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Analysis of the formation mechanisms and biological characteristics in WuLi carp-like fish derived from the hybrid of *Cyprinus carpio* var. Quanzhounensis (♀) × *Carassius auratus* red var. (♂)

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ABSTRACT

Distant hybridization is an effective strategy for creating polyploids and developing new germplasm resources in fish genetic breeding. The WuLi carp-like fish (2 n = 100, WCL) had been obtained through distant hybridization between female WuLi carp (*Cyprinus carpio* var. Quanzhounensis, 2 n = 100, WLC) and male red crucian carp (*Carassius auratus* red var., 2 n = 100, RCC), and extensive and in-depth studies had subsequently been conducted on it. Although some characteristics of the female parent WLC had been retained, significant morphological changes had occurred in the hybrid offspring. Systematic comparative analyses had been conducted on WCL, encompassing morphological traits, DNA content, chromosome number, 5S rDNA sequences, gonadal sections, microsatellite DNA markers, fluorescence in situ hybridization (FISH), and mitochondrial DNA. The results indicated that WCL had exhibited clear maternal inheritance in morphological traits, DNA quantification, chromosome configuration, 5S rDNA sequences, FISH signals, and mitochondrial DNA, with the FISH results being entirely consistent with those of the female parent. Microsatellite analysis had clearly confirmed the integration of DNA fragments from the male parent RCC into the WCL genome. Furthermore, observations of gonadal sections had revealed that both male and female WCL individuals had been fertile, with the number of females exceeding that of males. Based on a comprehensive analysis of the experimental results, WCL was inferred to be a natural gynogenetic offspring derived from hybridization, with its distinct morphological characteristics having possibly resulted from the insertion of paternal genetic material. The acquisition of WCL provides an ideal model for investigating the effects of hybridization on genomic composition in fish.

1. Introduction

Distant hybridization enables the transfer of one species' genome into another, leading to chromosomal-level changes in the offspring. These alterations are far greater than those achieved through close hybridization (Liu et al., 2022; Wang et al., 2019; Zhang et al., 2014). Compared to close hybridization, distant hybridization has significant advantages in fish genetic breeding by substantially increasing the probability of producing polyploid offspring (Hu et al., 2020; Liu et al., 2021, 2006). For instance, allotetraploid individuals have been

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successfully generated in hybridization combinations such as red crucian carp (*Carassius auratus* red var., 2 n = 100, RCC, ♀) × blunt snout bream (*Megalobrama amblycephala*, 2 n = 48, BSB, ♂) and common carp (*Cyprinus carpio,* 2 n = 100, COC, ♀) × blunt snout bream (*Megalobrama amblycephala*, 2 n = 48, BSB, ♂). Furthermore, fertile allotetraploid lines with bisexual fertility have been established through the self-crossing of these allotetraploid hybrids (Qin et al., 2016; Wang et al., 2020). Natural gynogenetic offspring may also arise during distant hybridization. Examples include natural gynogenetic RCC from the cross between RCC (♀) × blunt snout bream (♂)(Qin et al., 2020), and natural gynogenetic common carp from the cross between common carp (♀) × blunt snout bream (♂)(Wang et al., 2017). These natural gynogenetic offspring have the potential to establish pure genetic lines in future studies and can serve as high-quality germplasm resources for genetic breeding experiments (Xiao et al., 2011; Gui and Zhou, 2010; Liu et al., 2010; Wang et al., 2017).

Wuli carp (WLC) is a distinctive variety of carp endemic to China, belonging to the family *Cyprinidae* and classified as a warm-water fish. This fish is characterized by a robust body and a purplish-brown coloration with a dark back and bright luster. It is omnivorous, exhibits rapid growth, and has a strong reproductive capacity. Its meat is tender, finely textured, and flavorful, with a high protein content, soft bones, and the absence of a muddy odor. It is commonly cultivated in paddy fields (Liang et al., 2022; Xu et al., 2022; Zhou et al., 2020). Current research on WLC has primarily focused on rice-fish co-culture systems (Zhou et al., 2021) and quality traits (Wang et al., 2019). In this study, distant hybridization was employed to cross WLC with RCC, with the aim of producing offspring that exhibit heterosis. Based on this, a new WLC strain is expected to be developed, thereby contributing to the advancement of the rice-fish industry and the broader cyprinid aquaculture sector.

RCC, belonging to the genus *Carassius* within the family *Cyprinidae*, has a relatively flexible evolutionary status. It exhibits high gamete compatibility during hybridization with other genera within the same family (including species from *Cyprinus* or other *Carassius*), thereby demonstrating a greater success rate in overcoming interspecific reproductive barriers compared to many other cyprinid fish. This characteristic makes RCC an ideal paternal material for cultivating distant hybrid species (Zhong et al., 2002; Liu et al., 2017; Zeng et al., 2024). In this study, hybridization between WLC and RCC produced two types of offspring. One type displayed body color and scale patterns similar to those of the maternal WLC but lacked the barbels characteristic of the common carp. Consequently, a comprehensive analysis was conducted on this offspring, encompassing morphological traits, DNA content, chromosome number, 5S ribosomal DNA (5S rDNA) sequences, microsatellite DNA sequences, fluorescence *in situ* hybridization (FISH) analyses, and mitochondrial DNA. In eukaryotes, 5S rDNA is typically organized in tandem repeats, consisting of a 120-base pair (bp) coding sequence for 5S rRNA and a non-transcribed spacer (NTS) (Fujiwara et al., 2009). 5S rDNA has been commonly employed as a genetic marker for species identification and variation analysis (Martins and Wasko, 2004; Pasolini et al., 2006; Qin et al., 2015). The results of 5S rDNA sequencing, FISH, and mitochondrial DNA analyses collectively indicated that the key genetic material of this offspring was primarily derived from the maternal WLC. Therefore, this offspring was designated as WuLi Carp-like Fish (2 n = 100, WCL). Microsatellite DNA analysis, which compares alleles at multiple loci between the offspring and their parents, can be used to confirm the genetic origin of progeny. The detection of paternal-specific alleles is commonly used to assess whether introgression of paternal genetic material has occurred (Muneer, 2014; Liao et al., 2025; Xu et al., 2025). This study provides a systematic analysis of morphological and genetic characteristics of WCL, and the findings are expected to offer valuable insights into the genetic breeding of common carp.

2. Materials and methods

2.1. Ethics statement

All experimental fish used in this study were obtained from Hunan Normal University. The researchers strictly adhered to the ethical guidelines. The experimental fish were provided with adequate nourishment and maintained under specific pH (7.0–8.5), water temperature (22–24 °C), and dissolved oxygen levels (5.0–8.0 mg/L). For humane handling before necropsy, the selected fish were anesthetized with 100 mg/L MS−222 (Sigma-Aldrich, USA).

2.2. Preparation of hybrid offspring between WLC and RCC

Two to three months prior to the breeding season, female WLC and male RCC with well-proportioned body shapes, free from diseases and injuries, and exhibiting clear signs of sexual maturity were selected as broodstock and reared in dedicated ponds. One month before the breeding season, they were fed nutrient-rich bait and subjected to daily flowing water stimulation to promote gonadal maturation.

WLC were used as the female parent and RCC as the male parent. Female fish with distended, soft, and elastic abdomens were selected and artificially injected with a compound-inducing agent containing luteinizing-hormone-releasing hormone analog and chorionic gonadotropin. The dosage of luteinizing hormone-releasing hormone analog was 7.5–10 μg/kg, and that of chorionic gonadotropin was 100–150 IU/kg. The female broodstock were injected first, and 3 h later, the male broodstock, from which milt could be extruded with gentle abdominal pressure, were selected and injected with a mixture of luteinizing hormone-releasing hormone analog and chorionic gonadotropin at half the dosage administered to the females. The injection was performed via a single intraperitoneal injection at the base of the pectoral fin, where scales were absent. Artificial induction was performed during the breeding season when the water temperature was stabilized at 22–23 °C. After the injection of the compound-inducing agent, the broodstock were placed in spawning ponds at a female-to-male ratio of 1:3. Artificial fertilization was performed upon the onset of spawning. The fertilized eggs were then transferred to incubation ponds for flow-through water incubation. After complete hatching, the fry were reared, and the reared fish were tested and screened to obtain the targeted hybrid offspring.

The artificial fertilization process included the following steps: After the broodstock exhibited spawning behavior, they were captured using soft nets. Female WLC that were spawning smoothly were selected, and their eggs were stripped by gently pressing the abdomen into a clean container. Simultaneously, male RCC that were releasing milt smoothly were selected, and their milt was stripped into the same container. The eggs and milt were mixed thoroughly with a feather, and the mixture was quickly poured into a prepared culture dish for fertilization to occur.

The rearing process of the fry included the following steps: After the fry completely hatched, they were cultured in the incubation ponds for 2–3 days until the swim bladder became visible, after which they were transferred to fertilized ponds. Two days after the fry were introduced into the ponds, a soybean milk mixture was applied by sprinkling, ensuring even and fine distributed.

2.3. Measurement of countable traits

In this study, 90 experimental fish, including 30 WLC, 30 RCC, and 30 WCL, were randomly selected for detailed morphological observations. This study aimed to identify morphological differences between parental fish and their hybrid offspring, thereby establishing a reliable data foundation for future genetic and breeding research.

The measurable traits of the experimental fish were assessed, including body length (BL), total length (WL), body depth (BH), head length (HL), head depth (HD), caudal peduncle length (CPL), and caudal peduncle depth (CPD). These metrics were used to characterize the

overall morphology and body proportions. Additionally, a series of ratio indices, such as WL/BL, BH/BL, HL/BL, HD/BL, CPL/BL, and CPD/BL, was calculated to support a comprehensive evaluation of morphological features. These ratios are particularly valuable for interpreting adaptive morphologies and biological traits.

Countable traits were also recorded, such as the number of lateral line scales, scales above and below the lateral line, and the number of rays in the dorsal, pectoral, pelvic, and anal fins. As these traits often differ among species, this procedure aids in species identification.

All collected data were analyzed using one-way analysis of variance in the Statistical Package for the Social Sciences (version 17.0) to clarify intergroup differences. Duncan's test was applied for post-hoc multiple comparisons. Results are presented as mean ± standard error, and a significance level of $P < 0.05$ was adopted.

2.4. Study on the ploidy of WLC-like fish

To demonstrate the ploidy status of natural WLC-like Fish, 30 experimental fish were selected from the parental and offspring generations. Flow cytometry was used to assess the DNA content of blood cells, and chromosome sections were prepared to determine the chromosome numbers.

