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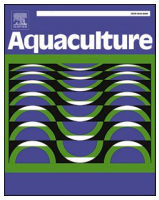

A new method for creating androgenetic diploid fish from tetraploid carp sperm and their genetic composition analysis

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ABSTRACT

The application of cold shock to inhibit the extrusion of the second polar body in fertilized eggs is one of the main methods to induce triploid in diploid fish. In this study, we originally planned to induce hexaploid by this method in tetraploid carp. However, among the surviving offspring, we observed only 50.7% tetraploid and 49.3% diploid individuals, which could potentially result from either androgenetic diploids or gynogenetic diploids. The absence of irradiation and the high proportion of diploids attracted us. However, the lack of sex-specific markers for tetraploid carp, makes it difficult to determine their origin. Fortunately, we also carried out the cross of yellow carp (*Cyprinus carpio* yellow var., 2n, ♀) × tetraploid carp (♂) and red crucian carp (*Carassius auratus* red var., 2n, ♀) × tetraploid carp (♂) with cold shock inhibiting the second meiosis, diploid offspring have also been found, and whether they have undergone gynogenesis or androgenesis can be judged based on phenotype and genotype. In terms of phenotypes, the key phenotypes of two diploids, such as the whisker length, number of lateral scales, and presence of melanin, are consistent with tetraploid carp. In terms of genotypes, through single-copy nuclear gene cloning and fluorescence *in situ* hybridization (FISH), it is supported that both diploids are androgenetic offspring. In addition, 14.9% of tetraploid offspring were found in red crucian carp (♀) × tetraploid carp (♂) with cold shock inhibiting the second meiosis. By the above-mentioned means of detection, it was found that they were created by inhibiting the extrusion of the second polar body of the fertilized eggs. From this it will be seen that the application of cold shock before the extrusion of the second polar body of fertilized eggs cause not only polyploidy, but also the elimination of both second polar body and female nucleus. Previous studies have found that cold shock can induce lethal androgenetic haploids in diploid fish, but these androgenetic diploid fish produced by tetraploid carp sperm were surviving. We summarize the mechanism behind the above phenomenon, which is mainly related to meiosis occurring at the edge of the cell membrane. Cold shock may disrupt the function of the spindle, resulting in the simultaneous retention or extrusion of female nucleus and second polar body. This study will provide valuable insights into androgenesis, polyploidization, and hybridization in fish genetic breeding.

1. Introduction

With the worldwide decline in wild fish stocks and the growing human population leading to an increased demand for aquaculture production, more and more biological technologies are being applied to fish genetic breeding in order to obtain new and improved varieties (Xu et al., 2015; Franek et al., 2020; Hu et al., 2021; Regan et al., 2021; Liu, 2022; Xu et al., 2022; Yang et al., 2022b). Among them, gynogenesis and androgenesis are two special reproductive modes in which eggs develop only by the female or the male pronucleus (Komen and Thorgaard, 2007), and they have received increasing attention due to their important contributions in the rapid establishment of pure lines (Xu et al., 2015; Li et al., 2018; Chi et al., 2020; Pang et al., 2022; Ocalewicz, 2023), monosex breeding (Li et al., 2015; Zhou et al., 2015; Li et al., 2022b; Zhang et al., 2023; Zhou et al., 2023), improved variety acquisition (Gong et al., 2019; Zhang et al., 2021; Wang et al., 2022; Wu et al.,

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2022; Li et al., 2023), and so on. In general, gynogenesis and androgenesis requires the activation of eggs with irradiated sperm or irradiation of the eggs before fertilization, respectively. Then, the extrusion of second polar body or the first cleavage is inhibited by physical shock (*e.g.*, cold, heat and pressure) to double chromosome (Komen and Thorgaard, 2007; Xu et al., 2015). However, due to factors such as radiation and physical shock causing damage, failure in haploid doubling, and failure to inactivate heterologous sperm, the survival rate of gynogenetic or androgenetic offspring is extremely low (Duan et al., 2007; Sun et al., 2007; Nagoya et al., 2010; Zhang et al., 2011; Zhou et al., 2015; Gong et al., 2019; Wu et al., 2019; Ocalewicz, 2023).

Common carp (*Cyprinus carpio*, 2n = 100) and crucian carp (*Carassius auratus*, 2n = 100) represent one of the most-produced fish in aquaculture worldwide (FAO Fisheries Statistics). Recent studies have shown that they originated from the same allotetraploid ancestor (originated from the hybridization between two ancient diploid species) (Fig. 1a) and have two sets of subgenomes (Fig. 1b) (Xu et al., 2019; Luo et al., 2020; Yang et al., 2022a). For a single-copy nuclear gene, allotetraploids have two copies (two subgenes), while autotetraploids have only one copy (Figs. 1c-d). Based on the phylogenetic relationship between subgene and mitochondria gene, it can be inferred whether the two sets of subgene in an allotetraploid belong to the maternal or paternal lineage, that is, the subgene consistent with the mitochondrial phylogeny is the maternal lineage, and the other set is the paternal lineage (Figs.1c-d). In this study, we denoted the maternal genome of common carp (AABB) as AA and the paternal genome as BB, while referring to the maternal genome of crucian carp (CCDD) as CC and the paternal genome as DD. For example, the multispecies phylogenetic tree divided the subgenomes of common carp and crucian carp into four distinct branches for the single-copy nuclear gene *RAG1* (recombination activating 1). Subgenes A and C formed a monophyletic group, while B and D clustered together as another monophyletic group (Fig. 1e) (data from Yang et al., 2022a). Furthermore, we constructed phylogenetic trees of *RAG1*, *IRBP2* (interphotoreceptor retinoid-binding protein 2), *EGR2B* (early growth response 2b) of yellow carp (a variant with recessive trait of common carp, abbreviated as YC) and red crucian carp (a variant with recessive trait of crucian carp, abbreviated as RCC) by monoclonal sequencing, and found that they all had two distinct branches, except that the *IRBP2* gene of RCC had only paternal copies (Figs.2a-b). Despite millions of years of evolution, common carp and crucian carp still can be clearly distinguished as two distinct subgenomes through the cloning of multiple single-copy nuclear genes, indicating that it is an effective method for identifying the genetic composition of hybrid. In previous studies (Wang et al., 2017; Wang et al., 2020), we obtained fertile tetraploid carp (4n = 200, abbreviated as 4N) through distant hybridization (due to traditional habits, we still classify ancient allotetraploids like common carp and crucian carp as diploids in this study). Through multiple single-copy nuclear gene cloning, we found that 4N (AABBCCDD) has two sets of subgenomes from common carp and two sets of subgenomes from crucian carp (Fig. 2c).

In our previous study (Gu et al., 2024), we proposed that tetraploid may create hexaploid by inhibiting the extrusion of the second polar body from their self-fertilized eggs. Cold shock can affect spindle function and cause polyploidization, which is frequently used in triploid and gynogenetic breeding, especially in eurythermic fish (Piferrer et al., 2009). Therefore, we originally intended to use 4N to create a hexaploid carp through cold shock. However, in actuality, we did not screen for hexaploids, but instead discovered a large number of diploid offspring in 3-month-aged fish (Figs. 3–4). In general, these diploid offspring can be generated through either gynogenesis or androgenesis without chromosome doubling (Thorgaard et al., 1990; Duan et al., 2007; Zhou et al., 2015). However, the absence of irradiation and the high proportion of diploids attracted us.

