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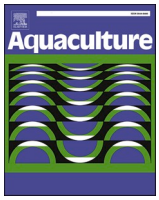

Different ploidy-level hybrids derived from female common carp × male topmouth culter

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ABSTRACT

Distant hybridization is an effective strategy for creating polyploids in fish genetic breeding. In this study, the cross of common carp (*Cyprinus carpio*, 2n = 100, COC, ♀) and topmouth culter (*Culter alburnus*, 2n = 48, TC, ♂), which belonged to different subfamilies, was conducted and obtained surviving hybrid offspring (CT hybrids). Based on the measurement of DNA content and chromosomal numbers, three ploidy levels of CT hybrids were identified: diploid hybrids (2n = 74, 2nCT), triploid hybrids (3n = 124, 3nCT), and tetraploid hybrids (4n = 148, 4nCT). Analyses of countable traits and measurable traits indicated a significant similarity between CT hybrids and COC in comparison to TC. The genetic variation analysis of 5S rDNA and *HoxD10a* gene in CT hybrids revealed the retention of all COC genotypes and the partial elimination of TC genotypes, as well as the generation of novel variant genotypes (derived from TC genotypes) and recombinant genotypes (derived from parental genotypes), which suggested that in CT hybrids, the paternal subgenome exhibited greater instability than the maternal subgenome. In addition, the droplet digital PCR (ddPCR) method, combining allele-specific probes targeting the *EPHX2* gene, was employed for the first time to investigate the genome composition of hybrids. The results showed that the ratios of COC-allele number to TC-allele number were approximately 1 in 2nCT, 2 in 3nCT, and 1.5 in 4nCT, respectively, which confirmed the hybridization origins for each type and effectively distinguished between the three CT hybrids. The obtainment of CT hybrids harbors potential benefits and application in aquaculture and provides an ideal model for understanding the influence of hybridization and polyploidization on the genomic composition of fish species.

1. Introduction

Distant hybridization, referring to crosses between two distinct species or higher-ranking taxa, facilitates genome transfer between species, resulting in phenotypic and genotypic changes in the progeny (Liu et al., 2022; Wang et al., 2019; Zhang et al., 2014) and potentially giving rise to the creation and utilization of heterosis (Houston et al., 2020; Wang et al., 2022b; Wu et al., 2022). Compared with close hybridization, distant hybridization offers a outstanding advantage in fish genetic breeding due to its significantly increased likelihood of producing polyploid progeny (Hu et al., 2018; Liu et al., 2021; Liu, 2010; Liu et al., 2007; Qin et al., 2014; Song et al., 2012). For instance, through distant hybridization between female red crucian carp (*Carassius auratus* red var., 2n = 100, RCC) and male common carp (*Cyprinus carpio*, 2n = 100, COC), a fertile allotetraploid hybrid lineage (4n = 200, F_3-F_{28}) was obtained, and then backcrossed with its diploid parents to generate triploid hybrids (3n = 150) that exhibit desirable traits such as accelerated growth rate, enhanced disease resistance, and high-quality meat (Chen et al., 2009; Cui et al., 2022; Liu, 2010; Liu et al., 2001; Shen et al., 2006). In another example involving the cross between female COC and male blunt snout bream (*Megalobrama amblycephala*, 2n = 48, BSB), an autotetraploid common carp lineage (4n = 200) was established for producing various innovative triploid hybrid varieties (Wang et al., 2020a, 2020b; Wang et al., 2017b).

The 5S ribosomal DNA (5S rDNA) in eukaryotes is organized in tandem arrays consisting of a 120 bp coding sequence (CDS) for 5S rRNA

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and a non-transcribed spacer (NTS) (Bogenhagen and Brown, 1981; Fujiwara et al., 2008), where the 5S rRNA acts as a crucial structural and functional component of the ribosome's large subunit, enhancing protein synthesis efficiency and accuracy with CDS regions exhibiting high conservation among species (Rebordinos et al., 2013); while NTS sequences vary among closely related species and are usually used as genetic markers for species identification or variation analysis (Martins and Galetti Jr, 2001; Martins and Wasko, 2004; Pasolini et al., 2006). Researches have demonstrated that hybridization events can impact the repeat units of 5S rDNA, resulted in the elimination of paternal specific units and the emergence of novel ones (Cao et al., 2020; He et al., 2013; Qin et al., 2010; Zhang et al., 2015). *Hox* genes, essential developmental regulatory genes, are organized in clusters on chromosomes, encoding transcription factors crucial for early embryonic development and stem cell differentiation (Gorski and Walsh, 2000), and despite undergoing multiple duplication, this gene family remains highly conserved in vertebrates, making it valuable for evolutionary and phylogenetic studies (Luo et al., 2007; Wang et al., 2021; Wang et al., 2017a).

TaqMan probes are short oligonucleotides labeled with a fluorescent reporter dye at the 5′-end and a quencher at the 3′ end (Behlke et al., 2005; Holland et al., 1991), commonly utilized in conjunction with quantitative real-time-PCR (qPCR) to specifically bind to the DNA target region for precise quantification (Xue et al., 2020). Compared to SYBR Green-based qPCR, TaqMan qPCR is more sensitive and can be used for multiple detections, rendering it particularly suitable for species identification researche (Tajadini et al., 2014; Ye et al., 2016). In this study, we developed allele-specific TaqMan probes to explore genomic composition and genetic variation in hybrid fish, integrating them with droplet digital PCR (ddPCR), a cutting-edge technology renowned for its superior accuracy compared to traditional qPCR (Campomenosi et al., 2016; Wang et al., 2022c; Zmienko et al., 2015). These techniques were utilized for the absolute quantification of parental alleles in hybrid fish genomes for the first time, allowing for a detailed understanding of the genetic composition and variation patterns in hybrid organisms.

The selection of appropriate parents and the screening of hybrid offspring with enhanced traits emerge as primary focal point in hybridization breeding. COC has one of the longest breeding histories of fish in China due to its gentle temperament, easy feeding, strong adaptability and widespread distribution, but its meat quality has generally declined due to the excessive pursuit of high productivity (Chen et al., 2009; Wang et al., 2020a, 2020b). Topmouth culter (*Culter alburnus*, 2n = 48, TC) is popular with customers for its tender meat and great taste, but has a poor resistance (Gong et al., 2021, 2022; Xiao et al., 2014). The hybridization of COC and TC aligns with the principle of selecting parents with complementary characteristics, potentially yielding progeny that exhibit favorable traits from both parental species. Additionally, while allopolyploid hybrids are commonly observed in crosses between species from distinct subfamilies with different chromosome numbers, there is no documented instance of hybrids resulting from the distant hybridization of COC and TC. In this study, we performed the cross between COC and TC and obtained three types of hybrid offspring (CT hybrids), including diploid hybrids (2n = 74, 2nCT), triploid hybrids (3n = 124, 3nCT), and tetraploid hybrids (4n = 148, 4nCT). Both of morphological and genetic characteristics of CT hybrids were investigated, and the results will be beneficial for fish genetic breeding and polyploidy study.

2. Materials and methods

2.1. Ethics statement

Administration of Affairs Concerning Animal Experimentation guidelines state that approval from the Science and Technology Bureau of China and the department of wildlife administration is not necessary when the fish in question are not rare or near extinction (first-class or second-class state protection level). Therefore, approval was not required for the experiments conducted in this paper.

2.2. Animals and crossing procedure

Specimens of COC and TC were obtained from the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, China. During the reproductive seasons (from May to June) in 2019–2022, ten mature female COC and ten mature male TC were selected as maternal and paternal parents, respectively. Embryos were produced by artificial insemination and hatched in culture dishes at a water temperature of 20–24°C. The hatched fry was transferred to a pond for further culture.

2.3. Measurement of DNA content and preparation of chromosome spreads

The DNA content of erythrocytes from CT hybrids and their parents was measured at one-day post-hatching for 100 hybrids and one-year post-hatching for all the living hybrids, respectively, using a flow cytometer (Cell Counter Analyzer, Partec, Germany). The whole body of the larva or approximately 0.5 mL of blood was collected from the caudal vein of the above fish into tubes or syringes containing 200–300 units of sodium heparin. Blood samples were treated following the method described in previously published papers (Liu et al., 2007; Wang et al., 2020a, 2020b), and red crucian carp was used as the control. All of the samples were measured under the same conditions. To estimate the ploidy of the hybrid offspring, we calculated the ratios of the DNA content of the hybrid offspring to the sum of that of COC and TC, and a $\chi 2$ test with Yate's correction were applied to test for deviation from expected ratio values.

Chromosome counts were conducted on kidney tissues from COC, TC, and CT hybrids to further determine their ploidy levels. Ten fish from each group, aged one-year, were sampled for the analysis. Preparations were made according to the method of Liu et al. (2007). The shape and number of chromosomes were analyzed under a microscope (Olympus). A total of 200 metaphase spreads of chromosomes were analyzed for each type of fish. Preparations were examined under an oil lens at a magnification of 330 × .

2.4. Morphological traits analyses and appearance of erythrocytes

At one year of age, twenty individuals in each group including COC, TC and CT hybrids were randomly selected for morphological examination. Seven measurable traits were recorded, including whole length (WL), body length (BL), body height (BH), head length (HL), head height (HH), caudal peduncle length (CPL), and caudal peduncle height (CPH) (accurate to 0.1 cm), and then the average values of WL/BL, BL/HL, BL/BH, HL/HH, BH/HH, and CPL/CPH were calculated. In comparing the countable characteristics of CT hybrids with their parents, the following characteristics are used: the number of scales with lateral-line canals (lateral-line scales), the number of scales in an oblique scale row on body above and below lateral line (scales above lateral line and scales below lateral line), the number of dorsal fins rays (dorsal-fin rays), the number of anal fins rays (anal-fin rays), and the number of pelvic fin rays (pelvic-fin rays). An independent-samples *t*-test in SPSS Statistics 17.0 software was used to assess the significant difference between the two given groups.

Approximately 0.5 mL of blood was drawn from each of the one-year-old COC, TC, and CT hybrids for analysis. 10 μL of blood was taken to make a blood smear. After air-drying, it was stained with Wright's solution for 3 min, and then added an equal proportion of phosphate buffer for 1 min. For each sample, 3 smears were made in the same manipulation. Each blood smear was meticulously examined under oil immersion to assess the shape of the erythrocyte nuclei in the peripheral blood, distinguishing between oval-shaped and dumbbell-shaped.