Sterile syringes pre-filled with 0.2 mL of anticoagulant were prepared to prevent blood clotting and maintain sample liquidity for further analysis. Blood samples were collected from the caudal vein to minimize harm to the fish and were immediately stored in an icebox to preserve cellular viability.

For nuclear staining, sterile EP tubes were prepared, each containing 0.3 mL of DAPI staining solution and 1 mL of 0.8% physiological saline solution. DAPI, a fluorescent dye with a specific affinity for DNA, was used to visualize nuclear morphology under a microscope. Blood was gradually added to the DAPI-containing tubes using a 1 μL micropipette until the solution turned light red, ensuring thorough mixing. After preparation, the tubes were kept in the dark for 10–15 min to facilitate DAPI penetration into the nuclei. The samples were then filtered through a 20 μm mesh to remove unbound dye and cellular debris, followed by dilution for flow cytometry analysis. The DNA content of wild-type WLC was used as a calibration control.

The chromosome section preparation protocol was based on an established method. On Day 1 at 20:30, the fish were injected with 4 mg/mL PHA (10 μg/g body weight; injection volume = 0.0025 × weight) near the pectoral fin. On Day 2 at 8:30, a second PHA injection (15 μg/g; 0.00375 × weight) was administered. A third injection of PHA (6 μg/g; 0.0015 × weight) and colchicine (2.5 mg/mL; 4 μg/g; 0.00075 × weight) was administered on the opposite side 3 h later. After 1 h, the fish were euthanized. Kidney tissues were aseptically extracted through cloacal dissection, rinsed with physiological saline, minced into a homogenate in a Petri dish, and transferred to a 15 mL EP tube. The homogenate was diluted to 4 mL with saline, vigorously pipetted (~200 times), adjusted to 12 mL, and re-pipetted. After 10 min of sedimentation, the supernatant was transferred to another tube. Then, 4 mL of KCl was added, mixed, diluted to 10 mL, and incubated for 60 min (with sediment removal every 10 min). The mixture was centrifuged at 1500 revolutions per minute (rpm) for 5 min. The pellet was fixed three times with Carnoy's fixative (methanol:glacial acetic acid = 1:3; initially 2 mL was added, then brought to 6 mL, mixed, held for 15 min, and centrifuged at 1500 rpm for 5 min per cycle). The final pellet was stored in a fixative at 4 °C. Fixed cells were dropped onto pre-chilled slides (−20 °C, 24 h), flame-dried, stained with Giemsa solution (containing Na_2HPO_4, NaH_2PO_4, and Giemsa stain) for 45 min, gently rinsed, air-dried, and examined under a microscope.

2.5. Study on the fertility of WLC-like fish

To evaluate the fertility of natural WCL, the gonadal structures of 30 individuals were examined using paraffin sections. The experimental procedures followed previously established methods (Qin et al., 2018). Age-matched female parental fish were used as the control group.

Paraffin-embedded gonadal sections were prepared from 12- and 18-month-old WCL to assess gonadal development. The detailed protocol included the following steps: Immediately after capture, the fish were surface-sterilized and dissected to obtain gonadal tissues, which were fixed in Bouin's solution (with one solution change within 1–3 days) and then preserved in 70% ethanol.

The following day at 8:00 AM, the tissues were dehydrated through a graded ethanol series (70–100%, 1 h per concentration), cleared in a xylene-ethanol mixture (1:1, 45 min), followed by pure xylene (≤15 min, repeated until transparent), and infiltrated with molten paraffin (60 °C, 2 h). One hour prior to infiltration, a 60 °C oven was preheated to melt paraffin. After infiltration, the tissues were embedded in paraffin blocks and cooled to solidify. The blocks were trimmed and sectioned at 5 μm using a microtome. Sections were floated in a 42 °C water bath, mounted onto glycerol-coated slides, and dried at 42 °C for 48 h.

For staining, the slides were dewaxed in xylene (6 h), rehydrated through a descending ethanol series (100–70%, 5 min per step), stained with hematoxylin (45 min), rinsed, and differentiated in 0.5% HCl (2 s) and 0.2% NaOH (10 s). The sections were then counterstained with eosin (1 min), dehydrated through an ascending ethanol series, cleared in xylene (5 min), and coverslipped using glycerol gelatin mountant under bubble-free conditions. The slides were air-dried before microscopic evaluation.

2.6. FISH experiment

In this experiment, the prepared chromosome fixative was used for routine slide dropping, after which the slides were baked in a 70 °C oven. Simultaneously, a water bath was preheated to 70 and 80 °C for further steps. Probe 263 was used in this study, which is an RCC-specific probe constructed previously by our laboratory using a BAC library. The methods for probe preparation and purification followed previously described protocols. The specific operational steps were as follows:

For the FISH experiment, a probe hybridization mixture was prepared. This mixture consisted of purified probe (5 μL), deionized formamide (4 μL), 50% dextran sulfate (3 μL), and 20 × saline sodium citrate (SSC, 2 μL). The slides containing chromosomes were then preheated in a 70 °C oven for 1–2 h to facilitate hybridization.

The slides were immersed in 2 × SSC solution for 30 min to reduce the background signals. They then dehydrated through a graded ethanol series (70% and 100%) for 5 min each, followed by a brief treatment in 70% 2 × SSC/deionized formamide solution for 2 min to further optimize the hybridization conditions.

After air-drying at room temperature, the denatured hybridization mixture was applied to the slides, covering the chromosomal areas, and the slides were sealed with coverslips. The sealed slides were placed in a humidified chamber and incubated overnight at 37 °C to allow probe-target binding.

Following hybridization, the slides were washed twice in 2 × SSC/deionized formamide solution at 43 °C for 5 min each, followed by additional washes in 2 × SSC and 1 × SSC for 5 min each. The slides were then air-dried at room temperature, and 8 μL of fluorescein isothiocyanate was added. The samples were incubated in the dark for 20–30 min.

The washing steps were repeated, and the slides were washed in buffer at 43 °C for 15 min and air-dried again at room temperature. Finally, 6 μL of DAPI with an antifade mounting medium was applied to enhance nuclear staining and reduce fluorescence quenching. After completing these steps, the slides were ready for observation under a fluorescence microscope to evaluate the hybridization efficiency and probe specificity.

2.7. Microsatellite experiment

The genetic loci in the 20 parent fish and 10 WCL were amplified by polymerase chain reaction (PCR) using 74 pairs of primers. Only the specific primer pair (SSR−4) primer pair (forward: ACTACTATTCATCAACCCAA; reverse: TGTCCTTCCTTTCTTCTT) fulfilled the specificity requirement.

First, a set of glass plates was thoroughly cleaned and dried, and then assembled with rubber gaskets. Then, 5–10 mL of heat-melted 10% agarose was carefully injected into the gap between the glass plates and the rubber frame to ensure no leakage. After solidification on the glass base, the base was connected to the electrophoresis stand, ensuring the correct alignment of the positive and negative electrodes; the positioning knobs were adjusted for proper orientation.

Polyacrylamide gel preparation: In a 50 mL centrifuge tube, 40 mL of 8% polyacrylamide gel solution was prepared. Then, 800 μL of 10% APS solution and 20 μL of TEMED were added and mixed quickly. Using a 5 mL syringe, the mixture was injected between the two glass plates, ensuring that no bubbles were introduced. A comb (1.0 or 1.5 mm) was inserted, and the gel was left undisturbed for approximately 30 min until polymerization was completed.

Microsatellite band separation: After the gel had solidified, the comb was carefully removed, and the wells were adjusted. A 1 × running buffer was poured into the chamber, ensuring that the buffer covered the wells on one side and submerged the electrodes on the other. Then, 2.5 μL of each sample was loaded sequentially onto the gel. After loading, the power supply was connected and set to 300 V. Electrophoresis was performed for 2.5–3 h, until the tracking dye migrated to the bottom of the gel.

Silver nitrate staining: Once electrophoresis was completed, the power supply was turned off and disconnected. The running buffer was collected in a container for reuse. Three containers were prepared: The first contained 10 g of acetic acid ($C_2H_4O_2$) in 500 mL of purified water; the second contained 0.5 g $AgNO_3$ dissolved in 500 mL of purified water; and the third contained a developing solution made of 9 g NaOH and 0.25 g Na_2CO_3 dissolved in 500 mL of purified water, with 2.5 mL CH_2O (formaldehyde) added. The glass plate assembly was placed in a $C_2H_4O_2$ solution, and the plates were gently separated. The gel was stained with $AgNO_3$ solution for 10 min. After staining, the samples were quickly rinsed with purified water and immersed in the developing solution for 10 min. Finally, the gel was rinsed with water, blotted dry, and the results were recorded and analyzed.

PCR was performed following the procedures and conditions documented in a previous study (Geng *et al.*, 2018). The amplification program initiated with a 5-min pre-denaturation at 94 °C, progressed through repeated cycles of denaturation (94 °C, 30 s), annealing (52.5 °C, 30 s), and extension (72°C, 30 s), followed by a final 7-min extension at 72 °C, and ended with indefinite storage at 4 °C. Amplified products were separated using 8% polyacrylamide gel electrophoresis to ensure accurate genotypic identification.

2.8. 5S rDNA detection experiment

Twenty parent fish and 10 WCL were selected for genetic analysis. First, the caudal fins of the fish were thoroughly washed with a 70% alcohol solution to remove potential microorganisms and impurities. After washing, autoclaved scissors were used to cut off the caudal fins, which were immediately placed into pre-labeled EP tubes. DNA was then extracted using the Omega tissue DNA kit. PCR amplification was performed using 5S rDNA primers (forward: GCTATGCCCGATCTCGTCTGA; reverse: CAGGTTGGTATGGCCGTAAGC). The thermal program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension step at 72 °C for 7 min.

The sequencing results of 5S rDNA were analyzed in detail using JAIView software (version 2.11.3.2).