For the XY sex determination system, the gynogenetic offspring are all female (XX), and the androgenetic offspring are super male (YY) or female (XX), so they can be distinguished by sex-specific molecular markers. However, due to the intricate genetic composition of 4N, the development of their sex-specific molecular markers is hindered, thus impeding us to discern their types. Fortunately, we also used 4N as the paternal parent, RCC and YC as the maternal parent, and applied cold shock to the fertilized eggs after hybridization. Interspecific hybridizations make it easier to determine the type of offspring due to differences in parental phenotype and genotype. We also found some diploid offspring in above hybridization (abbreviated as 2n (YC × 4N-CS) and 2n (RCC × 4N-CS)), which can be determined by phenotype and genotype whether they have undergone gynogenesis or androgenesis, refer to

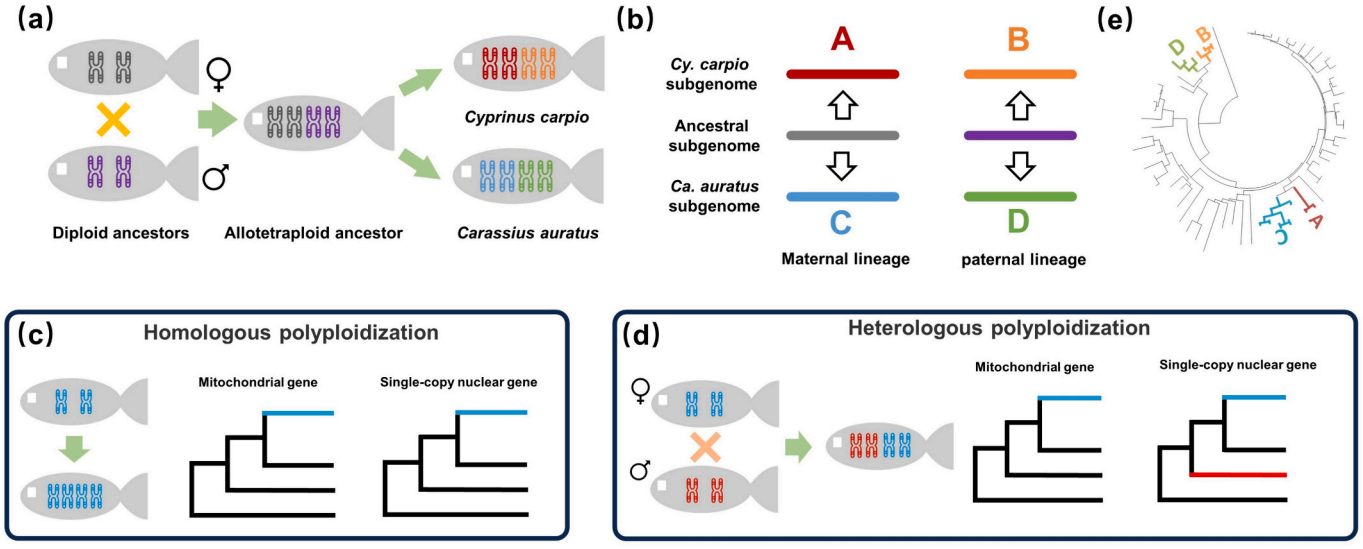

Fig. 1. The origin of *Cyprinus carpio* and *Carassius auratus*, and the phylogenetic differences between homologous polyploidization and heterologous polyploidization. (a) *Cy. carpio* and *Ca. auratus* originated from the same allotetraploid ancestor, which originated from the hybridization of two diploid ancestors. (b) Subgenomic differentiation between *Cy. carpio* and *Ca. auratus*. In this study, we denoted the maternal genome of *Cy. carpio* as A and the paternal genome as B, while referring to the maternal genome of *Ca. auratus* as C and the paternal genome as D. (c) Phylogenetic relationship between mitochondria and single-copy nuclear genes in homologous polyploidization. (d) Phylogenetic relationship between mitochondria and single-copy nuclear genes in heterologous polyploidization. (e) Phylogenetic relationship of a single-copy nuclear gene (*RAG1*) in Cyprinus species and Carassius species with other Cyprinidae species, data from Yang et al. (2022a).

