergoing cold shock treatment (Cheng et al., 2019; Yu et al., 2012).

Distant hybridization can integrate excellent traits from different species, form offspring with obvious hybrid vigor and even form new species (Qin et al., 2019; Wang et al., 2022; Wu et al., 2019). As a special method of distant hybridization, gynogenesis is one of the most effective techniques in fish germplasm improvement. For example, the hypoxia tolerance of blunt snout bream induced by the sperm of koi carp and topmouth culter was significantly stronger than that of common blunt snout bream (Gong et al., 2019). The growth rate, muscle texture, and antioxidant capacity of gynogenetic blunt snout bream induced by MD sperm were significantly better than those of common blunt snout bream (Wang et al., 2022; Wu et al., 2022). In our study, the developmental stages and morphology of GMD were consistent with those of MD, but the speed of GMD from fertilization to hatching was 5 h 20 min faster than that of MD. This may be related to the strict cold shock elimination mechanism in the process of gynogenesis. During this process, most embryos with low quality, weak activity, and weak stress resistance gradually died, while embryos with high quality, high activity, and strong stress resistance survived. In addition, it is also possible that after the embryo undergoes cold shock, the chromosomes double, the homozygosity of the embryonic genome is improved, and the germplasm is improved accordingly so that the development speed is faster (Mao et al., 2020).

5S rDNA, as a genomic DNA marker, is specific among species and is often used to elucidate the evolution and genetic relationship between species. When comparing the 5S rDNA sequences of the hybrid offspring with those of their parents, it was found that the coding region sequence and NTS sequence of the hybrid offspring inherited the genetic characteristics of their parents (Liu et al., 2021; Qin et al., 2010; Wu et al., 2019). In our study, the 5S rDNA sequence of GMD was consistent with that of MD in both the coding region and the NTS region, and there was no base difference. This shows that the 5S rDNA sequence of MD was completely inherited from MD. Fish mitochondria have the characteristics of maternal inheritance, so they are widely used in population