2.5. Genomic DNA extraction, PCR and sequencing

The total genomic DNA of COC, TC and CT hybrids (10 samples at one year old for each group) was extracted from the caudal fins using Tissue DNA Kits (OMEGA). A set of primers (5S—F, 5′-GCTATGCCC-GATCTCGTCTGA-3′; 5S-R, 5′-CAGGTTGGTATGGCCGTAAGC-3′) was designed and synthesized to directly amplify the 5S rDNA from genomic DNA by PCR according to previous studies (Qin et al., 2010; Wang et al., 2015). Similarly, a pair of primers (*HoxD10a*-F, 5'-GGAATG-CAAACCTGTGGACT-3′; *HoxD10a*-R, 5'-TCGGCTCATCTTCTT-GAGTTTCA-3′) was used to amplified the *HoxD10a* gene (Wang et al., 2020a; Wang et al., 2017a). The thermal program consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s and a final extension step at 72 °C for 7 min. Amplification products were separated on a 1.8% agarose gel using TAE buffer. The DNA fragments were purified using a gel extraction kit (Sangon, Shanghai, China), ligated to the pMD18-T vector (TaKaRa, Dalian, China), and transformed into *Escherichia coli DH5a* and purified. Finally, the inserted DNA fragments were sequenced using an automated DNA sequencer (ABIPRISM 3730). The sequences were aligned using Clustal W (Larkin et al., 2007), and analyzed using BLASTn (Johnson et al., 2008) and BioEdit (Hall et al., 2011) to determine the homology and variation regions. The software of MEGA11 (Tamura et al., 2021) was used to analyze sequences similarity and construct phylogenetic tree by neighbor-joining method. Nodal support values were assessed from 1000 nonparametric bootstrap replicates. Correlogram of sequences similarity was made using the corrplot package of R language. The sequences generated in this study have been submitted to GenBank under the following accession numbers: OM867554 - OM867560, OM867562 - OM867566, OM906829, and ON156355 - ON156367 for 5S rDNA; and OR242469 - OR242478 for *HoxD10a*. Refer to Tables 5 and 6 for additional details.

2.6. Designing TaqMan probe of parental alleles, qPCR and ddPCR experiments

After analyzing sequence alignments of several homologous genes of COC and TC, we finally selected *EPHX2* (*epoxide hydrolase 2*) gene to design TaqMan probe for distinguishing and quantifying the parental alleles. The universal primers (*EPHX2*-F, 5'-CTCAA-GAAGTAGGTGCAGTAA-3′; *EPHX2*-R, 5'-TGCCTTGACACTTTCTTCAT-3′) and allele-specific probes (*EPHX2*-COC-Probe, 5'-CTCAGCGATTG-GAAATTGACAGCAG-3′; *EPHX2*-TC-Probe, 5'-TGAAAACTGGCAGCA-GATTCAAAGA-3′) were designed by Primer Premier 5 and synthesized by Sangon. Thereinto, the 5 '-end of *EPHX2*-COC-Probe was labeled with fluorophore FAM, the 5 '-end of *EPHX2*-TC-Probe was labeled with fluorophore VIC. The 3 '-end of both probes was modified with quencher BHQ1. The qPCR experiment was carried out to detect the specificity of *EPHX2* allele probes on ABI 7900HT RTPCR System (Applied Biosystems, Carlsbad, CA, United States). To ensure reproducibility and reliability, three independent biological samples and three technical replicates of each biological sample were analyzed. The final reaction mix consisted of 10 μL SGExcel GoldStar TaqMan Mixture (Sangon, Shanghai, China), 0.5 μL forward and reverse primers, 30 ng DNA, and ddH_2O to bring up the reaction volume to 20 μL. The thermal program consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s.

For the quantification of *EPHX2* alleles in genomic DNA of COC, TC and CT hybrids, ddPCR was performed with the Naica crystal Digital PCR Systems (Stilla Technologies, Villejuif, France) by using the following optimized reaction mix: 2 μL of diluted DNA sample (10 ng/μL), 5 μL PerfeCTa MultiPlex qPCR ToughMix (Quantabio), 0.31 μL *EPHX2*-COC and *EPHX2*-TC probes, 2.5 μL forward and reverse primers, 2.5 μL Fluorescein (VWR), and nuclease-free water to bring up the total volume to 25 μL. Samples were loaded onto Sapphire chips (Stilla Technologies) and ddPCR was performed with the same cycle conditions as qPCR. After amplification, the experiment data was obtained using Naica Prism system (Stilla Technologies) and analyze with Crystal Miner software (Stilla Technologies). An independent-samples *t*-test was employed to test for deviation from expected ratio values.

3. Results

3.1. Formation of CT hybrids

The crossing procedure of female COC (2n = 100, Fig. 1A) × male TC (2n = 48, Fig. 1B) is outlined in Fig. 1. During the reproductive seasons (from May to June), we have repeated the distant hybridization experiment for four years (2019–2022), and successfully obtained three types of hybrid offspring including diploid hybrids (2nCT, 2n = 74, Fig. 1C), triploid hybrids (3nCT, 3n = 124, Fig. 1D), and tetraploid hybrids (4nCT, 4n = 148, Fig. 1E). The fertilization rate in this hybrid combination is high (approximately 90%), with nearly half of the fertilized eggs hatching into larvae. However, the survival rate of CT hybrid larvae was relatively low, with only about 30–50 out of 10,000 larvae growing into juvenile fish after one year. In the newly hatched larvae of CT hybrids, no polyploids were detected, as all samples measured by flow cytometry were identified as diploids (2nCT). Whereas, one year after hatching, the proportion of 2nCT significantly reduced to only 3%, with 53% 3nCT and 40% 4nCT being detected. Adult hybrids have not been obtained yet as the larvae of CT hybrids continue to die during the culture process.

3.2. Determination of ploidy

The ploidy of hybrid offspring was identified by measuring DNA content. The sum of the DNA content of COC and TC was used as the control, and the distribution of DNA content of hybrid offspring is presented in Table 1 and Fig. 1F-J. The mean DNA content of 2nCT was equal to half of the sum of COC and TC ($P > 0.05$), suggesting that 2nCT had one set of chromosomes from COC and one set of chromosomes from TC. The mean DNA content of 3nCT was equal to COC plus half of TC ($P > 0.05$), suggesting that 3nCT had two sets of chromosomes from COC and one set of chromosomes from TC. The mean DNA content of 4nCT was equal to the sum of COC and TC ($P > 0.05$), suggesting that 4nCT had two sets of chromosomes from COC and two sets of chromosomes from TC. To verify the ploidy of hybrid offspring, we further detected the chromosome number of and CT hybrids and their parents (Table 2). There were 100 and 48 chromosomes in COC and TC, respectively (Fig. 1K and L). Most of chromosomal metaphase spreads in 2nCT samples had 74 chromosomes (Fig. 1M), confirming that they were diploid hybrids (2n = 74). Most of chromosomal metaphase spreads in 3nCT samples had 124 chromosomes (Fig. 1N), and most of chromosomal metaphase spreads in 4nCT samples had 148 chromosomes (Fig. 1O), directly suggesting that 3nCT and 4nCT are triploid and tetraploid hybrids, respectively.

3.3. Morphological traits

The appearances of COC, TC and CT hybrids are presented in Fig. 1A-E. All progeny types exhibited hybrid morphological characteristics and inherited two pairs of barbels from their maternal parent, COC. We record both countable and measurable traits within each group and compared the values between CT hybrids and their parents. The results indicated no significant differences ($P > 0.05$) between CT hybrids and COC in most countable traits, such as the number of lateral-line scales, scales below lateral line, pelvic-fin rays, and anal-fin rays. However, there were significantly fewer dorsal-fin rays in 2nCT ($P < 0.05$) and more scales above lateral line in 4nCT ($P < 0.05$) (Table 3). Compared with TC, all countable traits in CT hybrids showed significant differences ($P < 0.05$), except for the number of pelvic-fin rays. The results of measurable traits in Table 4 showed distinct differences in the ratios of

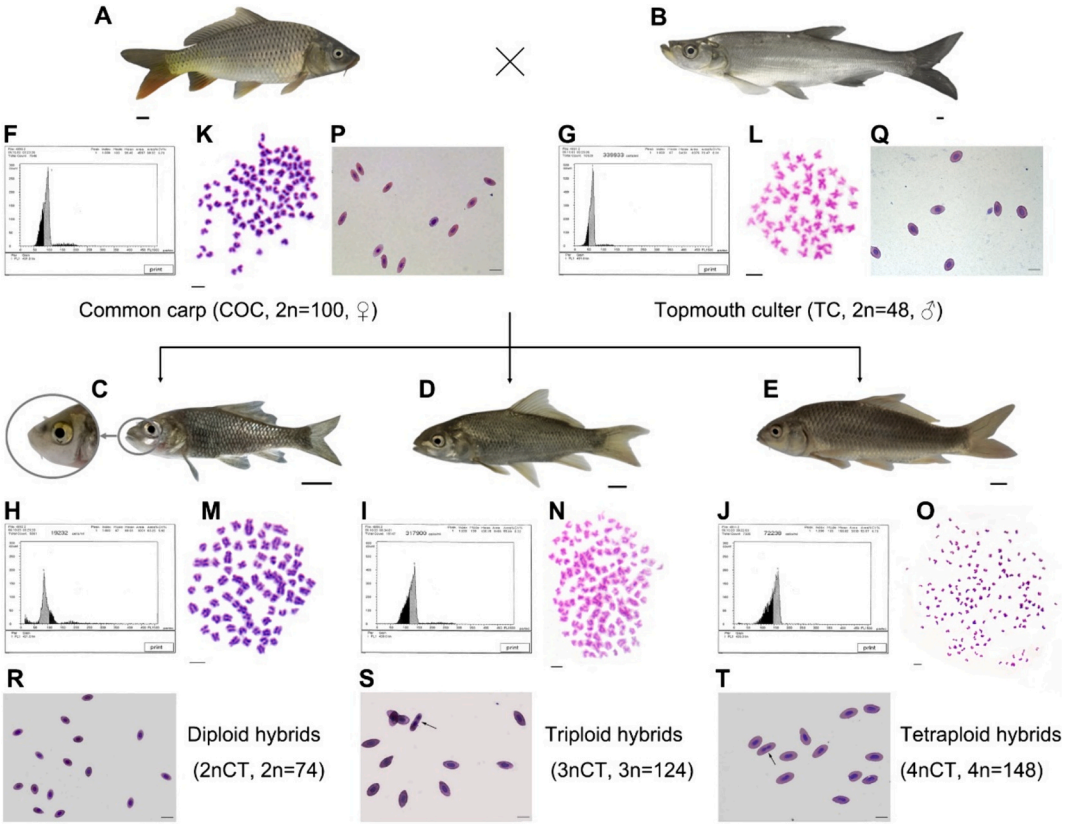

Fig. 1. The appearance, flow cytometric histograms, chromosomal trait, and erythrocytes of common carp (COC), topmouth culter (TC), and their hybrid offspring. (A-E) The appearance of COC (A), TC (B), diploid hybrids (2nCT) (C), triploid hybrids (3nCT) (D), and tetraploid hybrids (4nCT) (E). Bar = 1 cm. (F-J) Cytometric histograms of DNA fluorescence for COC, TC and their hybrid offspring. (F) The mean DNA content of COC (peak 1: 99.46). (G) The mean DNA content of TC (peak 1: 64.51). (H) The mean DNA content of 2nCT (peak 1: 88.66). (I) The mean DNA content of 3nCT (peak 1: 135.15). (J) The mean DNA content of 4nCT (peak 1: 159.82). (K—O) Chromosome spreads at metaphase in COC, TC and their hybrid offspring. (K) The metaphase chromosome spreads of COC (2n = 100). (L) The metaphase chromosome spreads of TC (2n = 48). (M) The metaphase chromosome spreads of 2nCT (2n = 74). (N) The metaphase chromosome spreads of 3nCT (3n = 124). (O) of 4nCT (4n = 148). Bar = 3 μm. (P-T) The appearance of erythrocytes in COC, TC and their hybrid offspring. Normal erythrocytes with one nucleus in COC (P), TC (Q), and 2nCT (R). Normal erythrocytes with one nucleus and unusual erythrocytes with two nuclei (arrows) in 3nCT (S) and 4nCT (T). Bar = 10 μm.