2.9. Mitochondrial DNA detection experiment

Maternal and offspring WCL, 10 each, were selected for the mitochondrial sequencing experiment. First, the caudal fins were thoroughly washed with a 70% alcohol solution to remove potential microorganisms and impurities from the samples. After washing, autoclaved scissors were used to excise the caudal fins. The caudal fins were immediately placed in pre-labeled EP tubes, and DNA was extracted using the Omega tissue DNA kit. PCR amplification was performed using the mitochondrial primers (Table 1). Twenty-two pairs of primers were used in this study as experimental references to ensure the precise amplification of the target DNA fragments. Following PCR amplification, the resulting products were subjected to gel extraction to purify the target DNA fragments, which were then ligated into the appropriate vectors and transformed into host cells for monoclonal screening and expansion. The screened samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for Sanger sequencing. The sequencing results were preliminarily assembled and aligned using CodonCode Aligner software (version 9.0.1) to reconstruct the mitochondrial genome. The assembled mitochondrial genome was annotated using the online annotation tool Mitofish(http://mitofish.aori.u-tokyo.ac.jp/), and a circular genome map was generated. The sequencing results were meticulously analyzed using JAIView software, and the complete mitochondrial genome sequence was similarly aligned and studied using the same software.

3. Results

3.1. WuLi carp-like hybrid offspring

Through the designed hybridization breeding experiment between WLC and RCC, with Fig. 1-C displaying WCL, Fig. 1-D of the WuLi Carp-Red Crucian Carp Hybrid had not been involved in this study. To evaluate the effectiveness of hybridization breeding, 1000 fish eggs were randomly extracted from the hybrid offspring embryos and subjected to detailed recordings and statistical analyses. Based on these data, the fertilization rate of the fish eggs was 93.03%, whereas the hatching rate was 87.13%.

3.2. Results of measurable and countable traits

In this study, a detailed statistical analysis was conducted on the countable traits of WCL, which included the number of lateral line scales, scales above and below the lateral line, and the counts of dorsal, pectoral, pelvic, and anal fin rays. By comparing the trait data of WLC (the maternal parent) and RCC (the paternal parent), a series of interesting genetic variation patterns was identified. Specifically, the number of lateral line scales in the WCL was intermediate between those of the two parents and exhibited significant differences from both ($P < 0.05$), indicating clear genetic intermediacy. The number of scales above the lateral line also differed significantly from both parental lines ($P < 0.05$), whereas the number of scales below the lateral line differed significantly only from the paternal parent RCC ($P < 0.05$), with no significant difference observed from the maternal parent WLC ($P > 0.05$).

Regarding the number of dorsal fin rays, a significant difference from the maternal parent WLC was found ($P < 0.05$), whereas no significant difference from the paternal parent RCC was detected ($P > 0.05$). Similarly, the statistical results for pectoral fin ray counts revealed a significant difference from the maternal parent WLC ($P < 0.05$) but no significant difference from the paternal parent RCC ($P > 0.05$). No significant differences were observed in the number of pelvic and anal fin rays between the WCL and either parental line ($P > 0.05$).

In this study, a detailed statistical analysis was conducted on the measurable traits of WCL, which included data on WL/BL, BH/BL, HL/BL, HD/BL, CPL/BL, and CPD/BL. For WCL, a significant difference was observed in WL/BL compared to WLC ($P < 0.05$) and in CPL/BL

Table 1
List of primer sequences.

Primers	Forward primer (5'−3')	Reverse primer (3'−5')
12SrRNA	ACAAAGCATAGCACTGAAGATG	TTTGCATGGATGTCTTCTCG
16SrRNA	AAACCCAGGGTGTGGCTGAG	CCGCGGCCGTTGAACTTGTAG
16SrRNA−2	CGCCTGTTTACCAAAAACATCG	CCGGTCTGAACTCAG ATCA
ND1	TCGACGAGGGGGTTTACGAC	AGTAGTTCCTAGTCCTAGGC
ND2	CGAGCCTCGTACCCACGAT	TTAGGCGGTGGGTTGT
ND2–2	TCCTGGTGCTTCCTTTAC	AGCGGTTCCTACTATTCC
COI	GCGAGCATCCATCTACTT	CGTCCATTCCGACAGTAA
COI−2	TCCTCCTTCTCCTATCACT	CTGGGACTGCGTCTATTT
COII	AATTGCTCTACCATCCCT	GCTCATTTATGTCCTCCT
ATPase	AAAGCGTTGGCCTTTTAAGC	GTTAGTGGTCATGGGCTTGGATC
CoNd3	CTTCTAAGCCTATACCTA	AGGCTGTGTGCGGTTGTA
CoNd3–2	CTAAGCCTATACCTACAAGAA	AACATAAGAGTGCGGAGA
ND4	CAGACCTCTGATTTCGGCTC	ACTTGGATTTGCACCAAGAG
ND4–2	TTTCTTTACGCTTCTTCC	GCAAATTGACCCTGTTAT
ND56–1	TCCAGCAATCTACGGGCACA	ATGGCGGAGGGAAGTCAGG
ND56–2	GGCTTCCTTGCGCTT	GCAGATGTGGGTAACGG
ND56–3	CTTATCGGATGATGGCACG	TGGCGGCTGATTGACCTA
ND56–4	CTAGCCAACATGACGAGC	TGAGAATGCGGTTGAAAT
cytb-l	AATGACTTGAAGAACCACCGT	CTCCGATCTTCGGATTACAAGAC
cytb−2	TTCTTTCCACCCATACTTT	AGGAACCAGATGCCAGTA
cytb−3	CACCTACTGTTTCTCCACG	TTTGTGCCTGCACTCTGA
CR	ACCCCTGGCTCCCAAAGC	ATCTTAGCATCTTCAGTG

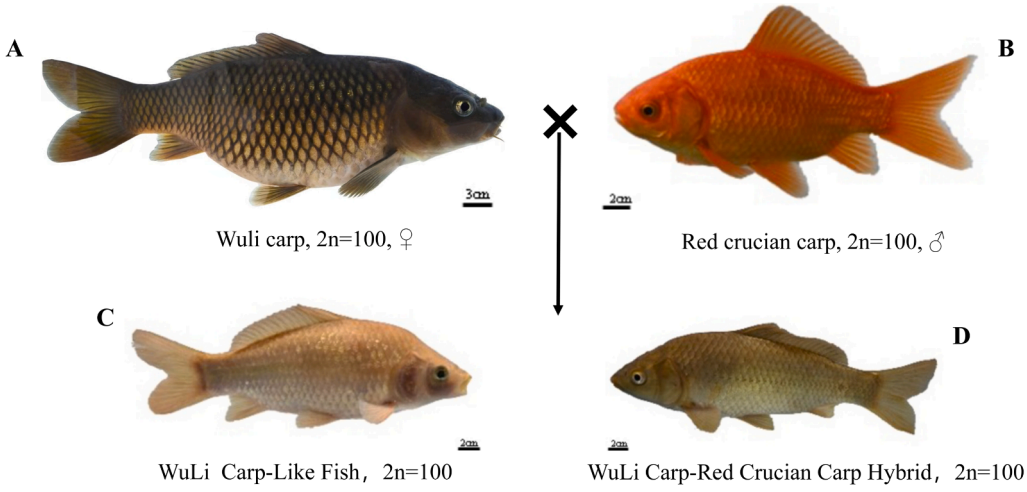

Fig. 1. Hybridization diagram of Paddy Field WLC × RCC. A: WLC; B: RCC; C: WCL; D: 2nWR.

compared to RCC ($P < 0.05$), while no significant differences were found in the remaining measurable traits when compared to either parent ($P > 0.05$).

3.3. Ploidy detection results of the WuLi carp-like fish

The mean DNA content of the parental lines and WCL was determined using flow cytometry. To ensure the accuracy of the experimental results, the DNA content of RCC was used as a reference control. During the experiment, cellular samples from the parental lines and hybrid offspring were comparatively analyzed alongside samples from the RCC. The experimental results are presented in Fig. 2 and Table 2. Through careful analysis of the data, we found that the mean DNA content of WCL was equal to half of the sum of the DNA contents of the parental lines ($P > 0.05$) (Tables 3 and 4).

To further verify the ploidy information of the WCL obtained through flow cytometry, a chromosomal experiment was conducted as a supplementary approach. This experimental method provided direct visual evidence of cellular ploidy by observing morphology and the number of chromosomes during cell division. During the experiment, carefully selected metaphase chromosome spreads, a stage in cell division where chromosome morphology is most distinct, and the number is most stable, were utilized to ensure the accuracy of the statistical results. Detailed observations and counting were performed on the chromosome spreads of WLC. The experimental results, as depicted in Fig. 3, revealed that WCL possessed 100 chromosomes, consistent with the characteristics of a diploid (2 n = 100). This finding was consistent with the flow cytometry results.

3.4. Fertility analysis results of the WuLi carp-like fish

To understand the fertility of WCL, gonadal paraffin sectioning and HE staining experiments were conducted. WCL individuals aged 12 and 18 months were used as experimental fish in this study. The results of the gonadal paraffin sections, as displayed in Fig. 4, revealed that in the ovarian sections of 12-month-old WCL, oogonial proliferation occurred, with a minority developing into Stage II oocytes. In the ovarian sections of 18-month-old WCL, most oocytes had developed to Stages III and IV, while a smaller portion remained at Stage II. In the testicular paraffin sections of male WCL, some spermatogonia developed into primary spermatocytes, and a significant proliferation of spermatids was observed in the 18-month-old testicular sections, but no sperm was