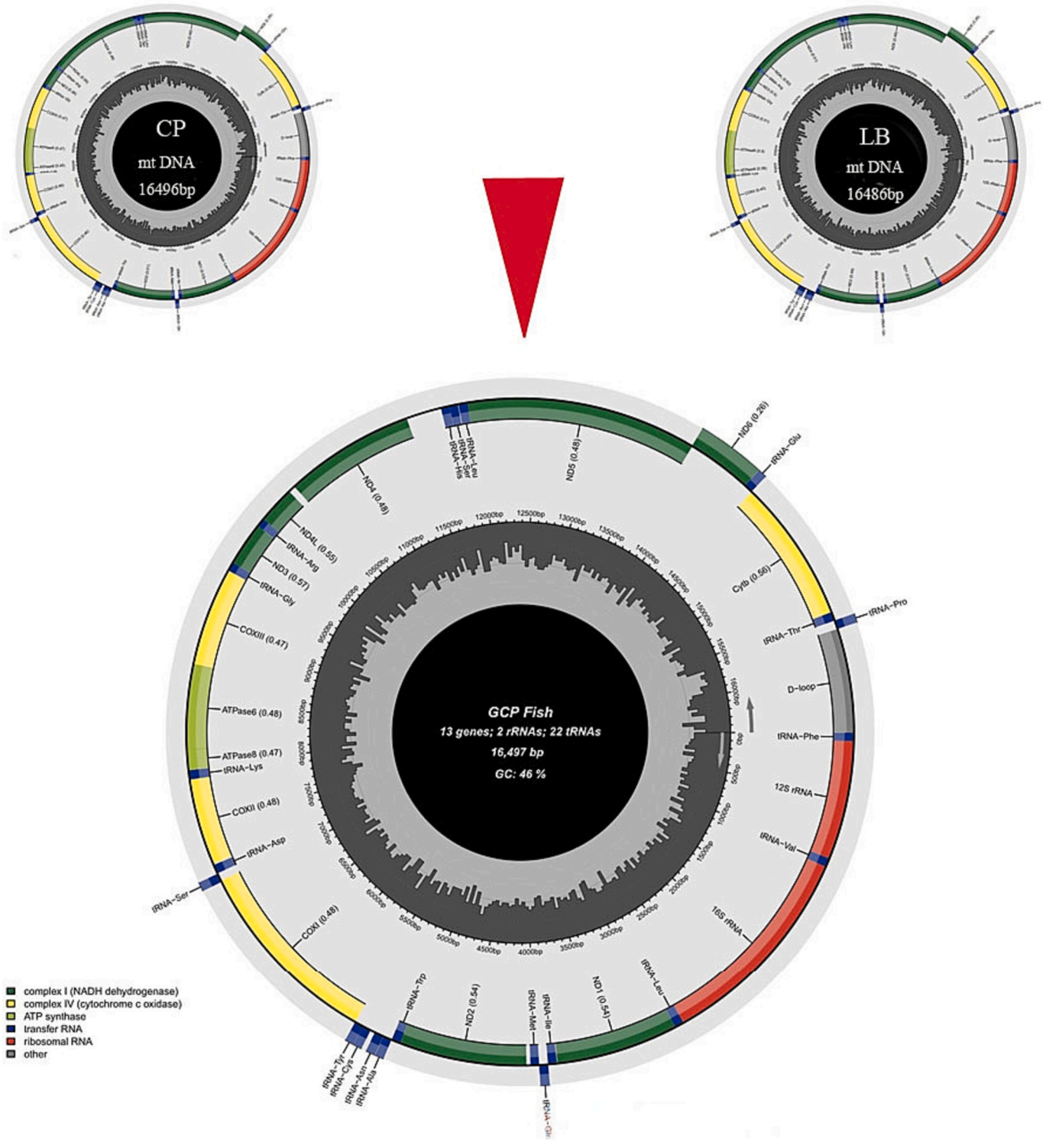

Fig. 8. Mitochondrial structures of MD, LB and GMD.

genetics and evolution analysis (Guo et al., 2004). In this study, the mitochondrial structure of GMD was consistent with that of MD, and the full length of its mitochondrial DNA sequence was very close to that of MD. Previous researchers explored the phylogenetic information contained in 13 protein-coding genes and believed that ND2, CO1, ND4, ND5, and Cytb had better phylogenetic information (Zardoya and Meyer, 1996). In recent years, many researchers have also used these protein-coding gene sequences to explore the phylogeny of fish and elucidate their origin (Giusti et al., 2017; Morgan et al., 2012). Our results showed that the identity rates of the four protein-coding genes (CO1, ND4, ND5, and Cytb) were all above 99%, which was in line with the characteristics of maternal inheritance. During the sequence analysis process, individual base differences were found, but there was no obvious pattern, which may be caused by the different sources of the parents and certain base errors in the sequencing process.