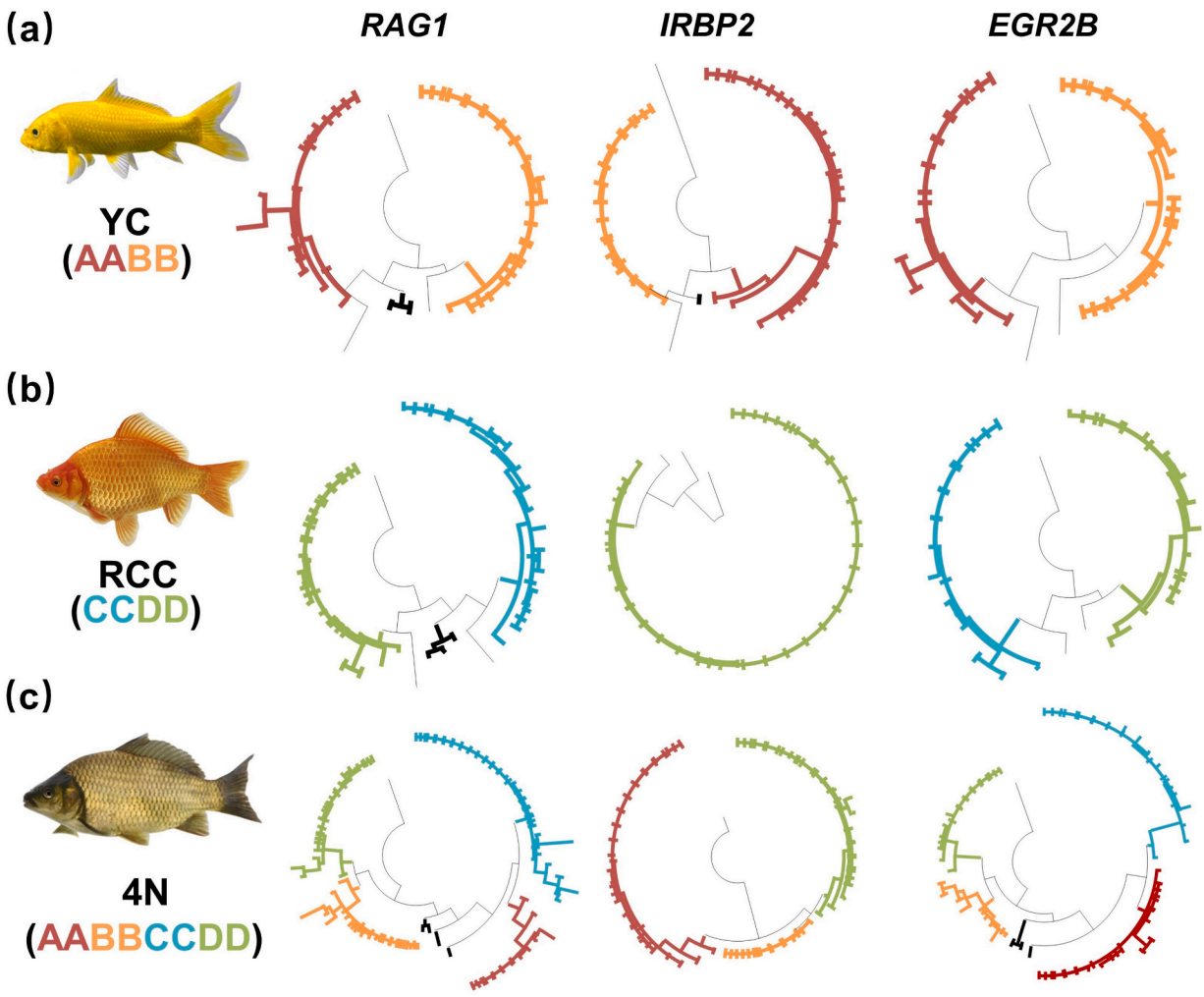

Fig. 2. The genetic composition of YC (yellow carp, *Cyprinus carpio* yellow var.), RCC (*Carassius auratus* red var.) and 4N (tetraploid carp) was analyzed by single-copy nuclear gene cloning. (a) Three single-copy nuclear genes (*RAG1*, *IRBP2*, *EGR2B*) of YC are divided into two sets (AABB) by phylogenetic analysis. (b) Three single-copy nuclear genes of RCC are divided into two sets (CCDD) by phylogenetic analysis except *IRBP2*. (c) Three single-copy nuclear genes of 4N are divided into four sets (AABBCCDD) by phylogenetic analysis except *IRBP2*. The black branch indicates the recombination sequence, and the clustering is not obvious. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

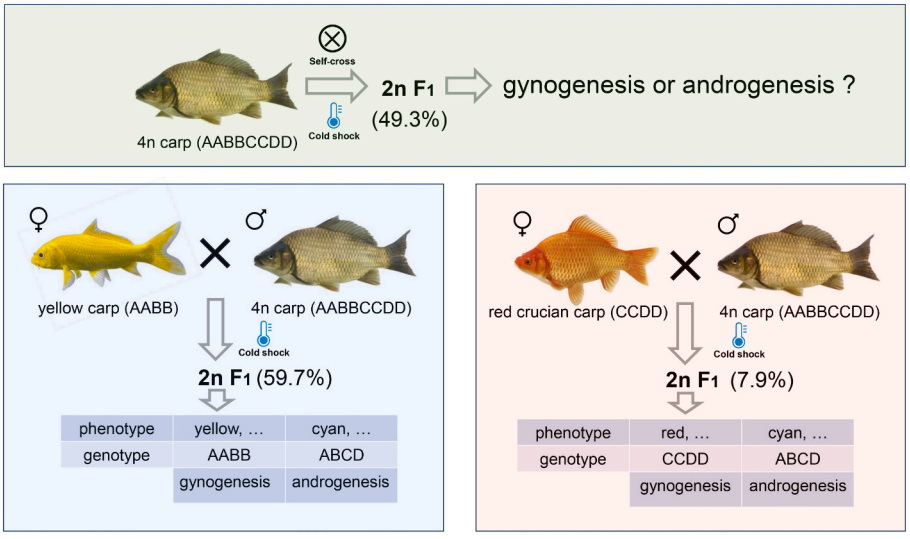

Fig. 3. The main research roadmap of this study.

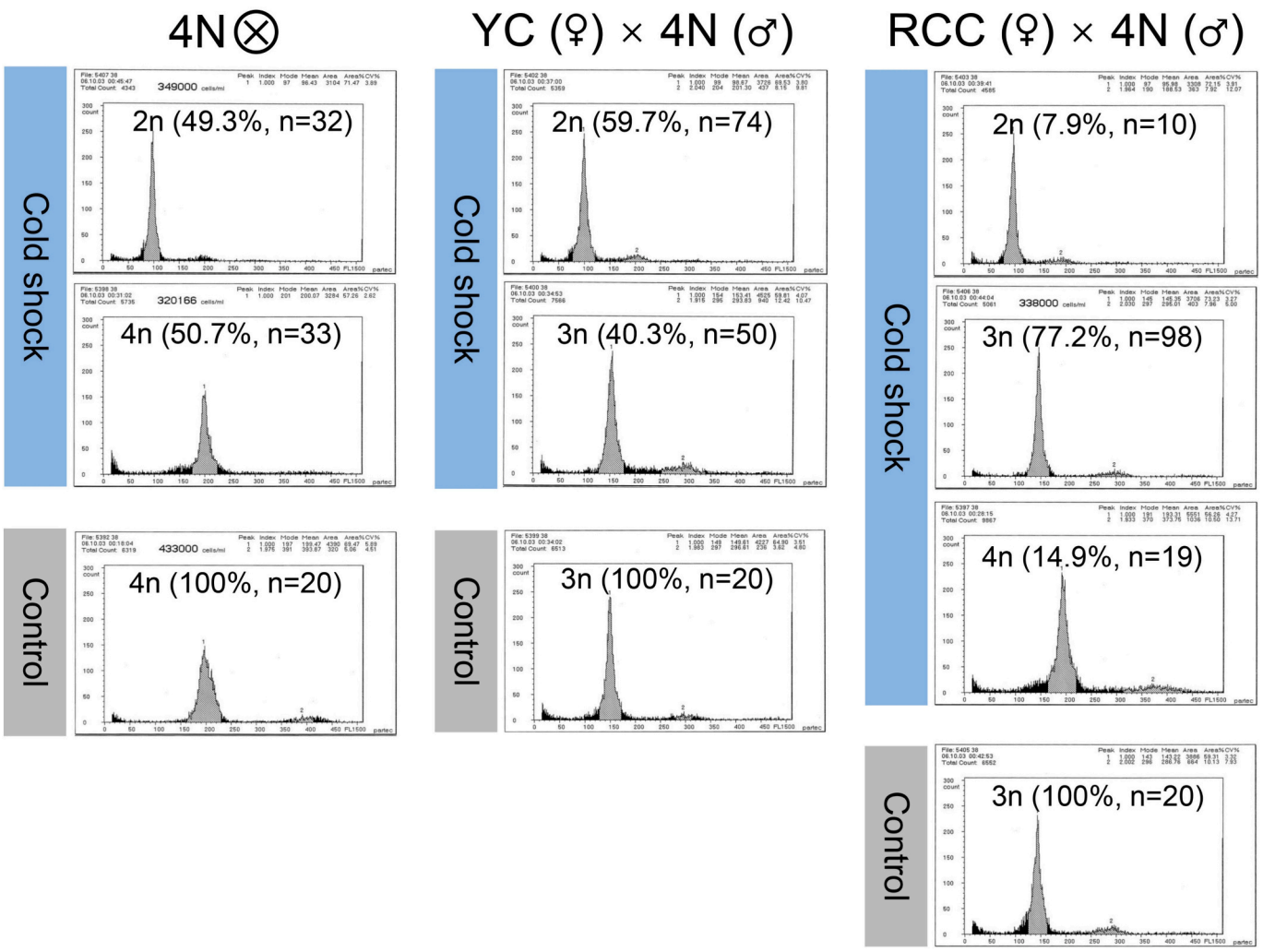

Fig. 4. The DNA content histograms of these offsprings with cold shock inhibiting the second meiosis from 4N self-cross, YC (♀) × 4N (♂), RCC (♀) × 4N (♂) and the control group without cold shock treatment. Diploid exhibiting a fluorescence level around 100, triploid around 150, and tetraploid around 200, consistent with their chromosome numbers.