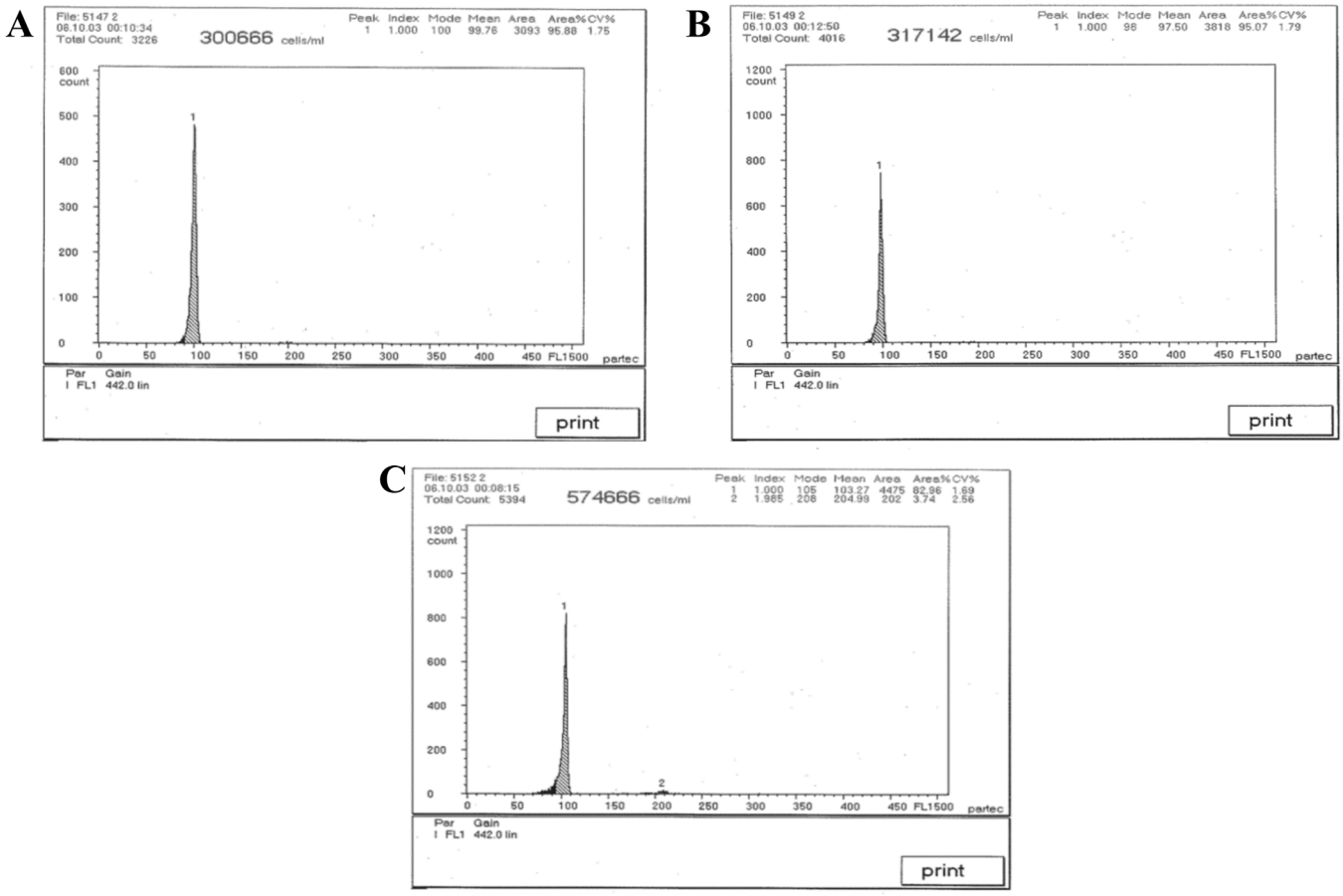

Fig. 2. Average DNA contents of the two-hybrid offspring and their parents. A: The average DNA content of WLC(1:99.76); B: The average DNA content of RCC (1:97.50); C: The average DNA content of WCL(1:103.27).

Table 2
Comparsion of the countable traits among WLC、RCC、and WCL.

Sample	Lateral line scales	Scales above the lateral line	Scales below the lateral line	Dorsal fin	Pectoral fin	Pelvic fin	Anal fin
WLC	34.50 ± 1.12^{d}	6.00 ± 0.00^{c}	5.50 ± 0.50^{a}	$III\sim16.70 \pm 0.46^{c}$	12.40 ± 0.66^{c}	8.40 ± 0.49	$III\sim5.70 \pm 0.46$
RCC	29.20 ± 0.87^{a}	6.30 ± 0.46^{c}	6.80 ± 0.40^{b}	$III\sim15.50 \pm 1.28^{b,c}$	10.80 ± 0.40^{b}	8.50 ± 0.50	$III\sim5.50 \pm 0.50$
WCL	31.40 ± 0.92^{c}	5.00 ± 0.00^{a}	5.50 ± 0.50^{a}	$III\sim14.70 \pm 1.19^{a,b}$	10.20 ± 1.66^{b}	8.50 ± 0.67	$III\sim5.10 \pm 0.83$

Note: The capital Roman numerals represent the hard fin rays, and the Arabic numerals represent the soft fin rays.
Note: The superscripts a, b, and c represent different significant differences respectively. If the subscripts are the same, there is no significant difference.

Table 3
Comparsion of the measurable traits among WLC、RCC、and WCL.

Sample	WL/SL	BH/SL	HL/SL	HD/SL	CPL/SL	CPH/SL
WLC	1.30 ± 0.05^{c}	0.34 ± 0.02^{a}	0.23 ± 0.01^{a}	0.19 ± 0.02	0.19 ± 0.02	0.13 ± 0.01^{a}
RCC	1.22 ± 0.02^{a}	0.38 ± 0.02^{a}	0.23 ± 0.03^{a}	0.20 ± 0.03	0.18 ± 0.01	0.17 ± 0.01^{b}
WCL	$1.26 \pm 0.03^{a,b}$	0.37 ± 0.06^{a}	0.25 ± 0.02^{a}	0.21 ± 0.05	0.16 ± 0.04	0.14 ± 0.01^{a}

Note: The superscripts a, b, and c represent different significant differences respectively. If the subscripts are the same, there is no significant difference. standard length (SL), total length (WL), body depth (BH), head length (HL), head depth (HD), caudal peduncle length (CPL), and caudal peduncle depth (CPH)

discharged from the testes.

3.5. Results of fluorescence in situ hybridization (FISH)

In the chromosome FISH experiment using the RCC-specific probe

Table 4
Average DNA contents of WLC、RCC and WCL.

Sample	Average DNA	Proportion	
		Observation	Anticipated value
WLC	99.76		
RCC	97.50		
WCL	103.27	$(WLC+RCC)/2nL= 1.91^{a}$	2

Note: a indicates that there is no significant difference between the observed and expected values of the ratio ($P > 0.05$).

263, different results were obtained. WLC, as the maternal parent, exhibited no signal points in FISH (Fig. 5-A), whereas the experimental results for the paternal parent RCC displayed 100 signal points, consistent with previous research (Fig. 5-B). Further observations of the WCL revealed that its hybridization results also exhibited no signals, aligning with the outcome observed for the maternal parent WLC (Fig. 5-C).

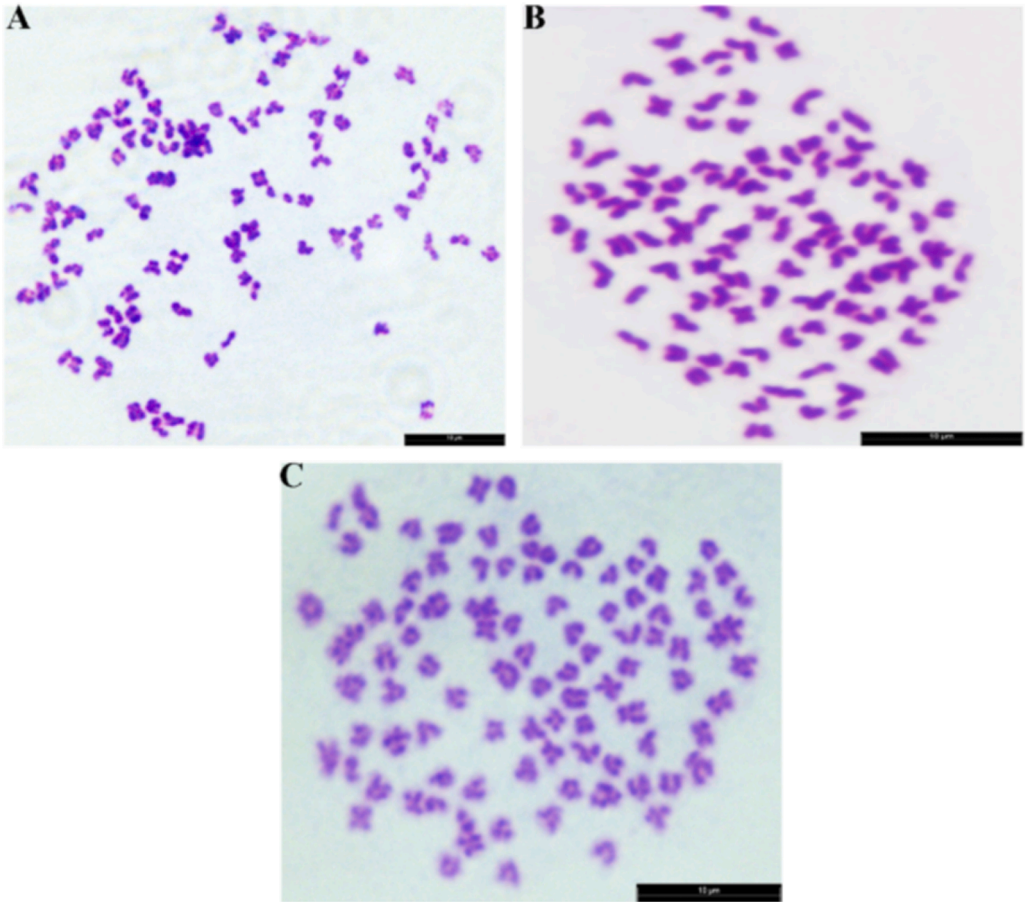

Fig. 3. Two new diploids and parental chromosomes. A: The chromosome is WLC; B: The chromosome is RCC; C: The chromosome is WCL.

3.6. Results of microsatellite analysis

In the detailed biological characterization analysis of WCL, a series of detection methods, including morphology, DNA content analysis, and FISH detection, were employed to investigate its genetic background. Preliminary results indicated that WCL exhibited high similarity to the maternal parent WLC in both morphological and genetic characteristics. However, direct evidence confirming that WCL is a hybrid offspring of WLC and RCC remains insufficient. To further verify the genetic origin of the WCL, a microsatellite analysis experiment was designed. In this analysis, 76 pairs of specific primers were used to conduct extensive genetic marker screening of samples from WLC, RCC, and WCL. Through meticulous comparison and analysis, a RCC-specific DNA insertion fragment was identified using a SSR−4. This insertion fragment was detected in both RCC samples and 2nL samples, but not in the maternal parent WLC. To validate this finding and verify its reliability by increasing the sample size, further testing was performed on more maternal parent WLC, paternal parent RCC, and offspring WCL. The results, as depicted in Fig. 6, confirmed that under the specific primer, the paternal RCC-specific insertion fragment (marked with a black line) was present in the WCL, while this feature was absent in the maternal parent WLC. Simultaneously, this specific DNA fragment was observed in the samples of the paternal parent RCC.