Many previous studies have held that the genetic material of

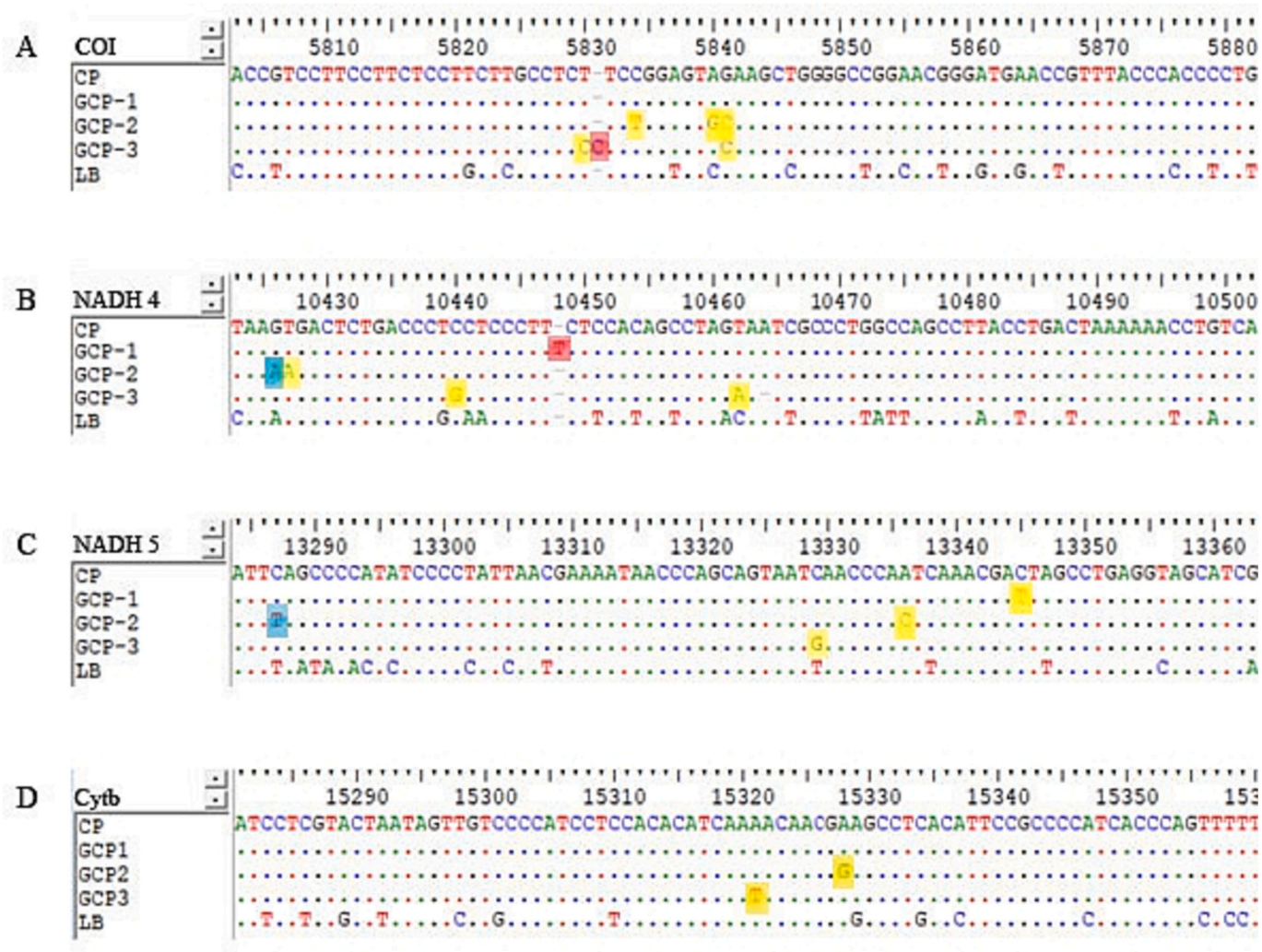

Fig. 9. Nucleotide sequence alignment of the mitochondrial protein-coding genes for MD, LB and the three GMDs (GMD1–3). A-D, Partial nucleotide sequence alignment for CO I, NADH 4 (ND4), NADH 5 (ND5) and Cytb. The dots indicate sequence identity, and the hyphens represent insertions/deletions. Yellow denotes bases that are different from both MD and LB; blue denotes the same base as LB; red denotes inserted bases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gynogenetic offspring all came from the maternal line until the discovery of the "heterosperm effect" (Gui and Zhou, 2010; Schartl et al., 1995). Heterologous sperm can exchange limited genetic material with the eggs while activating the development of the eggs, resulting in changes in the DNA molecular level of the offspring and even phenotypic changes. For example, faster growth is associated with stronger disease resistance (Li et al., 2014; Liu, 2022; Mao et al., 2020). In the microsatellite map, we found that there were bands consistent with LB in GMD, which indicated that GMD may also have inherited some DNA fragments from LB but not in MD. It may be that when LB sperm activated MD egg development, some DNA fragments were randomly integrated and inserted into the nuclear DNA of the eggs. The same situation as in this study was also found in previous studies on gynogenetic offspring (Gong et al., 2019; Lu et al., 2006; Mao et al., 2020).

In conclusion, we have obtained a new type of gynogenetic MD by stimulating gynogenesis with UV-inactivated largemouth bass sperm. By analyzing the DNA content, chromosome number, karyotype and genetic composition of GMD, we determined that GMD are gynogenetic diploid fish with 48 chromosomes. Its genetic composition is basically the same as that of the female parent, and there is also the phenomenon of the "heterosperm effect". In addition, sex-specific markers indicate that GMD are an all-female population. The normal development of GMD proves the possibility that heterologous sperm between subfamilies can stimulate MD gynogenesis. As a successful case, it enriches the theoretical knowledge of gynogenesis in cross-family species, and provides a certain reference for the subsequent gynogenesis of other species in cross-family or even more distant. In addition, the acquisition of GMD can be used to explore the specific molecular mechanism of the formation of gynogenetic offspring in cross-family species, such as growth and development, stress resistance, etc., which has certain value for scientific research and production practices.