Fig. 3. In addition, in the cross of RCC (♀) × 4N (♂) with cold shock, we also found some tetraploid offspring (abbreviated as 4n (RCC × 4N-CS)) (Fig. 4). Next, we compared their important phenotypes and analyzed their genetic composition through single-copy nuclear gene cloning and fluorescence *in situ* hybridization (FISH). This research will provide valuable insights for future studies on androgenesis, polyploidization and hybridization in fish.

2. Method

2.1. Ethics statement

The study received approval from the ethics committee of the Institute of Experimental Animals, Hunan Province, China. The fish used in the experiment were anesthetized with MS-222 (100 mg/L, 3-Aminobenzoic acid ethyl ester methanesulfonate) prior to dissection.

2.2. Fish production

4N, YC and RCC were obtained from the State Key Laboratory of Developmental Biology of Fish. In April 2023, the experiment of 4N self-cross, YC (♀) × 4N (♂), RCC (♀) × 4N (♂) treated by cold shock were carried out, and their control groups were not treated with cold shock. The cold shock conditions were as follows: 2 mins after fertilization in room temperature water (20–24 °C), the eggs were treated with cold water at 4–6 °C for 30 mins, and then transferred to room temperature water for incubation. Control groups were fertilized and hatched in room temperature water. In 4N self-cross, sexually mature male 4N (n = 20, 100–200 g, 1st age) and female 4N (n = 20, 150–350 g, 1st age) individuals were selected for artificial dry fertilization, which was completed in three times. Approximately 20,000 eggs were treated with cold shock, and the yield rate of hatchlings was about 3.00%. Approximately 30,000 eggs were used as control, and the yield rate of hatchlings was about 6.67%. In YC (♀) × 4N (♂), sexually mature male 4N (n = 10, 100–200 g, 1st age) and female YC (n = 3, 1000–1500 g, 3rd age) individuals were selected for artificial dry fertilization. Approximately 60,000 eggs were treated with cold shock, and the yield rate of hatchlings was about 50.00%. Approximately 20,000 eggs were used as control, and the yield rate of hatchlings was about 80.00%. In RCC (♀) × 4N (♂), sexually mature male 4N (n = 10, 100–200 g, 1st age) and female RCC (n = 3, 500–750 g, 2nd age) individuals were selected for artificial dry fertilization. Approximately 50,000 eggs were treated with cold shock, and the yield rate of hatchlings was about 40.00%. Approximately 20,000 eggs were used as control, and the yield rate of hatchlings was about 75.00%. These larvae fish of each group were reared in different tanks and fed twice a day.

2.3. Ploidy level determination

All groups of 3-month-old juvenile fish were sampled for DNA content determination. We cut off some of their tail fins and placed them on a 200-mesh square nylon mesh (40 mm × 40 mm) center. Next, fold the nylon mesh diagonally, wet the tissue with a small amount of ACD (anticoagulant citrate dextrose) solution, grind with tweezers, rinse with ACD solution, and collect single cell suspension (0.5 mL) using a 1.5 mL EP tube. Then, 0.5 mL DAPI solution (Biotium, USA) was added to the EP tube and stained in dark for 20 min. Finally, the DNA contents of all samples were measured using a flow cytometer (Cell Counter Analyser, Partec, Germany). The ploidy level can be determined based on its fluorescence level (FL), with diploid exhibiting an FL around 100, triploid around 150, and tetraploid around 200, consistent with their chromosome numbers.

2.4. Phenotype observation

The phenotypes of 2n (YC × 4N-CS) ($n = 10$), 2n (RCC × 4N-CS) ($n = 3$), 4n (RCC × 4N-CS) ($n = 10$) and their parents ($n = 10$) were compared using a stereomicroscope (Nikon SMZ25, Japan). These samples of 3-month-old juvenile fish were immobilized using 75% alcohol for optimal visualization. The disparity among 4N, YC and RCC larvae primarily stems from variations in pigmentary cell composition, whisker length and scale number, so we focused on the characteristics of their heads and scale number.

2.5. DNA extraction, PCR, cloning and sequencing

In this study, we selected three single-copy nuclear genes (*RAG1*, *IRBP2* and *EGR2B*) and two mitochondrial genes (*CYTB*, *cytochrome b*; *COI*, *cytochrome c oxidase I*) commonly employed in fish phylogenetic research. Each genetic population had 3 individual replicates. We have designed new primers of three single-copy nuclear genes based on previously reported sequences from Yang et al., 2022a, and new primers of *COI* based on previously reported sequences from NCBI (National Center for Biotechnology Information). The primers of *CYTB* have been reported (Wang et al., 2016). Refer to Table 1 for primer information of all genes.

Genomic DNA was extracted from fin tissues using the Tiangen DNeasy Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. PCRs were conducted in 50 μL volumes containing the following: 2 μL genomic DNA (100 ng/ul), 2 μL 10 μM primer, 21 μL ddH2O and 25 μL Tap Plus Master Mix (Tiangen, Beijing, China). The following conditions were used for PCRs: (1) predenaturation at 95 °C for 4 min; (2) denaturation at 95 °C for 30 s; (3) annealing at 50–58 °C (based on primers) for 30 s; (4) elongation at 72 °C for 1–1.5 mins (based on primers, 1 kb/min) (repeated 2 to 4 stages 38 times) and a final elongation at 72 °C for 7 min.

FastPure® Gel DNA Extraction Mini Kits (Tiangen, Beijing, China) were used to purify the amplified PCR products, according to the instructions of the manufacturer. The purified target gene was connected with the pLB-T vector (Tiangen, Beijing, China) and transformed into the receptive *Escherichia coli* DH5α cell (Tiangen, Beijing, China) by the heat shock method. The treated bacteria were inoculated into solid medium containing ampicillin for culture. After the colonies grew out, monoclonal colonies were selected for PCR and agarose gel electrophoresis verification, and positive results were sent to Bioengineering (Shanghai) Co., Ltd. for sequencing.

Table 1
Reference information for primers.

Primer		Sequence	Product length
RAG1	F	5'-AAARCCAAACTCTGAGCTCTC-3′	626
	R	5′- AGTTTCCATTCATCCTCATYAC-3'	
IRBP2	F	5'-ATCCTCRGCATTTCAGATC-3'	686
	R	5'-CCAGTGATGGGGTTAATAGAC-3'	
EGR2B	F	5'-GTGATCAACATMGTGAGCGC-3'	671
	R	5'-CGRCACTGGAAGGGTTTGTG-3'	
CYTB	F	5'-GACTTGAAAAACCACCGTTG-3'	1140
	R	5'-CTCCGATCTCCGGATTACAAGAC-3'	
COI	F	5'-TCTACYAACCACAAAGACATTGG-3'	1421
	R	5'-ACTTCTCGTTTAGCGGCRAAGGC-3'	

2.6. Sequences analysis

The *RAG1*, *IRBP2*, and *EGR2B* genes of common carp and crucian carp have been classified into two subgenes (AB and CD) in Yang et al., 2022a. We utilized the reported sequences as a reference to categorize the subgenes of 4N, YC, RCC, 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4n (RCC × 4N-CS) in MEGA (version 11, Mega Limited, Auckland, New Zealand). The aligned sequences were subjected to phylogenetic analysis using maximum likelihood (ML) methodology with default setting. The sequences that exhibit similarity to the reference sequences form monophyletic groups in phylogenetic trees, and were classified into four subgenes (A, B, C and D). The clustering characteristics of a small number of recombination sequences were not obvious, and they were grouped as E.