3.7. 5S rDNA sequencing results

In this study, JAIView software (version 2.11.3.2) was used to conduct a detailed comparative analysis of the 5S rDNA sequences of WLC, RCC, and the hybrid offspring WCL,Alignment was performed using the MAFFT program within JAIView. The analytical results revealed diversity in the classes of 5S rDNA monomers among the fish species. Specifically, two types of 5S rDNA monomers were identified in WLC: Class I (203 bp) and Class II (406 bp). Three types were found in RCC: Class I (203 bp), Class II (340 bp), and Class III (477 bp). Similarly, two types were detected in WCL: Class I (203 bp) and Class II (406 bp). As illustrated in Fig. 7, within the 203 bp sequence, differences in the coding region were observed between the paternal parent RCC and both the maternal parent and offspring, characterized by two base variations. However, no differences in the coding region were detected between offspring WCL and the maternal parent.

In the NTS region, the differences between RCC and WLC were more pronounced, involving multiple base changes, whereas WCL exhibited only individual base variations. In the 406 bp sequence, using the 340 bp sequence of the paternal parent RCC as a reference, differences in the coding region compared to the maternal parent and offspring were consistent with those observed in the 203 bp sequence. In the NTS region, the differences between the paternal parent and both the maternal parent and offspring were more pronounced. Among the base changes in the offspring, some sites, such as positions 115, 130, 140, 144, and 347, inherited bases from the paternal parent, whereas other sites, including 109, 118, 129, 134, 135, 199, 336, and 346, formed new bases unique to WCL. When comparing the 477 bp sequence of the paternal parent RCC with the 406 bp sequence, no new base changes were introduced in the coding region, but the differences in the NTS region compared to the maternal parent WLC increased. Among the base changes in the offspring, some sites, such as positions 120 and 250, inherited bases from the paternal parent, whereas other sites, including 114, 123, 133, 135, 139, 140, 149, and 408, formed new bases unique to the hybrid offspring WCL.

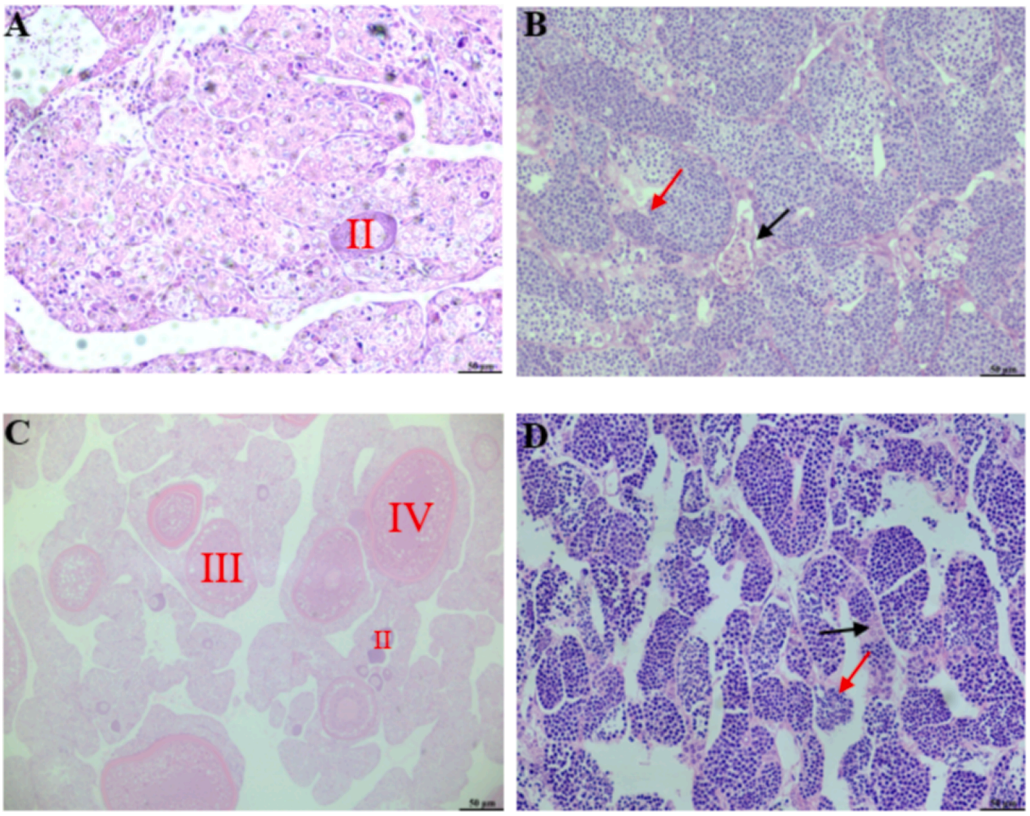

Fig. 4. HE staining results of WCL ovarian and testicular gonadal tissues in offspring. A: The ovarian histology of WCL at 12 months of age, in which oogonia proliferate profusely and very few develop into stage II oocytes; B: The testicular microstructure of WCL at 12 months of age, in which spermatogonia (indicated by the black arrows) are associated with spermatocytes (indicated by the red arrows); C: The ovarian microstructure of WCL at 18 months of age, in which oocytes of stages II, III, and IV develop in the ovary; D: The testicular microstructure of WCL at 18 months of age, in which spermatogonia (indicated by the black arrows) are associated with spermatocytes (indicated by the red arrows).

3.8. Mitochondrial sequencing results

The mitochondrial genomes of the WLC and its offspring were sequenced and analyzed. The sequencing results indicated that the mitochondrial genome length of both fish species was 16,582 bp, and each contained 13 mRNA, 22 tRNA, and 2 rRNA genes, as displayed in Fig. 8. To further investigate the genetic relationships among these fish species, complete mitochondrial genome sequences were compared and analyzed using the BLAST tool. The analysis revealed the following similarity patterns: WCL exhibited an extremely high similarity of 99.66% with the maternal parent WLC in terms of the complete mitochondrial genome sequence, whereas its similarity with the paternal parent RCC, whose mitochondrial genome was downloaded from NCBI, was relatively lower at 89.82%. Conversely, the complete mitochondrial genome sequence similarity between the maternal parent WLC and the paternal parent RCC was 89.74%. These results strongly suggest that the complete mitochondrial genome sequence of WCL primarily exhibits maternal inheritance.

4. Discussion

As common freshwater fish species in China, the hybridization and breeding of carp and crucian carp have consistently attracted considerable attention from aquaculture researchers. Due to their distinct biological characteristics and economic value, hybrid breeding can produce new varieties with desirable traits, thereby enhancing aquaculture efficiency and economic returns. In a previous study, we created an allotetraploid crucian-carp hybrid line and employed it to produce high-quality sterile triploid carp (Liu et al., 2001; Chen et al., 2009). Moreover, another research team successfully developed Furong carp-crucian using *Cyprinus carpio* Furong as the maternal parent and RCC as the paternal parent (Li et al., 2016). The maternal parent, Furong carp, exhibited desirable traits such as large body size, rapid growth, and strong disease resistance, whereas the paternal parent, RCC, possessed advantages including high reproductive capacity and delicious flesh quality. This new variety provides novel materials and perspectives for fish genetic breeding and fundamental biological research. These findings demonstrate the significant potential of distant hybridization in the genetic breeding of carp and crucian carp. Through hybrid breeding, new varieties with superior traits can be obtained, thereby enhancing the efficiency and economic benefits of aquaculture. Simultaneously, these new varieties offer fresh research materials and insights into the genetic characteristics and reproductive biology of fish. In conclusion, with the continuous development of aquaculture and the increasing demand for higher quality and nutritional value of aquatic products, research on the hybrid breeding of carp and crucian carp will continue to deepen.

In fish distant hybridization, when parental chromosome numbers differ, the F_1 generation often yields offspring of varying ploidy levels. For instance, both carp—bream and crucian carp—bream hybrid F_1 produced offspring of different ploidies (Qin et al., 2014; Wang et al., 2017). Conversely, when parental chromosome numbers are identical in fish distant hybridization, the F_1 generation typically produces a single type of offspring with a uniform ploidy level (Liu et al., 2020). Through flow cytometry and chromosomal analysis, WCL was confirmed as diploid with 100 chromosomes, a finding crucial for understanding its genetic background. Further histological examination of gonadal paraffin sections revealed normal germ cell development in both ovaries and testes; however, in 18-month-old WCL testicular sections, although abundant proliferating spermatocytes were observed, no sperm release

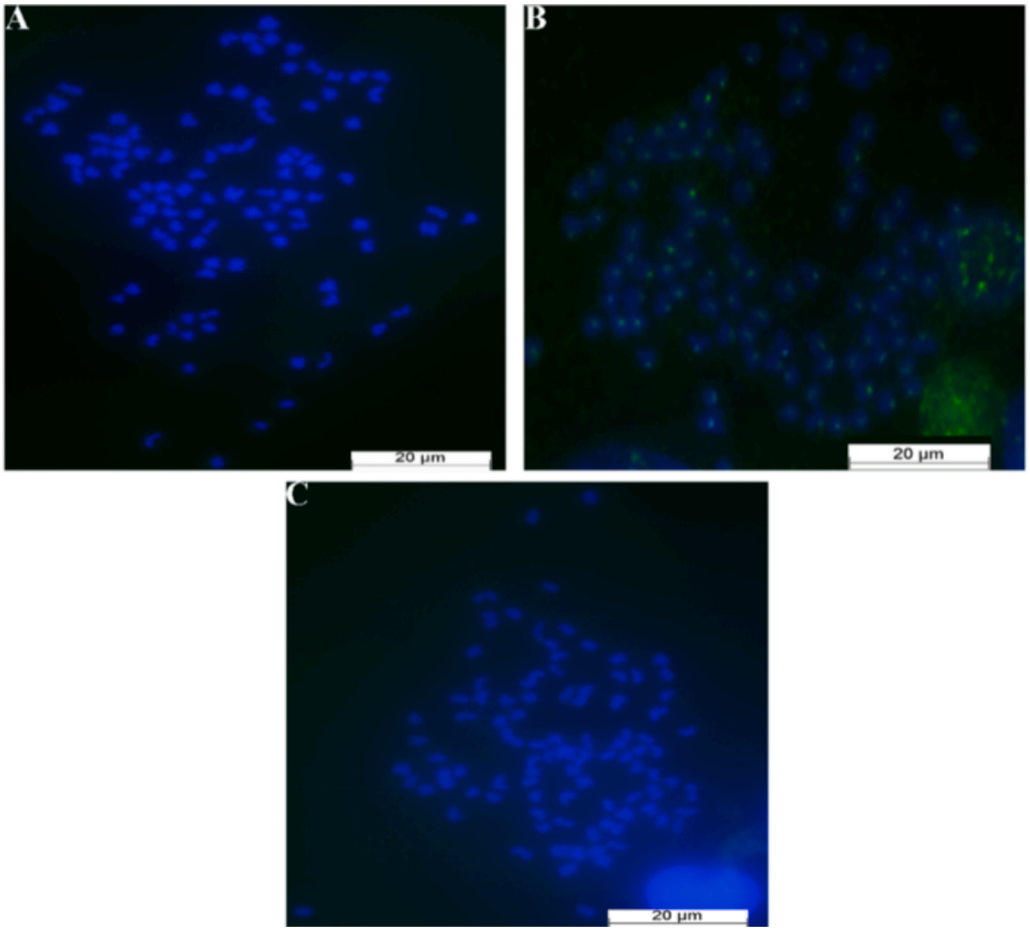

Fig. 5. Comparison of FISH results between WCL and parental lines. A: The FISH results of WLC; B: The FISH results of RCC; C: The FISH results of WCL.