Table 1
The mean DNA content measured by flow cytometer in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish type	DNA content [a]	Ratio	
		Observed	Expected
COC	99.46		
TC	64.51		
2nCT	88.66	2nCT/(1/2COC + 1/2TC) = 1.08 [b]	1
3nCT	135.15	3nCT/(COC + 1/2TC) = 1.03 [b]	1
4nCT	159.82	4nCT/(COC + TC) = 0.97 [b]	1

[a] The intensity of fluorescence (unit, channel).
[b] The observed ratio is not significantly different from the expected ratio ($P > 0.05$).

BL/BH and BH/HH in 2nCT and CPL/CPH in 4nCT compared to COC ($P < 0.05$). However, there were no significant differences between CT hybrids and COC for the remaining ratios, including WL/BL, BL/HL, and HL/HH ($P > 0.05$). When compared to TC, significant differences were observed in CT hybrids for most measurable traits, such as WL/BL, BL/HL, HL/HH, and BH/HH ($P < 0$ 0.05), except for the CPL/CPH ratio, which showed no difference between CT hybrids and TC ($P > 0.05$). Additionally, there was a noticeable disparity in the BL/BH ratio when comparing 3nCT or 4nCT with TC, while no differences were observed between 2nCT and TC (P < 0 0.05). Furthermore, based on examining the appearances of erythrocytes, we observed that in diploid samples (COC, TC and 2nCT) the nucleus of erythrocytes appeared normal and oval-shaped (Fig. 1P-R). However, in polyploid samples (3nCT and 4nCT), besides erythrocytes with an oval-shaped nucleus, we observed erythrocytes with a dumbbell-shaped nucleus (Fig. 1S and T). Previous studies have demonstrated that polyploid fish may exhibit deformed

Table 2
Examination of chromosome number in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish type	No. in metaphase	Distribution of chromosome number									
		<48	48	<100	100	<74	74	<124	124	<148	148
TC	200	16	184								
COC	200			24	176						
2nCT	200					27	173				
3nCT	200							21	179		
4nCT	200									35	165

Table 3
Comparison of the countable traits among common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish type	Lateral-line scales	Scales above lateral line	Scales below lateral line	Dorsal-fin rays	Pelvic-fin rays	Anal-fin rays
COC	34.63 ± 1.41 (33–37)	6.13 ± 0.64 (5–7)	5.63 ± 0.52 (5–6)	III + 18.38 ± 0.52 (III + 18–19)	8.75 ± 0.46 (8–9)	III + 6.75 ± 0.46 (6–7)
TC	81.50 ± 4.59 (75–88)	16.83 ± 1.17 (15–18)	8.83 ± 1.33 (7–11)	III + 7.83 ± 0.75 (7–9)	8.50 ± 0.55 (8–9)	III + 22.83 ± 1.72 (20–25)
2nCT	36.50 ± 2.38 (33–38)#	6.75 ± 0.50 (6–7)#	5.25 ± 0.50 (5–6)#	III + 16.25 ± 0.50 (III + 16–17)*,#	9.00 ± 0.00 (9)	III + 6.75 ± 0.50 (6–7)#
3nCT	36.33 ± 1.53 (36–38)#	7.67 ± 1.15 (7–9)#	5.67 ± 1.15 (5–7)#	III + 17.67 ± 1.53 (III + 16–19)#	9.00 ± 0.00 (9)	III + 7.33 ± 0.58 (7–8)#
4nCT	35.25 ± 2.76 (32–39)#	7.63 ± 1.30 (6–10)*,#	5.75 ± 1.04 (5–8)#	III + 18.00 ± 1.07 (III + 16–19)#	8.88 ± 0.35 (8–9)	III + 6.88 ± 0.64 (III + 6–8)#

Lateral-line scales: the number of scales with lateral-line canals; Scales above lateral line: the number of scales in an oblique scale row on body above lateral line; Scales below lateral line: the number of scales in an oblique scale row on body below lateral line; Dorsal-fin rays: the number of dorsal fins rays; Pelvic-fin rays: the number of pelvic fins rays; Anal-fin rays: the number of anal fins rays.
* indicates a significant difference when compared with COC ($P < 0.05$).
# indicates a significant difference when compared with TC ($P < 0.05$).

Table 4
Comparison of the measurable traits among common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish type	WL/BL	BL/HL	BL/BH	HL/HH	BH/HH	CPL/CPH
COC	1.22 ± 0.03	3.51 ± 0.14	2.91 ± 0.09	1.31 ± 0.10	1.58 ± 0.13	1.22 ± 0.03
TC	1.18 ± 0.02	4.58 ± 0.18	4.05 ± 0.38	1.88 ± 0.23	2.13 ± 0.09	1.35 ± 0.09
2nCT	1.27 ± 0.03#	3.32 ± 0.33#	3.80 ± 0.45*	1.48 ± 0.09#	1.31 ± 0.18*,#	1.13 ± 0.09
3nCT	1.28 ± 0.02#	3.41 ± 0.31#	3.12 ± 0.09#	1.50 ± 0.08#	1.63 ± 0.13#	1.36 ± 0.19
4nCT	1.26 ± 0.05#	3.72 ± 0.59#	3.20 ± 0.25#	1.27 ± 0.14#	1.46 ± 0.12#	1.50 ± 0.22*

WL: whole length; BL: body length; BH: body height; HL: head length; HH: head height; CPL: caudal peduncle length; CPH: caudal peduncle height.
* indicates a significant difference when compared with COC ($P < 0.05$).
# indicates a significant difference when compared with TC ($P < 0.05$).

erythrocytes, characterized by either two nuclei or a dumbbell-shaped nucleus (Hu et al., 2018; Lu et al., 2009; Wang et al., 2020a, 2020b). Hence, the presence of deformed erythrocytes with a dumbbell-shaped nucleus in the blood smear specimens of 3nCT and 4nCT provided additional confirmation for their polyploid nature.

3.4. Genetic variation of 5S rDNA

To reveal the genetic composition and molecular variation of hybrid offspring, DNA fragments of 5S rDNA were amplified and sequenced from COC, TC and CT hybrids. The sequencing results showed that there were two differently sized fragments (203 and 406 bp) in COC, three (188, 285, and 376 bp) in TC, five (188, 203, 225, 391 and 406 bp) in 2nCT, six (188, 199, 203, 376, 391 and 406 bp) in 3nCT, and seven (188, 198, 203, 288, 376, 391 and 406 bp) in 4nCT (Table 5). All fragments were confirmed to 5S rDNA by BLASTn analysis and split into the CDS region of and NTS region. In sequence alignments (Fig. 2A and B), the CDS regions of all 5S rDNA fragments showed highly conserved, but the NTS regions showed highly variable both in length and sequence. According to sequence similarity (Fig. 2C), the NTS regions were divided into five different types (NTS-I ~ V), which further constituted different types of 5S rDNA units. For example, in COC, the 203 bp fragment consisted of one CDS region (120 bp) and NTS-I (83 bp), and therefore it was designated as I type of 5S rDNA unit; the 406 bp fragment was just two repeats of 203 bp, and then it was designated as I + I type of 5S rDNA unit. The genetic variation of 5S rDNA in these fish samples was summarized and presented in Fig. 2D. In parents, COC contained one NTS type (NTS—I) that constituted two types of 5S rDNA units (I and I + I), and TC contained two NTS types (NTS-II and III) that constituted three types of 5S rDNA units (II, III and II + II). For CT hybrids, the results indicated that they not only inherited the 5S rDNA types from parents, but also generated some new variant and recombinant types. For example, all of hybrids (2nCT, 3nCT, and 4nCT) inherited I and I + I type from COC, and II type from TC, and at the same time generated two novel recombinant types of 5S rDNA units (I + II and II + I). Moreover, 2nCT lost two types of TC (III and II + II), but generated one novel variant type (IV) that characterized by a disparate NTS type (NTS-IV). NTS-IV was found only in 2nCT, and its first 42 bp was similar to NTS-III, but remaining part contained multiple mutations. 3nCT lost only one type of TC (II + II), and also generated one novel variant type (V) that characterized by a new NTS type (NTS-V). NTS-V was found in both 3nCT and 4nCT, and likely resulted from a deletion of 87 bp sequence in NTS-III. NTS-IV and V obviously derived from NTS-III of TC as they showed a higher similarity to NTS-III than other NTS types, with NTS-IV having more mutations than NTS-V. In addition to possessing V type of

Table 5
The sequence category of 5S rDNA units based on the types of non-transcribed spacer (NTS) in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish Species	Length (bp)			Types		GenBank accession number
	5S units	CDS	NTS	5S units	NTS	
COC	203	120	83	I	I	OM867564
	406	240	166	I & I	I	ON156355
TC	188	120	68	II	II	OM867565
	285	120	165	III	III	OM867566
	376	240	136	II & II	II	ON156356
2nCT	203	120	83	I	I	OM867554
	188	120	68	II	II	OM867555
	225	120	105	IV	IV	OM867556
	406	240	166	I & I	I	ON156357
	391	240	151	I & II	I, II	ON156358
	391	240	151	II & I	I, II	ON156359
3nCT	203	120	83	I	I	OM867557
	188	120	68	II	II	OM867558
	199	120	79	V	V	OM867559
	406	240	166	I & I	I	ON156360
	376	240	136	II & II	II	ON156361
	390	240	150	I & II	I, II	ON156363
	391	240	151	II & I	I, II	ON156362
4nCT	203	120	83	I	I	OM867560
	188	120	68	II	II	OM906829
	288	120	168	III	III	OM867562
	198	120	78	V	V	OM867563
	406	240	166	I & I	I	ON156364
	376	240	136	II & II	II	ON156365
	391	240	151	I & II	I, II	ON156366
	391	240	151	II & I	I, II	ON156367