2.7. Fluorescence in situ hybridization (FISH)

6-month-old juvenile fishes (10–20 g) of 2n (YC × 4N-CS), 2n (RCC × 4N-CS), 4n (RCC × 4N-CS) and their parents were used for this analysis. 4n (RCC × 4N-CS) had three replicates, while the other types have only one replicate, as their genotypes are relatively clear. Chromosome preparations from kidney were performed by air-drying preparation technology as previously described (Zhang et al., 2015). FISH was performed in accordance with a previously described method (Zhang et al., 2015). Amplification of the 263 bp centromere repeat sequence (Qin et al., 2015) from the genomic DNA of RCC was achieved using FISH probes labeled with Dig-11-dUTP, synthesized *via* a PCR DIG Probe Synthesis Kit (Roche, Germany). The primer pairs 5′-AAGCTTTTCTCTCTAGTAGAGAAAGC-3′ and 5′-TTGAGCAGATTTGGGCTTGATTTC-3′ (JQ086761) were employed for PCR amplification of the target sequence. The slides were observed under a Leica inverted DMIRE2 microscope image system (Leica, Germany), with image capture performed using the CW4000 FISH software (Leica). There are about 90–100 hybridization signals in the chromosomes of crucian carp, and none in other species (Qin et al., 2015; Zhang et al., 2015).

3. Result

3.1. Ploidy level

49.3% diploids and 50.7% tetraploids were detected in the juvenile fish of 4N self-cross with cold shock ($n = 65$), and 100% tetraploids were detected in 4N self-cross without cold shock ($n = 20$). 59.7% diploids and 40.3% triploids were detected in the juvenile fish of YC (♀) × 4N (♂) with cold shock ($n = 124$), and 100% tetraploids were detected in YC (♀) × 4N (♂) without cold shock ($n = 20$). 7.9% diploids, 77.2% triploids and 14.9% tetraploids were detected in the juvenile fish of RCC (♀) × 4N (♂) with cold shock ($n = 127$), and 100% tetraploids were detected in RCC (♀) × 4N (♂) without cold shock ($n = 20$) (Fig. 4). There was a significant difference in the proportion of offspring between RCC (♀) × 4N (♂) and YC (♀) × 4N (♂), which may be caused by interspecies differences.

3.2. Detection of phenotypes

YC juvenile fish lacked melanocytes and possessed longer mouth whiskers and snout whiskers. In contrast, RCC juvenile fish exhibited the

presence of melanocytes but lacked both mouth whiskers and snout whiskers. 4N juvenile fish exhibited the presence of melanocytes, and possessed significantly shorter mouth whiskers and snout whiskers. 2n (YC × 4N-CS) and 2n (RCC × 4N-CS) juvenile fish both exhibited the presence of melanocytes, and possessed significantly shorter mouth whiskers and snout whiskers. 4n (RCC × 4N-CS) exhibited the presence of melanocytes, but only possessed significantly shorter snout whiskers (Fig. 5). YC (34.40 ± 1.26, 33–36) possessed more lateral scales than RCC (29.10 ± 0.95, 28–30), and 4N (32.00 ± 1.00, 31–33) between them. The number of lateral line scales in 2n (YC × 4N-CS) (32.00 ± 1.00, 31–33) and 2n (RCC × 4N-CS) (32.00 ± 0.00, 32) was consistent with 4N, but 4n (RCC × 4N-CS) (30.00 ± 1.41, 28–32) possessed fewer lateral line scales than them (Table 2). These results indicated that these key characteristics of 2n (YC × 4N-CS) and 2n (RCC × 4N-CS) were consistent with those of 4N, while those of 4n (RCC × 4N-CS) were similar to RCC.

Table 2
Comparison of the number of scales among experimental fish.

Items	No. of lateral scales	No. of upper lateral scales	No. of lower lateral scales
YC	34.40 ± 1.26 (33–36)	5.37 ± 0.45 (5–6)	5.30 ± 0.43 (5–6)
RCC	29.10 ± 0.95 (28–30)	5.60 ± 0.34 (5–6)	6.37 ± 0.42 (6–7)
4N	32.00 ± 1.00 (31−33)	6.35 ± 0.39 (6–7)	6.67 ± 0.46 (6–7)
2n (YC × 4N-CS)	32.00 ± 1.00 (31–33)	5.90 ± 0.32 (5–7)	6.20 ± 0.67 (5–7)
2n (RCC × 4N-CS)	32.00 ± 0.00 (32)	6.33 ± 0.58 (6–7)	6.33 ± 0.58 (6–7)
4n (RCC × 4N-CS)	30.00 ± 1.41 (28–32)	5.60 ± 0.52 (5–6)	5.91 ± 0.43 (6–7)

3.3. Genetic composition

The RCC-specific 263 bp sequence fragment was used as probe to detect 2n (YC × 4N-CS), 2n (RCC × 4N-CS), 4n (RCC × 4N-CS) and their parents by FISH. The number of chromosomes in YC, RCC, 4N, 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4n (RCC × 4N-CS) were around 100, 100, 200, 100, 100, 200 respectively, and the number of hybridization signals were approximately 0, 90–100, 90–100, 45–50, 45–50, 135–150