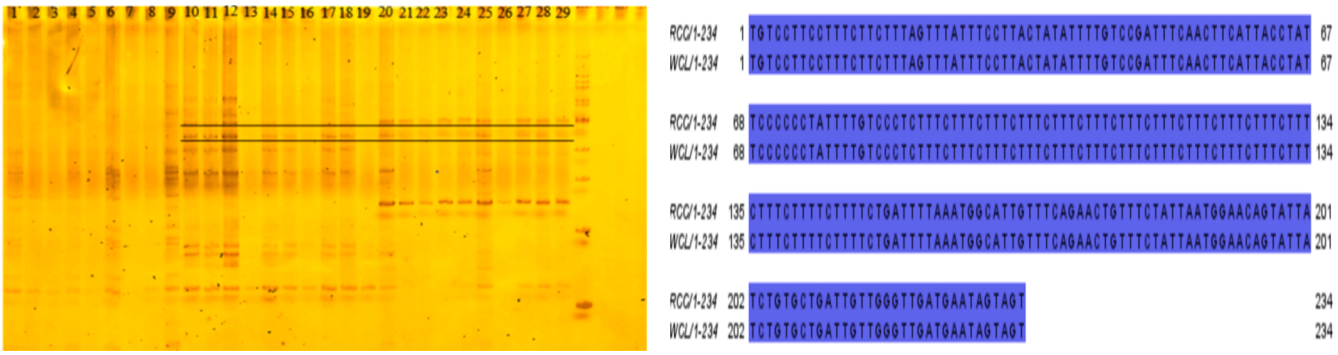

Fig. 6. WCL and parental Microsatellite results. From left to right, numbers 1–8 are the microsatellite results of the parent WLC, numbers 9–19 are the microsatellite results of the parent RCC, and numbers 20–29 are the microsatellite results of the zygote WCL, and the black marker line in the figure is the insertion fragment specific to the parent red crucian carp.

was detected, suggesting a potential delay or impairment in male reproductive maturation. Morphologically, WCL exhibited significant variations from both parents. In this cross, the maternal WLC possessed two pairs of barbels, whereas the paternal RCC had none. The resulting WCL lacked barbels entirely. WCL more closely resembled the maternal parent WLC in both body shape and coloration, and completely lacked barbels, a trait inherited from the paternal RCC. Similar barbel loss was noted in our team's earlier crucian carp × bream hybrids (Liu et al., 2025), leading to speculation that paternal gene insertion or specific recombination events during hybridization may have driven these morphological changes in WCL.

Morphological identification methods cannot be applied when offspring and parents do not exhibit significant differences in morphological features, DNA content, chromosome number, or other easily identifiable traits. In such cases, DNA molecular markers are used for identification. 5S rDNA is commonly used as a molecular marker. 5S rDNA is a transcriptional unit containing highly conserved sequences, and its conservation makes it a key DNA marker for distinguishing phylogenetic relationships among species (He et al., 2013; Martins and Galetti, 2001). Furthermore, FISH can be used to visually identify hybrid species using fluorescent signals. FISH is an important non-radioactive *in situ* hybridization technique, the fundamental principle of which involves using reporter-labeled nucleic acid probes, such as those tagged with Biotin or Digoxigenin, to recognize target DNA on chromosomes or

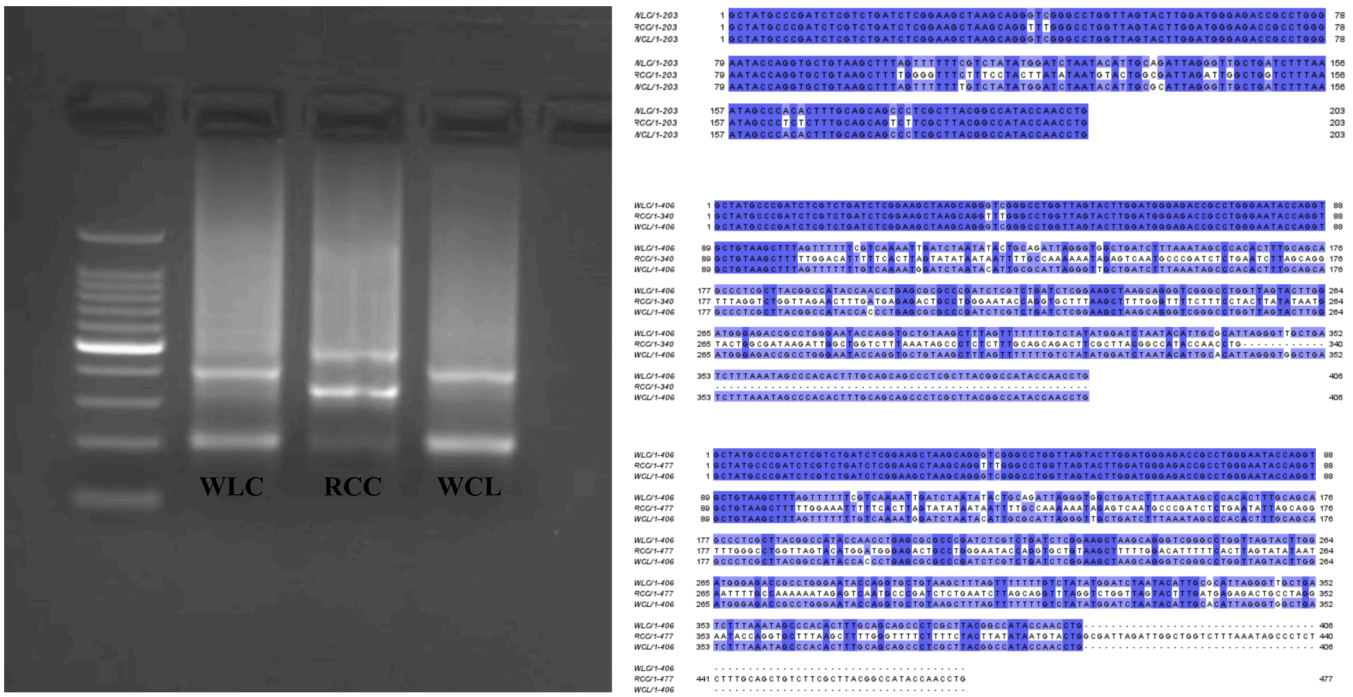

Fig. 7. Comparison of WCL and parental 5S rDNA sequences.

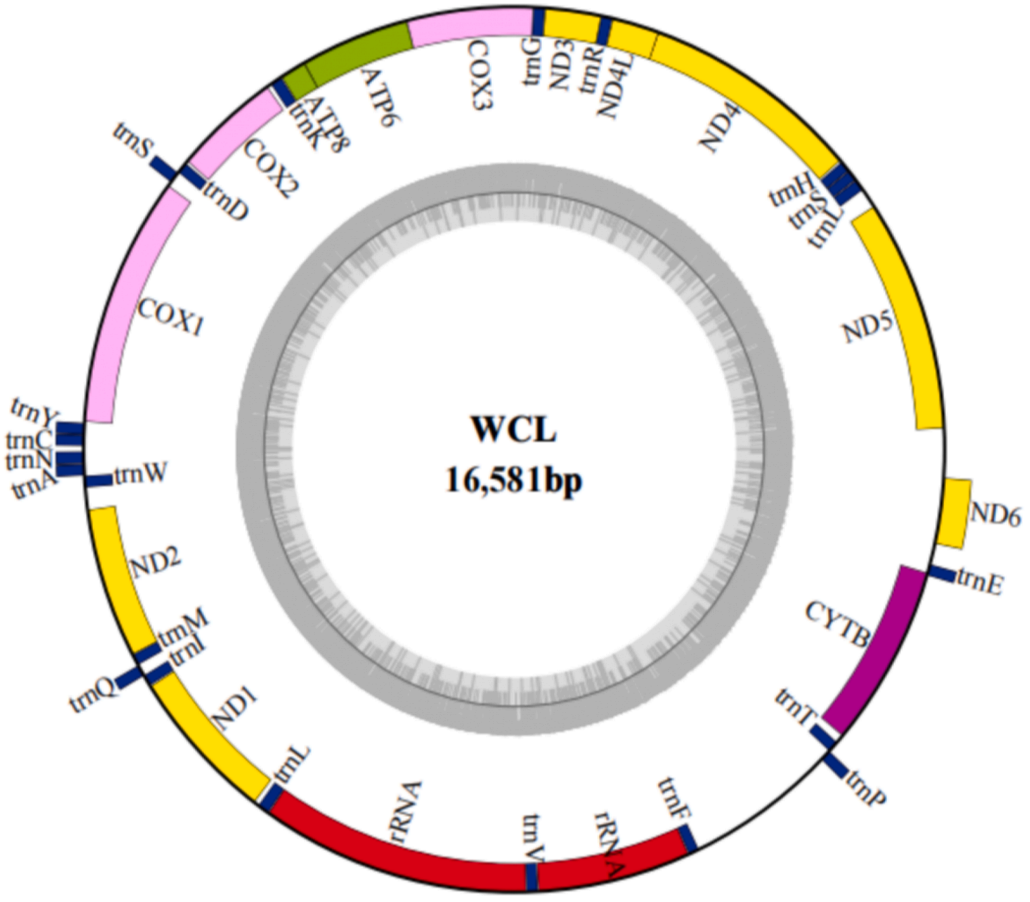

Fig. 8. Circular Maps of Mitochondrial Genomes from WCL.

DNA fiber segments. Hybridization occurs when the probe shares a complementary sequence with the target DNA.