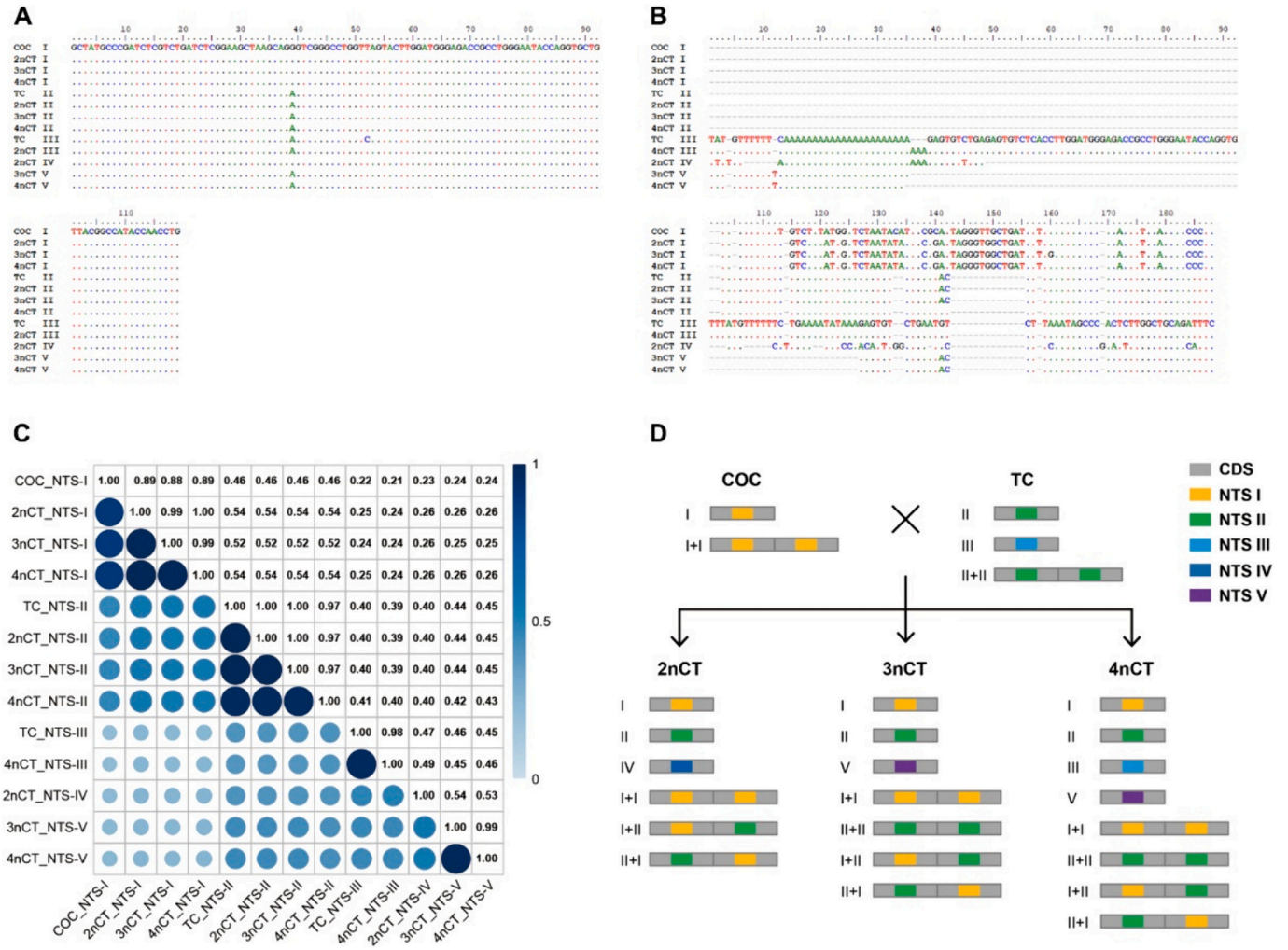

Fig. 2. Hereditary characteristics of 5S rDNA units in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT). (A) The alignment of the coding sequences (CDS) of 5S rDNA units. (B) The alignment of the non-transcribed spacer (NTS) sequences of 5S rDNA units. (C) Correlation diagram of different types of NTS identified based on sequences similarity. (D) Ideogram presenting the genetic constitution of 5S rDNA units in COC, TC, 2nCT, 3nCT and 4nCT. For each type of 5S rDNA units, the grey blocks represent the CDS region, and the colorful blocks represent various types of NTS regions.

5S rDNA unit, 4nCT inherited all 5S rDNA fragments from parents and had the most complicated variation pattern.

3.5. Genetic variation of *HoxD10a* gene

The *HoxD10a* gene was also been analyzed to study the genetic constitution and variation in CT hybrids. The sequencing results showed that both of COC and TC has only one type of *HoxD10a* gene fragment (designated I and II type, respectively). I Type of COC was inherited by all of CT hybrids, II type of TC was inherited only by 3nCT, and some variant types were derived in 2nCT (III type) and 4nCT (IV ~ VI type) (Table 6 and Fig. 3). To reveal the resource of variant types, all of *HoxD10a* gene fragments were divided into two exon and one intron regions based on BLASTn analysis. The alignment of the amino acid sequences showed that most of fragments were highly conserved in the exon regions, except Type IV of 4nCT, which producted a frameshift

Table 6
The sequence category of *HoxD10a* gene in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish Species	*HoxD10a* types	Length (bp)	Exon1 (bp)	Intron (bp)	Exon2(bp)	GenBank accession number
COC	I	1551	1–588	589–1320	1321–1551	OR242469
TC	II	1576	1–591	592–1345	1346–1576	OR242473
2nCT	I	1551	1–588	589–1320	1321–1551	OR242470
	III	1765	1–591	592–1534	1535–1765	OR242475
3nCT	I	1553	1–588	589–1322	1323–1553	OR242471
	II	1576	1–591	592–1345	1346–1576	OR242474
4nCT	I	1554	1–588	589–1323	1324–1554	OR242472
	IV$^{\Psi}$	1542	1-591	592-1312	1313–1405	OR242476
	V	1498	1–588	589–1267	1268–1498	OR242477
	VI	1481	1–591	592–1250	1251–1481	OR242478

$^{\Psi}$ indicates the pseudogene.

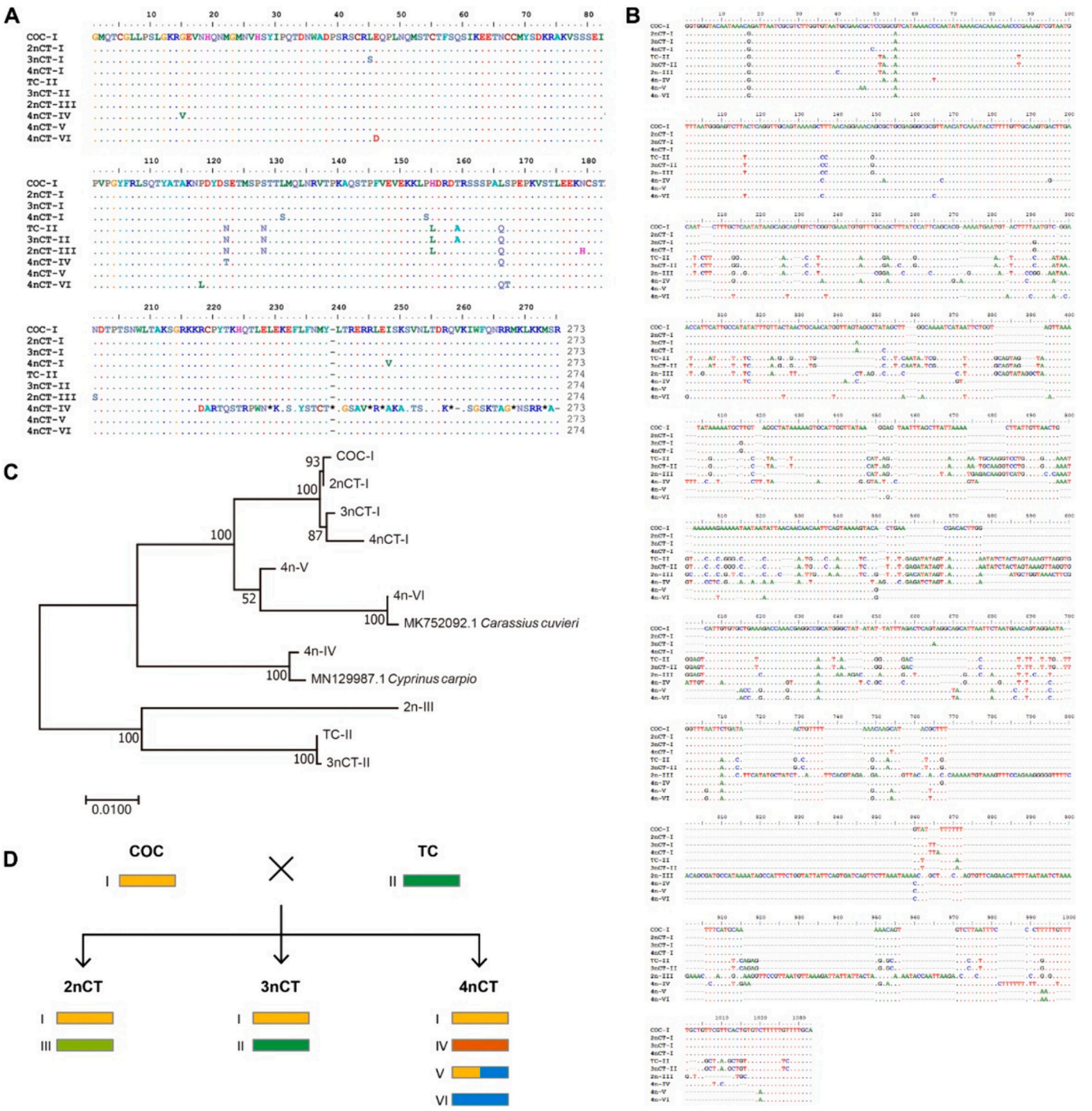

Fig. 3. Hereditary characteristics of *HoxD10a* gene in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT). (A) The alignment of the amino acid sequences of exon regions of *HoxD10a* gene. (B) The alignment of the nucleotide sequences of intron regions of *HoxD10a* gene. (C) Phylogenetic analyses of *HoxD10a* gene sequences by neighbor-joining method. Nodal support values were assessed from 1000 nonparametric bootstrap replicates. (D) Ideogram presenting the genetic constitution of *HoxD10a* gene in COC, TC, 2nCT, 3nCT and 4nCT. The blocks with different colors represent various types of the *HoxD10a* gene. The block containing two colors indicates that the *HoxD10a* sequence is a recombination type.

mutation by the deletion of one base and became a pseudogene. In comparison, intron regions of *HoxD10a* gene showed highly variable, especially III type of 2nCT. The sequences at both ends of 2nCT-III was consistent with II type of TC, while the middle region (1231–1518 bp) was consistent with a genome fragment of COC, which indicated that 2nCT-III resulted from genetic recombination of parental genomes. Base on the phylogenetic analysis performed by neighbor-joining method, the relationship among diverse types in 4nCT was clarified: the pseudogene of 4nCT-IV showed a high similarity to a COC type in GenBank (MN129987.1) that obviously differing from COC-I in this study; 4nCT-VI was unexpectedly found to be highly similar to the *HoxD10a* gene of *C. cuvieri* (MK752092.1); and 4nCT-V was also determined to be a recombinant type, whose former part (1–1092 bp) was similar to COC-I and latter part (1093–1498 bp) was consistent with 4nCT-VI. It is unexpected that neither II type nor any other derived types from TC have been detected in 4nCT.