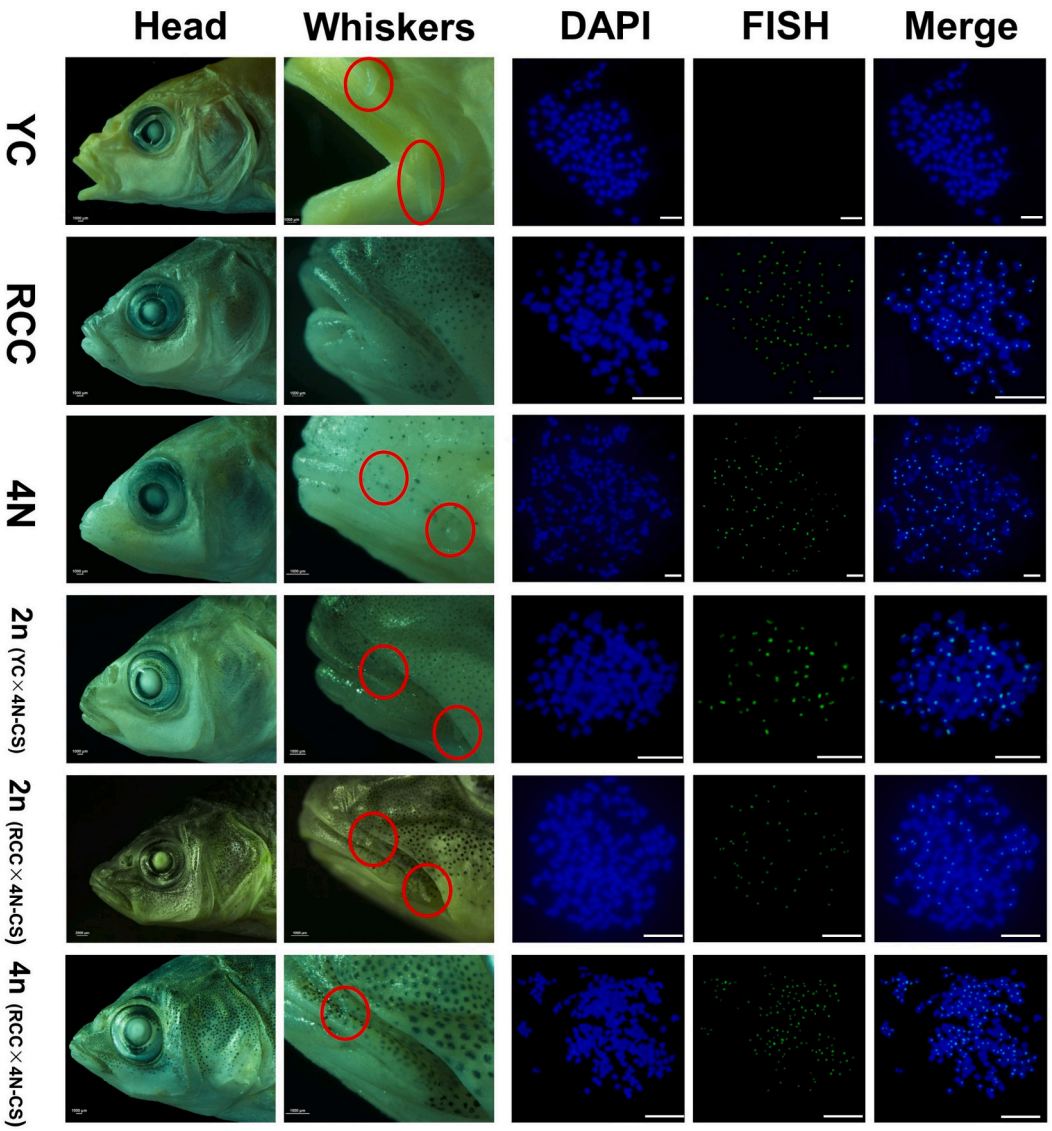

Fig. 5. Phenotypic and genotypic (based on FISH) analysis of experimental fish. In FISH, the RCC-specific 263 bp centromere repeat sequence was used as probe, it can display hybridization signal on almost all chromosomes of RCC, and none in other species. Some of the sister chromatids disengage to generate two hybridization signals in a chromosome. The scales are10 μm in FISH.

respectively (Fig. 5).

On the other hand, we had also demonstrated their genetic composition by cloning their single-copy nuclear genes. A total of 960 valid clone sequences were obtained from the three single-copy nuclear genes of YC, RCC, 4N, 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4n (RCC × 4N-CS). Among them, the three genes of YC were divided into two subgenes (A and B) (Fig. 2a). However, the *IRBP2* gene of RCC had only one type (D), and the *IRBP2* and *EGR2B* genes of RCC were divided into divided into two subgenes (C and D) (Fig. 2b). The *IRBP2* gene of 4N were divided into three subgenes (A, B and D), and the *IRBP2* and *EGR2B* genes of 4N were divided into four subgenes (A, B, C and D) (Fig. 2c). The compositions of the three genes of 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4n (RCC × 4N-CS) were consistent with that of 4N, while the subgene contents of crucian carp (CD) in 4n (RCC × 4N-CS) were higher than that in 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4N (Fig. 6 and Table 3). In summary, these results indicated that the genotypes of 2n (YC × 4N-CS) and 2n (RCC × 4N-CS) are ABCD, while the genotype of 4n (RCC × 4N-CS) is ABCCCDDD.

Through sequencing of two mitochondrial genes (*COI* and *CYTB*), it was found that the mitochondrial sequences of 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4n (RCC × 4N-CS) were consistent with their female parent, indicating that there was no occurrence of mixed fish fry (Fig. 6).

Table 3
Composition and abundance of three single-copy nuclear gene clones.

Items		*n*	A	B	C	D	E	A: B: C: D
RAG1	YC	3	10	15	/	/	2	/
	RCC	3	/	/	15	17	3	/
	4N	3	10	16	24	18	4	1.0: 1.6: 2.4: 1.8
	2n (YC × 4N-CS)	3	18	10	22	20	1	1.0: 0.6: 1.2: 1.1
	2n (RCC × 4N-CS)	3	16	11	24	18	/	1.0: 0.7: 1.5: 1.1
	4n (RCC × 4N-CS)	3	14	9	27	24	/	1.0: 0.6: 1.9: 1.7
IRBP2	YC	3	18	13	/	/	1	/
	RCC	3	/	/	/	30	/	/
	4N	3	20	8	/	18	/	1.0: 0.4: 0.0: 0.9
	2n (YC × 4N-CS)	3	27	18	/	21	/	1.0: 0.7: 0.0: 0.8
	2n (RCC × 4N-CS)	3	21	21	/	26	/	1.0: 1.0: 0.0: 1.2
	4n (RCC × 4N-CS)	3	19	21	/	29	/	1.0: 1.1: 0.0: 1.5
EGR2B	YC	3	11	12	/	/	/	/
	RCC	3	/	/	11	15	/	/
	4N	3	14	7	14	11	3	1.0: 0.5: 1.0: 0.8
	2n (YC × 4N-CS)	3	17	13	16	23	/	1.0: 0.8: 0.9: 1.4
	2n (RCC × 4N-CS)	3	15	14	11	25	1	1.0: 0.9: 0.7: 1.7
	4n (RCC × 4N-CS)	3	9	16	13	30	/	1.0: 1.8: 1.4: 3.3

The numbers in A-E represent the frequency of occurrence for each subgenomic clone sequence, where E indicates the recombination sequence with unclear clustering.

4. Discussion

4.1. Androgenetic diploids induced by cold shock from 4N sperm

Due to the small size of 3-month-old carp, they are not suitable for FISH. In order to determine their genetic composition earlier, we adopted a single-copy nuclear gene cloning method. 2n (YC × 4N-CS) and 2n (RCC × 4N-CS) have clonal sequences from A, B, C and D subgenomes (Fig. 6). Since the number of haploid chromosomes in cyprinid fish is generally around 25, regardless of the proportion of cloning sequences for A, B, C and D subgenes, we determined that two androgenetic diploids (2n = 100) have A, B, C and D subgenomes. Afterwards, we also verified the above results through FISH (Fig. 5). Consequently, we consider that the diploid offspring resulting from 4N self-crossing with cold shock are also generated through androgenesis. Theoretically, the ratio of clone sequences from A, B, C, and D subgenomes of two androgenetic diploids and 4N should be close to 1:1:1:1, but the actual ratio does not meet expectations (Table 3), which may be related to the preference amplification of primers. Therefore, single-copy nuclear gene cloning for identifying the genetic composition of hybrids is suitable for qualitative analysis, but not for quantitative analysis.