This technology enables researchers to detect and localize specific DNA sequences using fluorescent signals (Pack and Zhuang, 2001; Van

Beek et al., 2025). A comparison of the 5S rDNA sequences between the parents and offspring in this study revealed that WCL exhibited marked differences from the paternal parent, RCC. The 5S rDNA results, including NTS and coding regions, were largely consistent with those of the maternal parent WLC. Moreover, the 5S sequencing profiles also matched those of the maternal parent, displaying only two fragments of 203 and 406 bp. In the FISH experiment, the crucian carp-specific probe 263 was used to reveal differences in chromosomal sequence binding between the parents and offspring. The results indicated that the maternal parent WLC exhibited no signal points, demonstrating that probe 263 did not bind specifically to its chromosomal sequences. Conversely, the paternal parent RCC displayed distinct signal points, confirming the effective binding of probe 263 to RCC chromosomal sequences. WLC depicted no signal points, confirming that probe 263 did not specifically bind to its chromosomal sequences. These two sets of results indicate that the genetic material of WCL was primarily derived from the maternal parent. Furthermore, based on the 5S rDNA and FISH findings, even though WCL lacked barbels, it still belonged to the carp-type offspring.

As an offspring produced by hybridization, WCL has proven its maternal origin based on its 5S rDNA and FISH results. However, its morphology underwent noticeable changes, and therefore, it was speculated that the insertion of paternal genes caused such alterations. We plan to employ microsatellite experiments to confirm the insertion of paternal genes. Microsatellite technology, an important tool in fish genetics research, can identify paternal fragment insertions, such as determining whether specific DNA sequences originated from the paternal parent. This technique relies on the high polymorphism of SSRs, which are short, repetitive DNA sequences that vary among individuals and can serve as genetic markers (Umhang, et al., 2025; Xie et al., 2023; Ylmaz and Atasaral, 2024). We detected the presence of genetic fragments inserted from the paternal parent RCC into the offspring WCL. These microsatellite results indicate a genetic relationship between WCL and its paternal parent, RCC. Mitochondria, one of the vital organelles in eukaryotic cells, are regarded as the "powerhouses" of the cell. They possess an independent genetic system that includes a unique circular DNA molecule. The mitochondrial genome is relatively small, with molecular sizes ranging from 15,560 to 18,705 bp. Due to its compact size, tightly arranged genes, and high degree of conservation, mitochondrial DNA has often been employed in molecular phylogenetic analysis and population genetics studies (Wang et al., 2017; Wu et al., 2022).

In this study, primers were designed to sequence the full-length mitochondrial genomes of the maternal parent WLC and offspring WCL. The similarity between the complete mitochondrial genome sequences of WCL and the maternal parent WLC was extremely high, reaching 99.66%; whereas its similarity with the paternal parent RCC was relatively low at 89.82%. The similarity between the complete mitochondrial genome sequences of the maternal parent WLC and paternal parent RCC was 89.74%. The higher similarity of the offspring to the paternal parent compared to that between the maternal and paternal parents served as strong supplementary evidence to the earlier 5S rDNA, FISH, and microsatellite experiments. These results confirmed that, although most of the genetic composition of WCL was derived from the maternal parent, partial paternal gene insertions had occurred, and such insertions contributed to certain morphological and genetic alterations in WCL.

Through the aforementioned experiments, we demonstrated the genetic origin of WLC. Unlike ordinary hybrid offspring, WLC exhibit distinct characteristics. The results from the 5S rDNA, mitochondrial DNA, and FISH experiments collectively confirmed that most of its genetic material was derived from the maternal parent. Previous studies have demonstrated that distant hybridization in fish can produce natural gynogenetic offspring (Mishina et al., 2022; Uehara et al., 2025). Our research team has also observed this phenomenon in crucian carp × blunt snout bream, common carp × blunt snout bream, and grass carp × culter hybrids (Liu et al., 2010; Wang et al., 2017; Wu et al., 2019). Moreover, the natural Dongting Lake water system hosts a polyploid complex and exhibits natural gynogenesis (Xiao et al., 2011). Based on these findings, it is reasonable to infer that WCL is a naturally gynogenetic offspring. These results not only provide insights into the genetic composition of WCL but also establish a scientific foundation for the varietal improvement of WCL. It is anticipated that more new varieties with superior traits will emerge in future aquaculture practices, thereby promoting sustainable development and innovation in the common carp farming industry.

CRediT authorship contribution statement

Kun Zhang: Supervision, Methodology. **Qinbo Qin:** Supervision, Resources, Funding acquisition, Conceptualization. **Xidan Xu:** Supervision, Methodology. **Ming Ma:** Supervision, Methodology. **Zeyang Li:** Validation, Investigation. **Chongqing Wang:** Supervision, Methodology. **Yuhan Yang:** Validation, Investigation. **Yuchen Jiang:** Validation, Investigation. **Zhengkun Liu:** Validation, Investigation. **Jinhai Bai:** Validation, Investigation. **Yue Zhou:** Validation, Investigation. **Xu Huang:** Supervision, Methodology. **Wanjing Peng:** Validation, Investigation. **Ling Xiong:** Validation, Investigation. **Siyang Li:** Validation, Investigation. **Enkui Hu:** Validation, Investigation. **Qian Xiao:** Validation, Investigation. **Xiaowei Xu:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2026.103708.

Data availability

Data will be made available on request.

References

Chen, S., Wang, J., Liu, S., Qin, Q., Xiao, J., Duan, W., Luo, K., Liu, J., Liu, Y., 2009. Biological characteristics of an improved triploid crucian carp. Sci. China C. Life Sci. 52 (8), 733–738. https://doi.org/10.1007/s11427-009-0079-3. Epub 2009 Aug 29. PMID: 19727591.

Fujiwara, M., Inafuku, J., Takeda, A., et al., 2009. Molecular organization of 5S rDNA in bitterlings (*Cyprinidae*). Genetica 135 (3), 355–365. https://doi.org/10.1007/s10709-008-9294-2.

Gui, J.F., Zhou, L.L., 2010. Genetic basis and breeding application of clonal diversity and dual reproductive modes in polyploid gibel carp (*Carassius* gibelio). Sci. Sin. Vitae 40 (2), 97–103. https://doi.org/10.1360/zc2010-40-2-97.

He, W., Xie, L., Li, T., et al., 2013. The formation of diploid and triploid hybrids of female grass carp × male blunt snout bream and their 5S rDNA analysis. BMC Genet. 14, 110. https://doi.org/10.1186/1471-2156-14-110.

Hu, F., Wu, C., Zhou, Y., et al., 2020. Production of androgenetic, triploid and tetraploid hybrids from the interspecific hybridization of female Japanese crucian carp and male blunt snout bream (vol 491, pg 50, 2018). Aquaculture 527.

Li, Z., Wang, J., Ming, Z., Liu, M., Wang, D., Wu, Y., Li, C., Jiang, G., Liu, L., 2016. The Complete Mitochondrial Genome of the Hybrid Furong-Crucian [Cyprinus carpio Furong (♀) × *Carassius* auratus red var (♂)]. Mitochondrial DNA B Resour. 1 (1), 506–507. https://doi.org/10.1080/23802359.2016.1192511. PMID: 33473536; PMCID: PMC7800864.

Liang, Z., Zou, L., Tian, L., Liu, M., Li, C., Xiao, G., Cai, J., Zhang, Y., Li, S., An, M., et al., 2022. Genetic origin and differentiation of ten paddy field–farmed Cyprinus carpio strains in China. Aquaculture 561, 738573. https://doi.org/10.1016/j.aquaculture.2022.738573.

Liao, A., Zhang, S., Yu, Q., Wang, Y., Tan, H., Wu, P., Ding, Y., Hu, B., Liu, W., Tao, M., Zhang, C., Qin, Q., Luo, K., Tang, C., Liu, Q., Liu, S., 2025. Formation and characterization of artificial gynogenetic northern snakehead (Channa argus) induced by inactivated sperm of mandarin fish (Siniperca chuatsi). Aquaculture 595 (Part 1), 741488. https://doi.org/10.1016/j.aquaculture.2024.741488.

Liu, Q., Wang, J., Xiao, J., et al., 2017. Muscle nutrient of *Carassius* auratus cuvieri (♀) × *Carassius* auratus red var. (♂) and its parents. J. Fish. China. https://doi.org/10.11964/jfc.20160610428.

Liu, Q., Liu, J., Yuan, L., Li, L., Tao, M., Zhang, C., Qin, Q., Chen, B., Ma, M., Tang, C., Liu, S., 2020. The establishment of the fertile fish lineages derived from distant hybridization by overcoming the reproductive barriers. Reproduction 159 (6), R237–R249. https://doi.org/10.1530/REP-19-0576. PMID: 32191917.

Liu, Q.F., Luo, K.K., Zhang, X.Y., Liu, F.L., Qin, Q.B., Tao, M., Wen, M., Tang, C.C., Liu, S. J., 2021. Production of a new type of triploid fish derived from the diploid hybrid crucian carp (♀) × autotetraploid fish(♂). Reprod. Breed. 1 (2), 122–127. https://doi.org/10.1016/j.repbre.2021.07.003.

Liu, S., Qin, Q., Xiao, J., et al., 2006. The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176 (2), 1023. https://doi.org/10.1534/genetics.107.071373.

Liu, S., Qin, Q., Wang, Y., et al., 2010. Evidence for the Formation of the Male Gynogenetic Fish. Mar. Biotechnol. 12 (2), 160. https://doi.org/10.1007/s10126-009-9219-9.

Liu, S., Wang, S., Liu, Q., et al., 2022. The Research Advances in Animal Distant Hybridization and Polyploid Organisms. Fish. Distant Hybrid. 1–37. https://doi.org/10.1007/978-981-16-5067-3_1.

Liu, S.J., Liu, Y., Zhou, G.J., Zhang, X.J., Luo, C., Feng, H., He, X.X., Zhu, G.H., Yang, H., 2001. The Formation of Tetraploid Stocks of Red Crucian Carp×Common Carp Hybrids as an Effect of Interspecific Hybridization. Aquaculture 192 (2–4), 171–186. https://doi.org/10.1016/S0044-8486(00)00451-8.

Liu, Z.K., Deng, X.Y., Peng, W.J., Li, S.Y., Xiong, L., Hu, E.K., Tang, Y., Li, Z.Y., Xiao, Q., Yang, Y.H., Huang, X., Wang, C.Q., Xu, X.D., Xu, X.W., Zhang, K., Bai, J.H., Zhou, Y., Jiang, Y.C., Qin, Q.B., 2025. Gynogenetic Offspring from Distant Hybridization of Red Crucian Carp × Blunt Snout Bream: Insights into Barbel Origin. Aquaculture 609, 742791. https://doi.org/10.1016/j.aquaculture.2025.742791.