3.6. Quantitation of parental alleles

We further studied the genomic composition and genetic variation in hybrid fish by detecting the copy numbers and proportions of parental alleles. The *EPHX2* gene was selected as the target through analyzing sequence alignments of multiple homologous genes of COC and TC. Allele-specific probes were then designed with TaqMan technology (Fig. 4A). As probe specificity is essential for reliable interpretation of allele quantitative assay, the allele-specific probes were first validated by qPCR on genomic DNA of COC, TC and CT hybrids (Fig. 4B). The amplification curves showed that only COC-Probe signal was detected in COC, only TC-Probe signal was detected in TC, and both of COC-Probe and TC-Probe signals appeared in CT hybrids, which suggested that the probes had good specificity and can be used in the subsequent ddPCR experiments. As expected, the results of ddPCR showed that there were almost no positive droplets of the non-target probe in parents, while both types of target probes were detected in CT hybrids (Fig. 4C and D). Based on the allele-specific copy number estimated from positive droplets numbers of probes, we calculated the ratio of COC-allele number to TC-allele number (COC/TC ratio) in three types of CT hybrids (Table 7). In 2nCT, the average value of COC/TC ratios was approximately 1 ($P > 0.05$), indicating that one set of chromosomes originated from COC and another set from TC. In 3nCT, the COC/TC ratios were close to 2 ($P > 0.05$), suggesting that two sets came from COC and one set from TC. In comparison, in 4nCT that had two sets from COC and two sets from TC, the COC/TC ratios were around 1.5, which deviated from expected ratio of 1 ($P < 0.05$), implying that the genomic composition in 4nCT was more intricate and less stable than the other CT hybrids. The results of ddPCR experiment not only confirmed the source of hybridization of CT hybrids but also effectively distinguished different hybrid types.

4. Discussion

Distant hybridization is a valuable approach for creating novel fish resources in aquaculture, as it can effectively break down reproductive barriers between species and generate hybrid offspring with significant phenotypic and genotypic alterations (Chen et al., 2023; Wang et al., 2019; Yu et al., 2023). In this study, we performed an intersubfamily hybridization of COC (♀) × TC (♂) and successfully obtained three ploidy-level hybrid offspring: 2nCT, 3nCT, and 4nCT. Comprehensive analyses involving morphological, molecular, and cytological characteristics revealed distinct differences among the various CT hybrids.

To explore the genetic rules underlying distant hybridization, our research team has conducted over 30 distant crosses using freshwater fishes with either 100 (2nAA) or 48 (2nBB) chromosomes, including common carp, crucian carp, blunt snout bream, topmouth culter, Bleeker's yellowtail and other commercially valuable fishes (Liu, 2010; Liu et al., 2022; Wang et al., 2019). These studies found that successful production of diverse viable hybrid offspring was observed in crosses where fish with more chromosomes were used as the female parent and those with fewer chromosomes as the male parent (2nAA ♀ × 2nBB ♂). Conversely, hybrids resulting from reverse crosses (2nBB ♀ × 2nAA ♂) were found to be unlikely to survive. In addition, via the long-term and systematic breeding practice, our research team has demonstrated the prevalence of polyploid event resulting from distant hybridization in fish. Theoretically, the majority of hybrid offspring should be allodiploid hybrids (2nAB = 74), with one set of chromosomes from 2nAA and another set from 2nBB. However, triploid (3n = 124) or tetraploid (4n = 148) hybrids have been frequently observed in the hybridization of species with different chromosome numbers, such as in RCC (♀) × BSB (♂) (Liu et al., 2007), RCC (♀) × TC (♂) (He et al., 2012), and goldfish (♀) × Bleeker's yellowtail (♂) (Wang et al., 2022a). Polyploidization was assumed to arise from several mechanisms, including the inhibition of

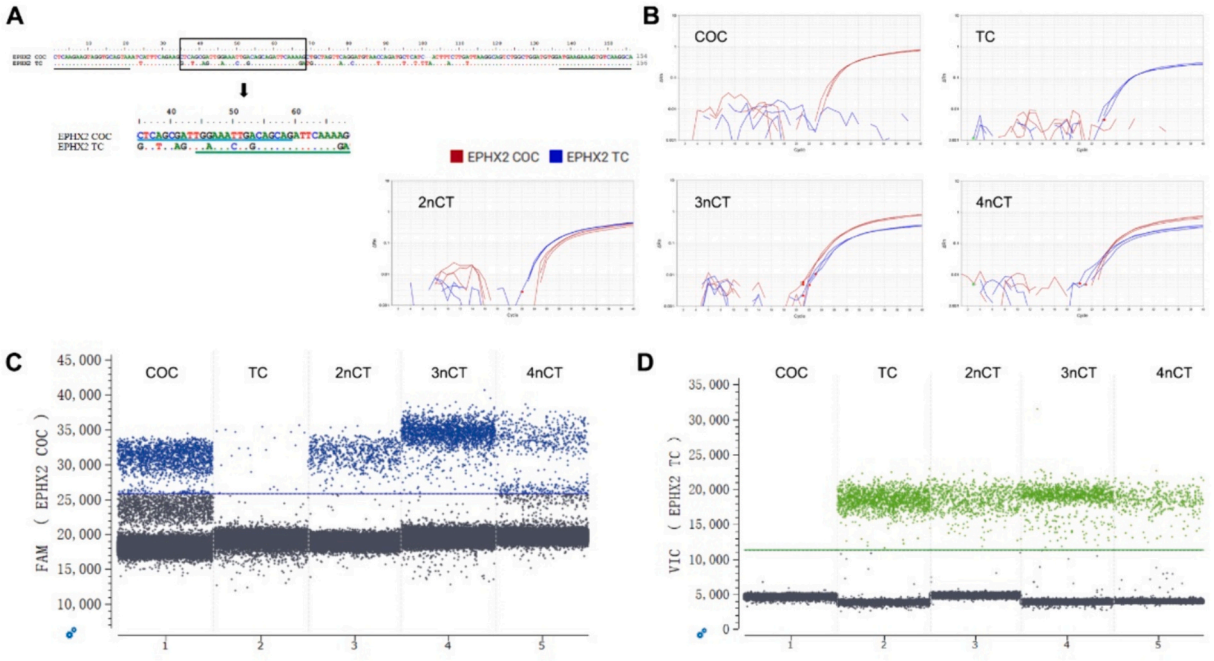

Fig. 4. The output generated by quantitative real-time-PCR (qPCR) and droplet digital PCR (ddPCR) experiments in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT) using allele-specific probes targeting the *EPHX2* gene. (A) The design of primers (indicated by a black line) and allele-specific probes (indicated by a shaded box) on the *EPHX2* sequences of COC and TC. (B) Specificity of *EPHX2* probes when used in qPCR for identifying COC, TC and their hybrid offspring. Red shows *EPHX2*-COC-Probe signals and blue shows *EPHX2*-TC-Probe signals. The amplification curves showed that only COC-Probe signal was detected in COC, only TC-Probe signal was detected in TC, and both of COC-Probe and TC-Probe signals appeared in hybrid offspring. (C, D) Quantification of *EPHX2* gene using ddPCR in COC, TC and their hybrid offspring. The horizontal lines are indicative of the threshold used to distinguish between positive and negative droplets in the analysis of ddPCR. Blue shows *EPHX2*-COC-Probe signals (C) and green shows *EPHX2*-TC-Probe signals (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 7
The quantitative results obtained from droplet digital PCR (ddPCR) using allele-specific probes targeting the *EPHX2* gene in common carp (COC), topmouth culter (TC), diploid hybrids (2nCT), triploid hybrids (3nCT), and tetraploid hybrids (4nCT).

Fish Species	Droplets numbers	Probe-COC positive droplets numbers	Probe-COC copies (cp/μL)	Probe-TC positive droplets numbers	Probe-TC copies (cp/μL)	The ratio of Probe-COC to Probe-TC	Significance of the difference from the expected ratio
COC	25,383	2017	1984	0	0		
	25,647	2280	2131	0	0		
	24,854	2113	2034	0	0		
TC	21,242	19	20.5	1246	1384		
	25,237	20	18.2	1521	1423		
	25,069	20	18.3	1506	1418		
2nCT	24,584	676	638.5	657	620.3	1.03	Expected ratio = 1;
	25,356	670	613.2	625	571.5	1.07	*P* value = 0.0872
	25,888	745	668.6	644	576.8	1.16	
3nCT	25,075	1926	1830	1127	1052	1.74	Expected ratio = 2;
	25,042	1761	1669	959	894.1	1.87	*P* value = 0.1033
	24,026	1771	1753	914	888.1	1.97	
4nCT	24,805	598	558.8	378	351.6	1.59	Expected ratio = 1;
	25,137	589	542.9	404	371	1.46	*P* value = 0.0005
	24,515	604	571.2	370	348.2	1.64	

the expulsion of the second pole body during fertilization (forming allotriploid, 3nAAB = 124), double fertilization (forming allotriploid, 3nABB = 98), and the hindrance of first mitotic division of the zygote (forming allotetraploid, 4nAABB = 148) (Ramsey and Schemske, 1998; Wang et al., 2022a). Furthermore, natural gynogenesis or androgenesis are frequently observed in fish distant hybridization (Komen and Thorgaard, 2007), resulting in the formation of autodiploid hybrids (2nAA = 100 and 2nBB = 48, respectively), and even other autodiploid species (autodiploid, 2nCC = 100) that indicated its evolutionary process. For example, F_1 hybrids of Japanese crucian carp (♀) × BSB (♂) contained a diploid androgenetic BSB (2n = 48) (Hu et al., 2018). Similarly, F_1 hybrids of COC (♀) × BSB (♂) contained a diploid gynogenesis COC (2n = 100), a diploid gynogenesis scattered mirror carp (2n = 100), and a new crucian carp-like homodiploid fish (2n = 100) (Wang et al., 2017b). The present study obtained three ploidy-level hybrids (2nCT, 3nCT and 4nCT) from the crosses of COC (♀) × TC (♂), mirroring the findings reported by He et al. (2012)in the crosses of RCC (♀) × TC (♂), thus demonstrating consistency with the fundamental principles of inheritance and reproduction in fish distant hybridization. The proportion of 2nCT hybrids in this study significantly decreased from nearly 100% in newly hatched larvae to only 3% in juvenile fish at one year of age. Previous studies have found that hybrid incompatibility stems from conflicts between maternally inherited cytoplasm and the hybrid genome, as well as from biased expression of parental alleles in hybrids (Vaid and Laitinen, 2019; Zhou et al., 2015). Therefore, we speculate that in 3nCT and 4nCT that inherited two sets of chromosomes from COC, the predominant influence of the maternal genome conferred developmental advantages to the hybrid offspring. Conversely, in 2nCT that only received one set chromosomes from COC and another from TC, the reduced number of orthologs resulted in increased hybridization incompatibility, leading to diminished survival rates.