Combining the phenotypic and genotypic evidences, we can determine that 2n (YC × 4N-CS) and 2n (RCC × 4N-CS) are androgenetic diploids from 4N sperm. Compared to the control group, cold shock induction is an essential prerequisite for achieving androgenesis. These observations suggest that cold shock inhibiting the second meiosis can

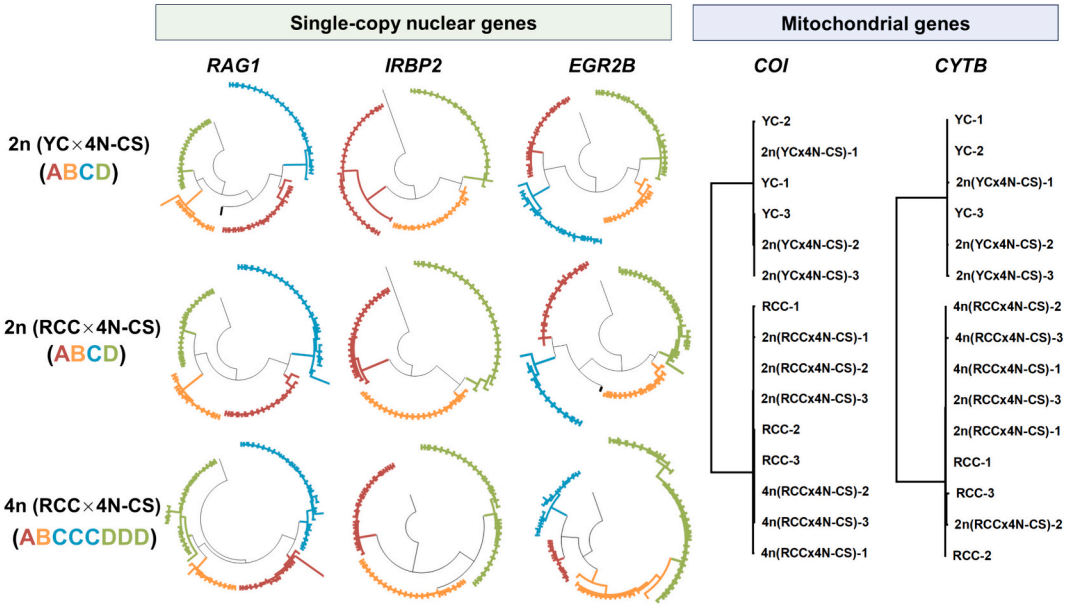

Fig. 6. Genotypic analysis of experimental fish based on three single-copy nuclear genes and mitochondrial genes.

not only inhibit the extrusion of the second polar body of fertilized eggs (Piferrer et al., 2009), but also lead to the elimination of both the second polar body and female nucleus simultaneously. However, the occurrence of androgenetic haploid induced by cold shock inhibiting the second meiosis is not a new finding. In numerous studies on artificial triploid breeding induced by cold shock in diploid fish, the occurrence of abnormal embryos exhibiting haploid-like external characteristics has frequently been documented shortly after fertilization. The presence of haploids in these aberrant embryos was subsequently confirmed through cytogenetic method in salmonids (Ueda et al., 1988), stickleback (Swarup, 1956, 1959), common carp (Gervai et al., 1980), loach (Suzuki et al., 1985; Morishima et al., 2011), bitterling (Ueda and Aoki, 1995), Japanese pufferfish (Zhou et al., 2019) and so on. These cases suggest that in cold shock induced triploid breeding, the occurrence of dead embryos is attributed not only to physical damage but also to androgenetic haploids. In this study, the androgenetic diploids produced from 4N sperm were surviving, so we found this phenomenon more easily. Similarly, using wild autotetraploid loaches to provide sperm can also induced surviving androgenetic diploids after cold shock treatment (Chen et al., 2021). Although salmon and trout are also wild autotetraploids, their polyploidy occurs earlier (90–102 Mya) and the genome tends to be diploid (Berthelot et al., 2014). As a result, they provide diploid gametes similar to haploid gametes, resulting in undoubled androgenetic offspring that are lethal (Ueda et al., 1988).

Next, we summarized the mechanisms behind the above phenomena. In fish, the second polar body is extruded after fertilization and occurs at the cell membrane edge, which is crucial for inducing androgenesis. Through histological sections of eggs revealed the female nucleus is presumably extruded together with the second polar body by the cold shock and thereby only sperm nucleus remains in the egg (Hou et al., 2015; Morishima et al., 2011). Sperm nucleus transforms to male pronucleus and then initiates androgenesis. In addition, it is also found that the female nucleus and the second polar body both were retained together, forming a triploid (Fig. 7) (Hou et al., 2015; Morishima et al., 2011). Thus, it can be seen cold shock may disrupt the function of the spindle, resulting in the concurrent retention or extrusion of both the female nucleus and the second polar body in fertilized eggs. However, under heat shock and pressure shock, the occurrence of androgenesis is rarely observed. Pressure shock probably involve an effect of pressure acting on the oolemma, literally resisting the extrusion of the second polar body (Piferrer et al., 2009). Compared to cold shock, the microtubules implicated in chromosome migration exhibit greater sensitivity to heat shock (Dustin, 1984), which potentially disrupting the migration of the egg nucleus prior to the extrusion of the second polar body.

In our previous study, 1.7% surviving offspring was obtained using the irradiation of eggs of RCC and untreated diploid sperm from the tetraploid crucian carp (4n = 200) (Zhou et al., 2015). In these offspring, a male fish with white semen (distinguished from other male fish with water-like semen) was found. Furthermore, self-crossing and hybridization of the male fish with diploid female RCC and tetraploid crucian carp sired all male triploids and tetraploids, respectively, which proves that the male fish selected was a super-male YY crucian carp and produced unreduced diploid sperm (Zhou et al., 2023; Zhou et al., 2015). From this, it can be seen that YY androgenetic diploid fish may have important application value. However, the survival rate of these androgenetic diploids was very low due to irradiation of eggs. In this study, we obtained a higher yield of androgenetic diploid by cold shock, especially in the cross of YC (♀) × 4N (♂), which can provide more individuals for YY androgenetic diploid screening. Eliminating maternal nuclear genome through radiation can form radiation-induced chromosome fragments in androgenetic offspring (Michalik et al., 2015; Ocalewicz et al., 2013), which may lead to their death. According to the mechanism of cold shock eliminating the maternal nuclear genome (Fig. 7), it may be eliminated more completely, which may be a reason for the higher survival rate of offspring induced by cold shock than radiation. In summary, the use of cold shock to eliminate the maternal nuclear genome may have less damage to the eggs and more simplified technology compared to UV radiation.

For diploid fish, inducing androgenetic haploids is meaningless, and it is necessary to double the paternal genome to form androgenetic diploids. The usual approach is to inhibit the first cleavage of the eggs through heat or pressure shock (Komen and Thorgaard, 2007; Hou et al., 2014; Kaspar et al., 2022), which can cause secondary damage to eggs. However, under the same treatment, undoubled androgenetic haploid embryos survive better than androgenetic diploid embryos until the eye stage in salmonid fish (Michalik et al., 2014; Michalik et al., 2015; Ocalewicz et al., 2013). Additionally, triploids generated through inhibiting the extrusion of the second polar body with heat and pressure shock exhibit a high survival rate, but tetraploids generated through inhibiting first cleavage with heat shock and pressure shock exhibit an extremely low survival rate (Piferrer et al., 2009) and a high risk of diploid/tetraploid chimerism (Zhang and Onozato, 2004). The above results indicate that genetic defects form inhibiting first cleavage may be the main factor causing the low yield of androgenetic diploids, rather than the physical damage from heat or pressure shock. On the other hand, androgenetic diploids that doubling by heat or pressure shock are also known as androgenetic double haploids due to their two sets of genomes are identical (Komen and Thorgaard, 2007). Theoretically,