Martins, C., Wasko, A.P., 2004. Organization and evolution of 5S ribosomal DNA in the fish genome. Focus. Genome Res.

Martins, Cesar, Galetti Jr., Pedro Manoel, 2001. Organization of 5S rDNA in species of the fish Leporinus: two different genomic locations are characterized by distinct nontranscribed spacers. Genome 44 (5), 903–910. https://doi.org/10.1139/g01-069.

Mishina, T., Nomoto, K., Machida, Y., Hariu, T., Watanabe, K., 2022. Origin of scarlet gynogenetic triploid *Carassius* fish: Implications for conservation of the sexual-gynogenetic complex. PLoS. One 17 (10), e0276390. https://doi.org/10.1371/journal.pone.0276390. PMID: 36264937;.

Muneer, P.M.A., 2014. Application of Microsatellite Markers in Conservation Genetics and Fisheries Management: Recent Advances in Population Structure Analysis and Conservation Strategies. Genet. Res. Int. 2014, 691759. https://doi.org/10.1155/2014/691759. PMID: 24808959; PMCID: PMC3997932.

Pack, S.D., Zhuang, Z., 2001. Fluorescence in situ hybridization. Methods Mol. Med. 50, 35–50. https://doi.org/10.1385/1-59259-084-5:35.

Pasolini, P., Costagliola, D., Rocco, L., et al., 2006. Molecular Organization of 5S rDNAs in Rajidae (Chondrichthyes): Structural Features and Evolution of Piscine 5S rRNA Genes and Nontranscribed Intergenic Spacers. J. Mol. Evol. 62 (5), 564–574. https://doi.org/10.1007/s00239-005-0118-z.

Qin, Q,, Huo, Y,, Liu, Q,, Wang, C,, Zhou, Y,, Liu, S,, 2018. Induced gynogenesis in autotetraploids derived from *Carassius auratus* red var. (♀) × Megalobrama amblycephala (♂). Aquaculture 495, 710–714. https://doi.org/10.1016/j.aquaculture.2018.06.028.

Qin, B., Qin, J., et al., 2015. Organization and variation analysis of 5S rDNA in gynogenetic offspring of *Carassius* auratus red var. (♀) × *Megalobrama amblycephala* (♂). BMC Genet. 16, 57. https://doi.org/10.1186/s12863-015-0186-z.

Qin, Q., Lai, Z., Cao, L., Xiao, Q., Wang, Y., Liu, S., 2016. Rapid genomic changes in allopolyploids of *Carassius* auratus red var. (♀) × *Megalobrama amblycephala* (♂). Sci. Rep. 6, 34417. https://doi.org/10.1038/srep34417. PMID: 27703178; PMCID: PMC5050501.

Qin, Q., Wang, Y., Wang, J., et al., 2014. Abnormal chromosome behavior during meiosis in the allotetraploid of *Carassius auratus* red var. (♀) × *Megalobrama amblycephala* (♂) [J]. BMC Genetics 15, 95.

Qin, Q., Wang, C., Zhou, Y., Qin, H., Zhao, C., Yang, L., Yu, T., Liu, S., 2020. Rapid Genomic and Epigenetic Alterations in Gynogenetic *Carassius* auratus Red Var. Derived from Distant Hybridization. Mar. Biotechnol. (NY) 22 (3), 433–442. https://doi.org/10.1007/s10126-020-09963-6. Epub 2020 Apr 5. PMID: 32249338.

Uehara, R., Takeda, S., Oku, D., Sasaki, R., Murakami, M., Shiba, H., Katakura, F., Moritomo, T., 2025. Establishment of a novel clonal GFP-expressing transgenic ginbuna crucian carp. Dev. Comp. Immunol. 162, 105290. https://doi.org/10.1016/j.dci.2024.105290. Epub 2024 Nov 19. PMID: 39571925.

Umhang, G., Bastid, V., Rinaldi, L., Pepe, P., Ciccone, E., Sotiraki, S., Ligda, P., Oudni-M′rad, M., M'rad, S., Lahmar, S., Said, Y., Elmehatli, K., Ahmad, H., Kuzmina, T., Yevstafieva, V., Aghayan, S.A., Gevorgyan, H., Simsek, S., Ponce-Gordo, F., Benchikh El Fegoun, M.C., El Berbri, I., Fassi Fihri, O., Saarma, U., Grenouillet, F., Boué, F., Aguayo, J., 2025. A panel of five microsatellites as an alternative to mitogenome sequencing for investigating the genetic diversity of Echinococcus granulosus sensu stricto from global to farm scale. Infect. Genet. Evol., 105868 https://doi.org/10.1016/j.meegid.2025.105868.

Van Beek, J.M., Robles, G., Mewalal, R., Blaby, I., Hatzenpichler, R., 2025. A collection of archaeal 16S rRNA Clone-FISH cultures for probe validation in fluorescence in situ hybridization experiments. Microbiol. Resour. Announc. 14 (12). https://doi.org/10.1128/mra.01131-25.

Wang, S., Ye, X., Wang, Y., et al., 2017. A new type of homodiploid fish derived from the interspecific hybridization of female common carp×male blunt snout bream. Sci. Rep. 7 (1), 4189. https://doi.org/10.1038/s41598-017-04582-z.

Wang, S., Zhou, P., Huang, X.X., Liu, Q.L., Lin, B.W., Fu, Y.Q., Gu, Q.H., Hu, F.Z., Luo, K. K., Zhang, C., Tao, M., Qin, Q.B., Liu, S.J., 2020. The establishment of an autotetraploid fish lineage produced by female allotetraploid hybrids × male homodiploid hybrids derived from Cyprinus carpio (♀) × *Megalobrama amblycephala* (♂). Aquaculture 515, 734583. https://doi.org/10.1016/j.aquaculture.2019.734583.

Wang, T., Huang, K., Sun, L., Zuo, T., Lin, Y., Liang, Y., 2019. Analysis of Nutritional Components and Safety Evaluation of Hehua Carp Muscle. J. South. Agric. 50, 1579–1586. https://doi.org/10.3969/j.issn.2095-1191.2019.07.24.

Wu, C., Huang, X., Hu, F.Z., Ouyang, Y.X., Zhao, L., Wang, S., Li, W.H., Fan, J.J., Zhang, C., Ren, L., Qin, Q.B., Luo, K.K., Tao, M., Liu, S.J., 2019. Production of diploid gynogenetic grass carp and triploid hybrids derived from the distant hybridization of female grass carp and male topmouth culter. Aquaculture 504, 462–470. https://doi.org/10.1016/j.aquaculture.2018.12.056.

Wu, T.H., Yang, C.H., Pai, T.W., Ho, L.P., Wu, J.L., Chou, H.Y., 2022. Identification of fish species through tRNA-based primer design. BMC Bioinforma. 22 (10), 633. https://doi.org/10.1186/s12859-022-04717-8. PMID: 36474163; PMCID: PMC9724258.

Xiao, J., Zou, T., Chen, Y., Chen, L., Liu, S., Tao, M., Zhang, C., Zhao, R., Zhou, Y., Long, Y., You, C., Yan, J., Liu, Y., 2011. Coexistence of diploid, triploid and tetraploid crucian carp (*Carassius* auratus) in natural waters. BMC Genet. 12, 20. https://doi.org/10.1186/1471-2156-12-20. PMID: 21276259; PMCID: PMC3040159.

Xie, D., Yang, N., Xu, W., et al., 2023. Development and Application of Potentially Universal Microsatellite Markers for Pheasant Species. Animals 13 (23), 14. https://doi.org/10.3390/ani13233601.

Xu, X., Hu, E., Xiao, Q., Huang, X., Wang, C., Xu, X., Zhang, K., Zhou, Y., Bai, J., Liu, Z., Jiang, Y., Tang, Y., Deng, X., Li, S., Peng, W., Xiong, L., Yang, Y., Li, Z., Ma, M., Qin, Q., Liu, S., 2025. Formation and Biological Characteristics Analysis of Artificial Gynogenetic WuLi Carp Induced by Inactivated Sperm of *Megalobrama amblycephala*. Biol. (Basel) 14 (8), 994. https://doi.org/10.3390/biology14080994. PMID: 40906134; PMCID: PMC12383469.

Xu, Y.L., Chen, Z., Qin, J.Q., Wen, L.T., Pan, X.H., Zhou, K.Q., Huang, Y., Lin, Y., Du, X.S., 2022. Estimation of Genetic Parameters for the Main Growth Traits of Quanzhou Hehua Carp. Guangxi Sci. 29, 801–808. https://doi.org/10.13656/j.cnki.gxkx.20220919.023.

Ylmaz, D., Atasaral, E., 2024. Microsatellite-based Parentage Assignment in Tiger Trout (Salmo trutta × Salvelinus fontinalis). Turk. J. Fish. Aquat. Sci. 24 (6). https://doi.org/10.4194/trjfas25096.

Zeng, Q., Chen, Y., Wang, M., et al., 2024. Study on sperm cryopreservation of hybrid fish derived from *Carassius* cuvieri (♀) × *Carassius* auratus red var (♂). Reprod. Breed. 4 (4), 234–242. https://doi.org/10.1016/j.repbre.2024.09.002.

Zhang, Z.H., Chen, J., Li, L., et al., 2014. Research advances in animal distant hybridization. Sci. China Life Sci. 57 (9), 889–902. https://doi.org/10.1007/s11427-014-4707-1.

Zhong, L.J., Zhang, J.X., Liu, J.S., et al., 2002. Studies on the gonadal development in allotetraploid hybrids of *Carassius* auratus red var. (♀) × Cyprinus carpio L. (♂). Acta Hydrobiol. Sin.

Zhou, A., Sun, D., Chen, Y., Chen, S., Li, Q., Chen, Y., Zou, J., 2021. Analysis on the Aquaculture Technology and Benefits of Lian Shan Paddy Field Hehua Carp. Sci. Fish. Farming 2, 82. https://doi.org/10.14184/j.cnki.issn1004-843x.2021.02.050.

Zhou, K.Q., Pan, X.H., Lin, Y., Du, X.S., Huang, Y., Pan, Z.Z., Wen, L.T., Qin, J.Q., Zhang, F., Chen, Z., 2020. Analysis of the Relationship between Morphological Traits and Body Mass of Guangxi Hehua Carp. Henan Agric. Sci. 49, 159–165. https://doi.org/10.15933/j.cnki.1004-3268.2020.09.020.