Author statement

S.L and P·W conceived and designed the experiments. P·W, Y.Z, W.J and C·W performed the experiments including breeding fish, DNA extraction, and bioinformatics analysis. M.W worked for fish sex-specific primers. R.Z and C.T are responsible for the procurement of reagents. All authors were involved in preparing and writing the manuscript and approved the final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

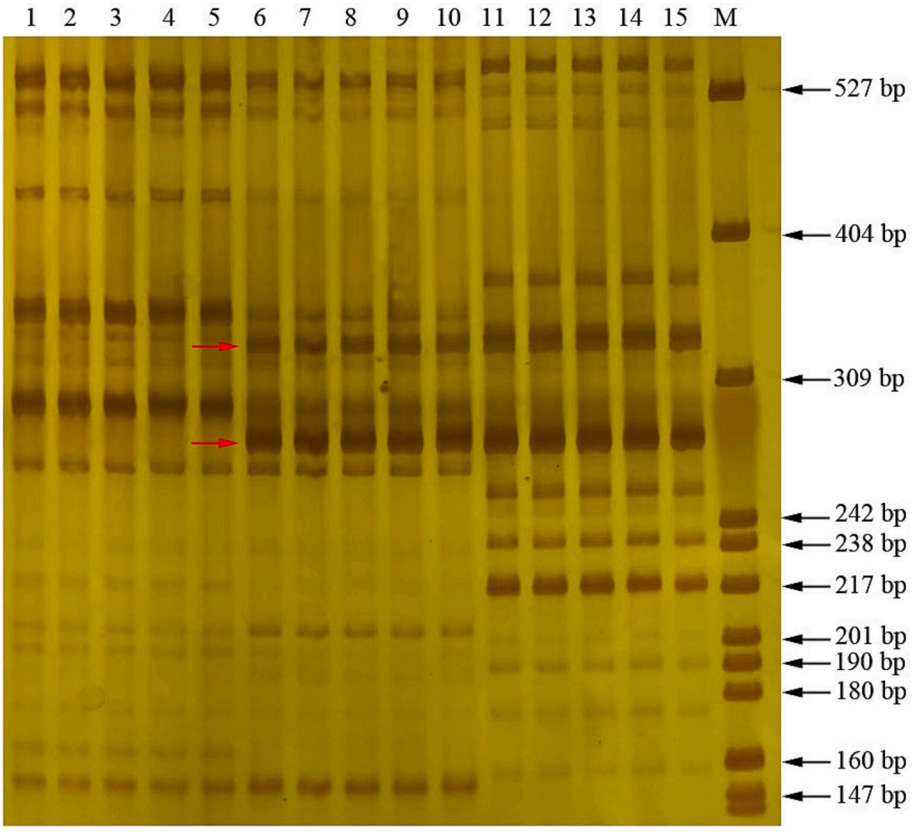

Fig. 10. Microsatellite patterns of MD (No. 1–5), GMD (No. 6–10) and LB (No. 11–15) fish. Electropherogram of microsatellite DNA patterns produced by the primer pair JZL108 in MD, GMD and LB. Lanes 1–5 represent MD, Lanes 6–10 represent GMD, and Lanes 11–15 represent LB. The red arrow indicates the DNA bands derived from LB. M represents the pBR322 DNA/Mspl marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

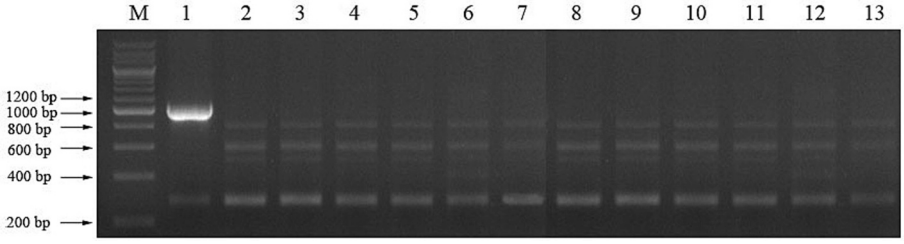

Fig. 11. Sex-specific labeled agarose gel chart. Lane 1 represents the male control, and Lanes 2–13 represent GMD.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739969.

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