Through distant hybridization, the hybrid offspring tended to blend parental traits, resulting in significant phenotypic changes (Groszmann et al., 2015; Li et al., 2016). This study analyzed countable and measurable traits, revealing that only three morphological characteristics (two in 2nCT and one in 4nCT) of CT hybrids exhibited significant divergence from both parents ($P < 0.05$). Most traits of CT hybrids predominantly showed inheritance from COC, with exceptions of two traits (one in 2nCT and one in 4nCT) displaying inherited from TC. Overall, 2nCT and 4nCT exhibited obvious phenotypic variation, while 3nCT exhibited more COC-like characteristics. The CT hybrids with different ploidy levels displayed significant distinctions in their morphological characteristics, likely influenced by the number and proportion of chromosomes inherited from the maternal parent.

Abnormal erythrocyte division has been frequently observed in the peripheral blood of polyploid fish, such as polyploidy hybrids from various fish distant hybridization (Hu et al., 2020; Liu et al., 2007; Lu et al., 2009; Wang et al., 2020a, 2020b), triploid Atlantic salmon (Sadler et al., 2000), and polyploidy rainbow trout (Han et al., 2007; Liu et al., 2012). Therefore, this phenomenon has been regarded as a distinctive characteristic distinguishing polyploids from diploid fish (Han et al., 2007). The dumbbell-shaped nucleus was suggested to be associated with the cell division of immature erythrocytes, representing an intermediate stage in which immature erythrocytes undergo amitosis (Benfey, 1999; Liu et al., 2012; Wang et al., 2010). This phenomenon was rarely observed in healthy fish, but the occurrence significantly increased in fish during anemic or stimulated states (Ellis, 1984; Houston and Murad, 1995). Compared with diploids, polyploid fish have undergone changes in cell morphology, physiological function, and developmental processes, which may trigger previously dormant erythrocytes in the peripheral blood to reacquire their ability to divide, thereby facilitating material exchange within the nucleus in response to the respiratory pressure induced by polyploidization (Dorafshan et al., 2008; Sadler et al., 2000).

The sequence of 5S rDNA is specific to each species and frequently used as a molecular genetic marker for detecting genetic variation that arise from hybridization or polyploidization (He et al., 2013; Martins and Galetti Jr, 2001; Martins and Wasko, 2004). In fish, the 5S rDNA of hybrid offspring was reported to retain more than one class of 5S rDNA repeats from the parents, while also undergoing elimination, variation, or recombination of parent-specific 5S rDNA. For instance, Qin et al. (2010) analyzed the 5S rDNA of hybrid offspring derived from a cross between RCC (♀) and BSB (♂), and observed that triploid hybrids (3nRB) retained all parental types, while in tetraploid hybrids (4nRB), they found only part RCC types and a novel variant type, but no BSB types. He et al. (2012) found that in the cross of RCC (♀) × TCB (♂), diploid hybrids (2nRT) inherited all three 5S rDNA types from RCC and only one type from TC, triploid hybrids (3nRT) retained two RCC types and one TC type, and tetraploid hybrids (4nRT) inherited only two types from RCC but generated a new variant type. In this study, 2nCT possessed the lowest diversity of types, while 4nCT demonstrate the highest diversity of types, suggesting a potential correlation between the complexity of 5S rDNA variations and the ploidy level or genome size in hybrids. In addition, all of the CT hybrids fully inherited both types of COC and only type II of TC. Type II + II of TC was lost in 2nCT, and type III of TC was lost in both 2nCT and 3nCT, which gave rise to two new variant types (NTS-IV and V). These results demonstrated that in CT hybrids, the number and composition of 5S rDNA inherited from the female parent remained relatively consistent, while those inherited from the male parent were prone to mutation or loss. Therefore, we propose that in the hybrid, the maternal subgenome potentially exerts dominance in the genomic conflict and exhibits greater stability compared with the

paternal subgenome.

Next, we employed the *HoxD10a* gene as an additional molecular marker for detecting genetic variations in CT hybrids. The sequence of 4nCT-VI showed a higher similarity to that of *C. cuvieri* compared to parental species, and similar results were found in previous study. For example, the COC type of 5S rDNA was detected in 4nRB, which were tetraploid hybrids derived from crossing RCC (♀) with BSB (♂), as well as in 4nRT, which were tetraploid hybrids derived from crossing RCC (♀) with TC (♂) (He et al., 2012; Qin et al., 2010). Additionally, in diploid hybrids NCRC that derived from crossing COC (♀) with BSB (♂), numerous types of *Hox* genes were found to have a higher similarity to that of crucian carp than that of parents (Wang et al., 2021). Studies have shown that COC and crucian carp can readily interbreed with each other in natural waters, potentially resulting in the prevalence of gene introgression between the two species and the mutual conversion of genotypes in hybrid offspring (Hänfling et al., 2005; Haynes et al., 2012). In conclusion, the genetic recombination and mutation of the *HoxD10a* gene further confirm the instability characteristic of the paternal subgenome in CT hybrids, which may play a crucial role in the formation and evolution of hybrid species.

The oligonucleotide probes, which are highly sensitive and selective, are frequently used in studies on species identification and the origin of polyploid species in fish. For instance, Qin et al. (2016) conducted fluorescence in situ hybridization (FISH) experiments using specific probes from crucian carp, which provided compelling cell biological evidence supporting the homologous origin of triploid crucian carp from Dongting Lake. However, there were no available FISH probes for many other species such as COC, and this hindered the application of FISH in studies on fish distant hybridization. Here, we reported a novel approach that used specific oligonucleotide probes with ddPCR technology to investigate the genetic composition of hybrid fish. The *EPHX2* gene, selected as the target for designing parental allele-specific probes, is a nuclear gene encoding a member of the epoxide hydrolase family associated with familial hypercholesterolemia (Hanif et al., 2020). Only one copy of *EPHX2* gene was detected in both COC and TC, with numerous sequence differences that were suitable for designing specific probes. The ddPCR results showed that the COC/TC ratios in 4nCT approached 1.5, surpassing the anticipated value of 1, indicating a higher proportion of COC alleles than TC alleles. This discrepancy could be attributed to the genotype of TC, which potentially harbors more mutations that prevent binding with the probe compared to COC. This result was consistent with the analyses of 5S rDNA and *HoxD10a* gene, which demonstrated a high level of instability in the paternal subgenome along with an increased mutation frequency. This occurrence is suggested to relate to the significant genomic shock caused by hybridization and polyploidization, a phenomenon that may be particularly pronounced in tetraploid hybrids (Liu et al., 2016). In the cross of RCC and TC, the results of FISH experiments using probes from 5S rDNA of TC showed that both types of TC probes were absent in 4nRT hybrids, with only one type detected in 2nRT and 3nRT hybrids (He et al., 2013). Therefore, we propose that the hybrids, particularly tetraploid hybrids, may exhibit a tendency to lose more paternal subgenome, manifested as the partial or complete elimination of paternal genotypes. Furthermore, our method proved effective in distinguishing different types of CT hybrids and could serve as a valuable tool for identifying the ploidy level of hybrid offspring in further researches.

In summary, diploid, triploid, and tetraploid hybrids were successfully produced by the different subfamily fish crossings of COC (♀) × TC (♂). A comparative analysis of the biological characteristics and genetic constitution of the CT hybrids and their parent species revealed that CT hybrids closely resembled COC in both appearance and at the molecular level, suggesting that the maternal subgenome plays a dominant role in shaping the newly established hybrid genome. The formation of these various ploidy offspring not only harbors potential benefits for application in fish genetic breeding but also serves as a valuable model for exploring the molecular mechanisms underlying hybridization and polyploidization.

CRediT authorship contribution statement

Conghui Yang: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Chenghua Dai:** Writing – original draft, Visualization, Investigation, Formal analysis. **Yating Zhu:** Validation, Investigation. **Xuexue Huang:** Investigation. **Xiaowei Xu:** Investigation. **Yi Zhou:** Validation, Funding acquisition. **Shi Wang:** Validation, Funding acquisition. **Qingfeng Liu:** Validation, Funding acquisition. **Shaojun Liu:** Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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References

Behlke, M.A., Huang, L., Bogh, L., Rose, S., Devor, E.J., 2005. Fluorescence and fluorescence applications. Integrat. DNA Technol. 1–13.

Benfey, T.J., 1999. The physiology and behavior of triploid fishes. Rev. Fish. Sci. Aquac. 7 (1), 39–67. https://doi.org/10.1080/10641269991319162.

Bogenhagen, D.F., Brown, D.D., 1981. Nucleotide sequences in *Xenopus* 5S DNA required for transcription termination. Cell 24 (1), 261–270. https://doi.org/10.1016/0092-8674(81)90522-5.

Campomenosi, P., Gini, E., Noonan, D.M., Poli, A., D'Antona, P., Rotolo, N., Dominioni, L., Imperatori, A., 2016. A comparison between quantitative PCR and droplet digital PCR technologies for circulating microRNA quantification in human lung cancer. BMC Biotechnol. 16 (1), 60. https://doi.org/10.1186/s12896-016-0292-7.

Cao, L., Zhao, C., Wang, C., Qin, H., Qin, Q., Tao, M., Zhang, C., Zhao, R., Liu, S., 2020. Evolutionary dynamics of 18S and 5S rDNA in autotriploid *Carassius auratus*. Gene 737, 144433. https://doi.org/10.1016/j.gene.2020.144433.

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.