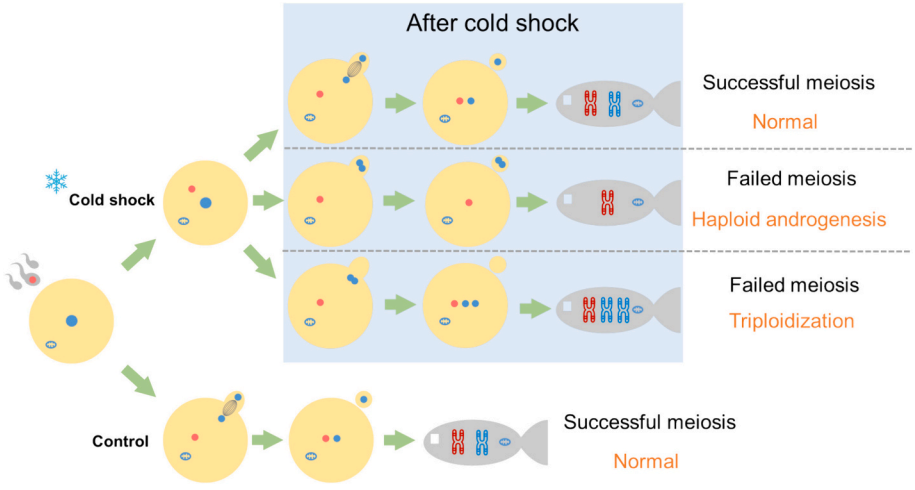

Fig. 7. Presumptive cytological mechanisms for androgenesis and triploidization induced by cold shock inhibiting the second meiosis, summarized from Morishima et al. (2011) and Hou et al. (2015).

androgenetic double haploids are expected to experience inbreeding depression due to the manifestation of homozygous deleterious mutations. A considerable decrease in androgenetic double haploids survival could be attributed to the impact of these mutations during early embryo development. To avoid this, the activation of heterologous eggs by two haploid sperms, known to enhance heterozygosity, was attempted. Unexpectedly, dispermic induction of androgenesis also resulted in equally low hatching success (Araki et al., 1995; Kirankumar and Pandian, 2004); however, the causes for this outcome are still unknown. Although a large number of studies have achieved favorable fry yields, the survival rate of androgenetic diploids in the first year often sharply decreases. In summary, the low survival rate of androgenetic diploids is the main factor limiting the application of fish androgenesis.

At present, the development and growth of these androgenetic diploids obtained in this study is normal, which suggests the use of artificially created tetraploids to provide diploid sperm may be a great way to address the low survival rate of androgenetic diploids, as it avoids all the above problems from doubling paternal genome. The quality of artificially created tetraploid fish eggs is generally poor, as described in this study, using the same or related diploid species to provide high quality eggs can improve the yield of androgenetic diploids.

4.2. A new tetraploid carp was obtained

4n (RCC × 4N-CS) may originate from two results. (1) Cold shock inhibits the extrusion of the second polar body of the fertilized eggs, resulting in tetraploid formation (ABCCCDDD); (2) Cold shock first induces androgenetic diploid and then inhibits the first cleavage, resulting in tetraploid formation (AABBCCDD). Theoretically, the possibility of first hypothesis is high because we applied cold shock early, but the second hypothesis is also possible due to sustained cold shock (Morishima et al., 2011; Piferrer et al., 2009). Although 4n (RCC × 4N-CS) has more cloned sequences from crucian carp than 2n (YC × 4N-CS), 2n (RCC × 4N-CS) and 4N (Table 3), their genetic composition cannot be determined. Therefore, when the fish grew to 6-month-old, we conducted FISH experiments, and the results supported the first hypothesis. Of course, both conditions may also occur simultaneously, but we only detected the first one.

It is difficult to obtain surviving and fertile tetraploid offspring by inhibiting the first cleavage with physical shock in diploid fish (de Alvarenga et al., 2020; Liu, 2022; Piferrer et al., 2009; Yuan et al., 2023), which may be due to diploid–tetraploid mosaicism in different organs or high homology resulting in the formation of quadrivalents during meiosis (Tian et al., 2011; Yamaki and Arai, 2000; Zhang and Onozato, 2004). Tetraploid offspring can also be obtained through distant hybridization, as a result of the production of unreduced gametes or inhibition of primary mitosis (Liu, 2022). However, the likelihood of obtaining allotetraploids through distant hybridization is exceedingly low, necessitating the screening of tetraploid offspring across many populations (Liu, 2022).

In the cross between RCC (♀) × 4N (♂) with cold shock inhibiting the second meiosis, we discovered that the genetic composition of 4n (RCC × 4N-CS) is ABCCCDDD. This implies that using tetraploid fish as a male parent to cross with another diploid female fish, and then inhibiting the extrusion of the second polar body of the fertilized egg, resulting in obtaining allotetraploid. These diploid female parents can be replaced, so one tetraploid can obtain a variety of allotetraploids through the above approach. In this study, due to the genotype of 4N is heterologous, the 4n (RCC × 4N-CS) may exhibit sterility. However, if autotetraploid (AAAA) are used, the allotetraploids (AABB) can be obtained, which are theoretically fertile and can be stably inherited. Although obtaining autopolyploids through physical shock is difficult, there have been a few successful examples, such as yellowtail tetra (*Astyanax altiparanae*) (do Nascimento et al., 2020), brook trout (*Salvelinus fontinalis*) (Weber et al., 2015) and rainbow trout (*Oncorhynchus mykiss*) (Weber and Hostuttler, 2012), and their male tetraploid can produce diploid sperm. Wild autotetraploid loach can also produce diploid sperm (Chen et al., 2021). Some studiesshowed that the triploid is not reproductive barrier but serve as a bridge in the formation of polyploid species, that is, a triploid may produce 3n unreduced gametes that combine with the 1n normal gametes from a diploid to produce tetraploid (Li et al., 2022a; Lu et al., 2023; Piferrer et al., 2009). In addition, we have successfully created fertile autotetraploid zebrafish through disrupting meiotic crossover formation based on *CNTD1* (cyclin n-terminal domain containing 1) gene knockout (Ou et al., 2024).

5. Conclusion

Due to the lethality of haploids, cold shock induced androgenetic haploids are often overlooked. Fortunately, 4N can provide 2n sperm, so we find this phenomenon more easily. Compared to UV radiation, cold shock causes less damage to eggs. Using diploids with better egg quality as maternal parent can significantly increase the yield of androgenetic diploids of 4N. In addition, taking a crossing between the tetraploid (♂) and another diploid fish (♀) can create new-type allotetraploid by inhibiting the extrusion of the second polar body of their fertilized eggs. Cold shock may disrupt the function of the spindle, resulting in the concurrent retention or extrusion of both the female nucleus and the second polar body.

Author contributions

Haoran Gu: idea, experimental design, the manuscript writing, experiment, improvement and modification.
Qilong Liu: experiment.
Yi Fan: experiment.
Qiong Liu: experiment.
Yating Zhu: experiment.
Conghui Yang: improvement and modification.
Shi Wang: provide parent fish, improvement, fund and modification.
Shaojun Liu: idea, improvement, fund and modification.

CRediT authorship contribution statement

Haoran Gu: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yi Fan:** Methodology, Investigation. **Qiong Liu:** Methodology, Investigation. **Yating Zhu:** Methodology, Investigation. **Conghui Yang:** Writing – review & editing. **Shi Wang:** Writing – review & editing, Resources, Funding acquisition. **Shaojun Liu:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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