Chen, Y., Xiong, Z., Qin, P., Liu, Q., Fan, Y., Xu, Q., Wang, X., Yang, Z., Li, W., Wen, M., Hu, F., Luo, K., Wang, S., Liu, S., 2023. A comparative study of muscle nutrition and intermuscular bone number in improved diploid carp. Reproduct. Breed. 3 (3), 118–124. https://doi.org/10.1016/j.repbre.2023.08.001.

Cui, J., Luo, M., Gao, X., Zhang, H., Zhang, X., Ren, L., Liu, S., 2022. The formation and biological characterization of two allotriploid fish derived from interploid crosses. Reproduct. Breed. 2 (1), 22–29. https://doi.org/10.1016/j.repbre.2022.02.004.

Dorafshan, S., Kalbassi, M.R., Pourkazemi, M., Amiri, B.M., Karimi, S.S., 2008. Effects of triploidy on the Caspian salmon *Salmo trutta caspius* haematology. Fish Physiol. Biochem. 34 (3), 195–200. https://doi.org/10.1007/s10695-007-9176-z.

Ellis, A.E., 1984. Bizzare forms of erythrocytes in a specimen of plaice, *Pleuronectes platessa L.* J. Fish Dis. 7 (5), 411–414. https://doi.org/10.1111/j.1365-2761.1984.tb01207.x.

Fujiwara, M., Inafuku, J., Takeda, A., Watanabe, A., Fujiwara, A., Kohno, S.-I., Kubota, S., 2008. Molecular organization of 5S rDNA in bitterlings (Cyprinidae). Genetica 135 (3), 355–365. https://doi.org/10.1007/s10709-008-9294-2.

Gong, D., Xu, L., Liu, Q., Wang, S., Wang, Y., Hu, F., Wu, C., Luo, K., Tang, C., Zhou, R., Zhang, C., Tao, M., Wang, Y., Liu, S., 2021. A new type of hybrid bream derived from a hybrid lineage of *Megalobrama amblycephala* (♀) × *Culter alburnus* (♂). Aquaculture 534, 736194. https://doi.org/10.1007/s11427-021-2005-5.

Gong, D., Tao, M., Xu, L., Hu, F., Wei, Z., Wang, S., Wang, Y., Liu, Q., Wu, C., Luo, K., Tang, C., Zhou, R., Zhang, C., Wang, Y., Liu, S., 2022. An improved hybrid bream derived from a hybrid lineage of *Megalobrama amblycephala* (♀) × *Culter alburnus* (♂). Sci. China Life Sci. 65 (6), 1213–1221. https://doi.org/10.1007/s11427-021-2005-5.

Gorski, D.H., Walsh, K., 2000. The role of homeobox genes in vascular remodeling and angiogenesis. Circ. Res. 87 (10), 865–872. https://doi.org/10.1161/01.RES.87.10.865.

Groszmann, M., Gonzalez-Bayon, R., Lyons, R.L., Greaves, I.K., Kazan, K., Peacock, W.J., Dennis, E.S., 2015. Hormone-regulated defense and stress response networks contribute to heterosis in *Arabidopsis* F1 hybrids. Proc. Natl. Acad. Sci. 112 (46), E6397–E6406. https://doi.org/10.1073/pnas.1519926112.

Hall, T.A., Biosciences, I., Carlsbad, C., 2011. BioEdit: an important software for molecular biology. GERF Bull. Biosci. 2 (1), 60–61.

Han, Y., Wang, K., Zhang, L.L., Liu, M., 2007. Comparison on erythrocyte and some hematology indices of diploid and triploid rainbow trout (*Oncorhynchus mykiss*). Freshw. Fish. 37 (6), 52–55. https://doi.org/10.3969/j.issn.1000-6907.2007.06.013.

Hänfling, B., Bolton, P., Harley, M., Carvalho, G.R., 2005. A molecular approach to detect hybridisation between crucian carp (*Carassius carassius*) and non-indigenous carp species (*Carassius spp.* and *Cyprinus carpio*). Freshw. Biol. 50 (3), 403–417. https://doi.org/10.1111/j.1365-2427.2004.01330.x.

Hanif, A., Edin, M.L., Zeldin, D.C., Nayeem, M.A., 2020. *Ephx2*-gene deletion affects acetylcholine-induced relaxation in angiotensin-II infused mice: role of nitric oxide and CYP-epoxygenases. Mol. Cell. Biochem. 465 (1–2), 37–51. https://doi.org/10.1007/s11010-019-03665-x.

Haynes, G.D., Gongora, J., Gilligan, D.M., Grewe, P., Moran, C., Nicholas, F.W., 2012. Cryptic hybridization and introgression between invasive cyprinid species *Cyprinus carpio* and *Carassius auratus* in Australia: implications for invasive species management. Anim. Conserv. 15 (1), 83–94. https://doi.org/10.1111/j.1469-1795.2011.00490.x.

He, W., Qin, Q., Liu, S., Li, T., Wang, J., Xiao, J., Xie, L., Zhang, C., Liu, Y., 2012. Organization and variation analysis of 5S rDNA in different ploidy-level hybrids of red crucian carp × topmouth culter. PLoS One 7 (6), e38976. https://doi.org/10.1371/journal.pone.0038976.

He, W., Xie, L., Li, T., Liu, S., Xiao, J., Hu, J., Wang, J., Qin, Q., Liu, Y., 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.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5′——3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. 88 (16), 7276–7280. https://doi.org/10.1073/pnas.88.16.7276.

Houston, A.H., Murad, A., 1995. Erythrodynamics in fish: recovery of the goldfish *Carassius auratus* from acute anemia. Can. J. Zool. 73 (3), 411–418. https://doi.org/10.1139/z95-046.

Houston, R.D., Bean, T.P., Macqueen, D.J., Gundappa, M.K., Jin, Y.H., Jenkins, T.L., Selly, S.L.C., Martin, S.A.M., Stevens, J.R., Santos, E.M., Davie, A., Robledo, D., 2020. Harnessing genomics to fast-track genetic improvement in aquaculture. Nat. Rev. Genet. 21 (7), 389–409. https://doi.org/10.1038/s41576-020-0227-y.

Hu, F., Wu, C., Zhou, Y., Cao, L., Xiao, J., Wang, S., Wu, Y., Ren, L., Liu, Q., Li, W., Wen, M., Tao, M., Qin, Q., Zhao, R., Luo, K., Liu, S., 2018. Production of androgenetic, triploid and tetraploid hybrids from the interspecific hybridization of female Japanese crucian carp and male blunt snout bream. Aquaculture 491, 50–58. https://doi.org/10.1016/j.aquaculture.2018.03.014.

Hu, F., Zhong, H., Fan, J., Wu, C., Wang, S., Wang, Y., Luo, K., Zhao, R., Liu, J., Qin, Q., Tang, C., Liu, S., 2020. A new type of tetraploid fish derived via female autotetraploid × male allotetraploid hybridization. Aquaculture 524, 735244. https://doi.org/10.1016/j.aquaculture.2020.735244.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T.L., 2008. NCBI BLAST: a better web interface. Nucleic Acids Res. 36 (suppl_2), W5–W9. https://doi.org/10.1093/nar/gkn201.

Komen, H., Thorgaard, G.H., 2007. Androgenesis, gynogenesis and the production of clones in fishes: a review. Aquaculture 269 (1–4), 150–173. https://doi.org/10.1016/j.aquaculture.2007.05.009.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23 (21), 2947–2948. https://doi.org/10.1093/bioinformatics/btm404.

Li, D., Zeng, R., Li, Y., Zhao, M., Chao, J., Li, Y., Wang, K., Zhu, L., Tian, W.-M., Liang, C., 2016. Gene expression analysis and SNP/InDel discovery to investigate yield heterosis of two rubber tree F_1 hybrids. Sci. Rep. 6 (1), 24984. https://doi.org/10.1038/srep24984.

Liu, S., 2010. Distant hybridization leads to different ploidy fishes. Sci. China Life Sci. 53 (4), 416–425. https://doi.org/10.1007/s11427-010-0057-9.

Liu, S., Liu, Y., Zhou, G., Zhang, X., Luo, C., Feng, H., He, X., Zhu, G., 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, S., Qin, Q., Xiao, J., Lu, W., Shen, J., Li, W., Liu, J., Duan, W., Zhang, C., Tao, M., Zhao, R., Yan, J., Liu, Y., 2007. The formation of the polyploid hybrids from different subfamily fish crossings and its evolutionary significance. Genetics 176 (2), 1023–1034. https://doi.org/10.1534/genetics.107.071373.

Liu, Y., Wang, B., Fan, Z., Liu, H., 2012. Expression of proliferation cell nuclear antigen (PCNA) in peripheral erythrocytes of triploid rainbow trout *Oncorhynchus mykiss* (Walbaum). J. Anim. Vet. Adv. 11 (23), 4451–4454. https://doi.org/10.3923/javaa.2012.4451.4454.

Liu, S., Luo, J., Chai, J., Ren, L., Zhou, Y., Huang, F., Liu, X., Chen, Y., Zhang, C., Tao, M., Lu, B., Zhou, W., Lin, G., Mai, C., Yuan, S., Wang, J., Li, T., Qin, Q., Feng, H., Luo, K., Xiao, J., Zhong, H., Zhao, R., Duan, W., Song, Z., Wang, Y., Wang, J., Zhong, L., Wang, L., Ding, Z., Du, Z., Lu, X., Gao, Y., Murphy, R.W., Liu, Y., Meyer, A., Zhang, Y.-P., 2016. Genomic incompatibilities in the diploid and tetraploid offspring of the goldfish × common carp cross. Proc. Natl. Acad. Sci. 113 (5), 1327–1332. https://doi.org/10.1073/pnas.1512955113.

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

Liu, S., Wang, S., Liu, Q., Wu, C., Zhou, Y., Tao, M., Zhang, C., Qin, Q., Luo, K., 2022. The research advances in animal distant hybridization and polyploid organisms. In: Liu, S. (Ed.), Fish Distant Hybridization. Springer, Singapore, pp. 1–37.

Lu, W., Liu, S., Long, Y., Tao, M., Zhang, C., Wang, J., Xiao, J., Chen, S., Liu, J., Liu, Y., 2009. Comparative study of erythrocytes of polyploid hybrids from various fish subfamily crossings. Cell Tissue Res. 336 (1), 159–163. https://doi.org/10.1007/s00441-009-0759-0.

Luo, J., Stadler, P.F., He, S., Meyer, A., 2007. PCR survey of hox genes in the goldfish *Carassius auratus auratus*. J. Exp. Zool. (Mol. Dev. Evol.) 308B (3), 250–258. https://doi.org/10.1002/jez.b.21144.

Martins, C., Galetti Jr., P.M., 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.

Martins, C., Wasko, A.P., 2004. Organization and evolution of 5S ribosomal DNA in the fish genome. In: Williams, C.R. (Ed.), Focus on Genome Research. Nova, New York, pp. 335–363.

Pasolini, P., Costagliola, D., Rocco, L., Tinti, F., 2006. Molecular organization of 5S rDNAs in Rajidae (chondrichthyes): structural features and evolution of piscine 5S rRNA genes and nontranscribed intergenic spacers. J. Fish Dis. 62 (5), 564–574. https://doi.org/10.1007/s00239-005-0118-z.

Qin, Q., He, W., Liu, S., Wang, J., Xiao, J., Liu, Y., 2010. Analysis of 5S rDNA organization and variation in polyploid hybrids from crosses of different fish subfamilies. J. Exp. Zool. (Mol. Dev. Evol.) 314B (5), 403–411. https://doi.org/10.1002/jez.b.21346.

Qin, Q., Wang, Y., Wang, J., Dai, J., Xiao, J., Hu, F., Luo, K., Tao, M., Zhang, C., Liu, Y., Liu, S., 2014. The autotetraploid fish derived from hybridization of *Carassius auratus red var.* (female) × *Megalobrama amblycephala* (male). Biol. Reprod. 91 (4), 93. https://doi.org/10.1095/biolreprod.114.122283.

Qin, Q., Wang, J., Hu, M., Huang, S., Liu, S., 2016. Autotriploid origin of *Carassius auratus* as revealed by chromosomal locus analysis. Sci. China Life Sci. 59 (6), 622–626. https://doi.org/10.1007/s11427-016-5040-7.

Ramsey, J., Schemske, D.W., 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Evol. Syst. 29 (1), 467–501. https://doi.org/10.2307/221716.

Rebordinos, L., Cross, I., Merlo, A., 2013. High evolutionary dynamism in 5S rDNA of fish: state of the art. Cytogenet. Genome Res. 141 (2–3), 103–113. https://doi.org/10.1159/000354871.

Sadler, J., Wells, R.M.G., Pankhurst, P.M., Pankhurst, N.W., 2000. Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. Aquaculture 184 (3–4), 349–361. https://doi.org/10.1016/S0044-8486(99)00321-X.

Shen, J., Liu, S., Sun, Y., Zhang, C., Luo, K., Tao, M., Zeng, C., Liu, Y., 2006. A new type of triploid crucian crap-red crucian carp (♀) × allotetraploid (♂). Prog. Nat. Sci. 16 (12), 1348–1352. https://doi.org/10.1080/10020070612330152.

Song, C., Liu, S., Xiao, J., He, W., Zhou, Y., Qin, Q., Zhang, C., Liu, Y., 2012. Polyploid organisms. Sci. China Life Sci. 55 (4), 301–311. https://doi.org/10.1007/s11427-012-4310-2.

Tajadini, M., Panjehpour, M., Javanmard, S.H., 2014. Comparison of SYBR green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. Adv. Biomed. Res. 3 (1), 85. https://doi.org/10.4103/2277-9175.127998.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38 (7), 3022–3027. https://doi.org/10.1093/molbev/msab120.

Vaid, N., Laitinen, R.A.E., 2019. Diverse paths to hybrid incompatibility in *Arabidopsis*. Plant J. 97 (1), 199–213. https://doi.org/10.1111/tpj.14061.

Wang, B., Liu, Y., Chen, X., Fan, Z., 2010. Amitosis-like nuclear division in erythrocytes of triploid rainbow trout *Oncorhynchus mykiss*. J. Fish Biol. 76 (5), 1205–1211. https://doi.org/10.1111/j.1095-8649.2010.02556.x.

Wang, J., Xiao, J., Zeng, M., Xu, K., Tao, M., Zhang, C., Duan, W., Liu, W., Luo, K., Liu, Y., Liu, S., 2015. Genomic variation in the hybrids of white crucian carp and red crucian carp: evidence from ribosomal DNA. Sci. China Life Sci. 58 (6), 590–601. https://doi.org/10.1007/s11427-015-4835-2.

Wang, Y.D., Qin, Q.B., Yang, R., Sun, W.Z., Liu, Q.W., Huo, Y.Y., Huang, X., Tao, M., Zhang, C., Li, T., Liu, S.J., 2017a. *Hox* genes reveal genomic DNA variation in tetraploid hybrids derived from *Carassius auratus* red var. (female) × *Megalobrama amblycephala* (male). BMC Genet. 18, 86. https://doi.org/10.1186/s12863-017-0550-2.

Wang, S., Ye, X., Wang, Y., Chen, Y., Lin, B., Yi, Z., Mao, Z., Hu, F., Zhao, R., Wang, J., Zhou, R., Ren, L., Yao, Z., Tao, M., Zhang, C., Xiao, J., Qin, Q., Liu, S., 2017b. 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., Tang, C., Tao, M., Qin, Q., Zhang, C., Luo, K., Zhao, R., Wang, J., Ren, L., Xiao, J., Hu, F., Zhou, R., Duan, W., Liu, S., 2019. Establishment and application of distant hybridization technology in fish. Sci. China Life Sci. 62 (1), 22–45. https://doi.org/10.1007/s11427-018-9408-x.

Wang, S., Xu, X., Luo, K., Liu, Q., Chen, L., Wei, Z., Zhou, P., Hu, F., Liu, Z., Tao, M., Liu, S., 2020a. Two new types of triploid hybrids derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂). Aquaculture 528, 735448. https://doi.org/10.1016/j.aquaculture.2020.735448.

Wang, S., Zhou, P., Huang, X., Liu, Q., Lin, B., Fu, Y., Gu, Q., Hu, F., Luo, K., Zhang, C., Tao, M., Qin, Q., Liu, S., 2020b. 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, S., Liu, Q., Huang, X., Yang, C., Chen, L., Han, M., Shu, Y., Wang, M., Li, W., Hu, F., Wen, M., Luo, K., Wang, Y., Zhou, R., Zhang, C., Tao, M., Zhao, R., Tang, C., Liu, S., 2021. The rapid variation of *Hox* clusters reveals a clear evolutionary path in a crucian carp-like homodiploid fish lineage. Reproduct. Breed. 1 (3), 149–156. https://doi.org/10.1016/j.repbre.2021.09.002.

Wang, J., He, W., Wang, W., Luo, Z., Han, L., Xiang, C., Chai, M., Li, T., Li, J., Luo, K., Zhao, R., Liu, S., 2022a. A novel allotriploid hybrid derived from female goldfish × male bleeker's yellow tail. Front. Genet. 13, 880591 https://doi.org/10.3389/fgene.2022.880591.

Wang, Y., Luo, Y., Geng, C., Zhao, R., Tan, H., Yao, J., Wang, S., Luo, K., Qin, Q., Zhang, C., Tao, M., liu, S., 2022b. Production of a diploid hybrid with fast growth performance derived from the distant hybridization of *Hypophthalmichthys nobilis* (female) × *Megalobrama amblycephala* (male). Reproduct. Breed. 2 (2), 56–64. https://doi.org/10.1016/j.repbre.2022.05.002.

Wang, D., Wang, S., Du, X., He, Q., Liu, Y., Wang, Z., Feng, K., Li, Y., Deng, Y., 2022c. ddPCR surpasses classical qPCR technology in quantitating bacteria and fungi in the environment. Mol. Ecol. 22 (7), 2587–2598. https://doi.org/10.1111/1755-0998.13644.

Wu, C., Li, J., Cai, C., Qin, Q., Huang, C., Chen, Z., Hu, F., Hu, J., Huang, H., Luo, J., Cao, L., Chen, Q., Huang, X., Tang, C., Cai, Y., Cai, J., Cai, S., Cai, H., Chen, Y., Yang, Y., Ma, M., Chen, B., Liu, S., 2022. A new type of hybrid golden pompano "Chenhai no. 1" produced by the hybridization of (*Trachinotus ovatus* ♀× *Trachinotus blochii* ♂) ♀ × *T. ovatus* ♂. Reproduct. Breed. 2 (3), 78–82. https://doi.org/10.1016/j.repbre.2022.06.001.

Xiao, J., Kang, X., Xie, L., Qin, Q., He, Z., Hu, F., Zhang, C., Zhao, R., Wang, J., Luo, K., Liu, Y., Liu, S., 2014. The fertility of the hybrid lineage derived from female *Megalobrama amblycephala* × male *Culter alburnus*. Anim. Reprod. Sci. 151 (1–2), 61–70. https://doi.org/10.1016/j.anireprosci.2014.09.012.

Xue, B., Li, Y., Wang, X., Li, R., Zeng, X., Yang, M., Xu, X., Ye, T., Bao, L., Huang, Y., 2020. TaqMan-MGB probe quantitative PCR assays to genotype and quantify three mtDNA mutations of Leber hereditary optic neuropathy. Sci. Rep. 10 (1), 12264. https://doi.org/10.1038/s41598-020-69220-7.

Ye, J., Feng, J., Liu, S., Zhang, Y., Jiang, X., Dai, Z., 2016. Identification of four squid species by quantitative real-time polymerase chain reaction. Mol. Cell. Probes 30 (1), 22–29. https://doi.org/10.1016/j.mcp.2016.01.001.

Yu, P., Zhong, H., Chen, H., Liu, M., Zhou, Y., Cao, X., Wu, C., Sun, Y., Wang, S., Gong, D., Gong, Q., Wen, M., Hu, F., Liu, S., 2023. Study of biological characteristics of an improved Japanese white crucian carp lineage derived from *Carassius cuvieri* (♀) × *Megalobrama amblycephala* (♂). Aquaculture 577, 739955. https://doi.org/10.1016/j.aquaculture.2023.739955.

Zhang, Z., Chen, J., Li, L., Tao, M., Zhang, C., Qin, Q., Xiao, J., Liu, Y., Liu, S., 2014. Research advances in animal distant hybridization. Sci. China Life Sci. 57 (9), 889–902. https://doi.org/10.1007/s11427-014-4707-1.

Zhang, C., Ye, L., Chen, Y., Xiao, J., Wu, Y., Tao, M., Xiao, Y., Liu, S., 2015. The chromosomal constitution of fish hybrid lineage revealed by 5S rDNA FISH. BMC Genet. 16, 140. https://doi.org/10.1186/s12863-015-0295-8.

Zhou, Y., Ren, L., Xiao, J., Zhong, H., Wang, J., Hu, J., Yu, F., Tao, M., Zhang, C., Liu, Y., Liu, S., 2015. Global transcriptional and miRNA insights into bases of heterosis in hybridization of Cyprinidae. Sci. Rep. 5, 13847. https://doi.org/10.1038/srep13847.

Zmienko, A., Samelak-Czajka, A., Goralski, M., Sobieszczuk-Nowicka, E., Kozlowski, P., Figlerowicz, M., 2015. Selection of reference genes for qPCR-and ddPCR-based analyses of gene expression in senescing barley leaves. PLoS One 10 (2), e0118226. https://doi.org/10.1371/journal.pone.0118226.