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ORIGINAL ARTICLE

Two New Types of Homodiploid Fish and Polyploid Hybrids Derived from the Distant Hybridization of Female Koi Carp and Male Bighead Carp

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Abstract

Bighead carps (*Hypophthalmichthys nobilis*) and silver carps (*Hypophthalmichthys molitrix*) represent an important component of freshwater ichthiofauna in its native range, though they might become mass propagation in other systems (North America) and the reason of concern for fisheries management. Therefore, understanding their reproductive traits and particularly in the context of hybridization with other cyprinids was of value to explain their rapid propagation as well as potential benefits for aquaculture due to their unique diet, behavior, growth potential, and tolerance to deteriorating environmental conditions in freshwater ecosystems. Distant hybridization is an effective tool to create different ploidy offspring with changed phenotypes and genotypes. In this study, we reported distant hybridization of female koi carp (*Cyprinus carpio haematopterus*, KOC, $2n=100$) × male bighead carp (*Hypophthalmichthys nobilis*, BIC, $2n=48$) and the spontaneous occurrence of two new "crucian" carp-like homodiploid fish (2nGCC-L; 2nCCC-L; $2n=100$), a new type of triploid hybrid (3nKB, $3n=124$), and a new type of tetraploid hybrid (4nKB, $4n=148$). The body color of 2nGCC-L and 2nCCC-L were gray and multicolor, respectively. Both phenotypes were similar to the crucian carp (*Carassius auratus*). The difference was that their heads were rounder than those of the crucian carp and they had higher backs. Compared with the KOC with two pairs of barbels and BIC without barbel, 2nGCC-L, 2nCCC-L, and 4nKB had no barbel, but 3nKB had one pair of barbels. Microsatellite patterns and 5S rDNA sequences confirmed that 2nGCC-L, 2nCCC-L, and 3nKB were of hybrid origin. In regard to feeding, KOC was omnivorous and BIC was a typical filter-feeder. However, the 2nGCC-L, 2nCCC-L, and 3nKB were omnivorous. The formation of four kinds of new offspring is a groundbreaking finding in fish genetic breeding and evolutionary biology.

Keywords Hybridization · Homodiploid · Triploid · Tetraploid · Microsatellite · 5S rDNA

Introduction

Understanding and predicting consequences of climate changes are of particular interest to fish acclimatization (via purposeful introductions) (Coulter et al. 2020). For instance, silver and bighead carps that are of domesticated origin and were released from fish farms in North America, and subsequently become established in the Mississippi River system, are undergoing extensive process of hybridization producing fertile progeny (Lamer et al. 2014; Liss et al. 2016). First and second generations of early hybrids (F_1 and F_2) of these species, and F_1 backcross, move faster to mass propagation (Coulter et al. 2020). In 2013 early hybrids and advanced complex introgression reached the proportion of 0.3–0.55 of the parental, bighead carp, and silver carp populations in this new (40–50 years) environment (Coulter et al. 2020).

In the present study, we explore the potential hybridization between even more distant fish species that although it has not been described to occur in the wild, we hypothesize that it has the potential to become a common alteration in many ecosystems (Houde et al. 2010). With the significant changes in the river environment regarding hydrology,

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chemistry, and temperature paralleled by the general plasticity of cyprinid fish species, new habitats can generate new hybridization (speciation) (Chunco and Amanda 2014; Stebbins 1985).

Distant hybridization refers to hybridization between species, genera, and even more distantly related species according to a new biological classification. As one of the basic means of fish breeding, distant hybridization is widely used in fish culture practice. Fish are the most easily hybridized vertebrates (Schwartz 1972). The distant hybridization of fish helps to enhance certain traits, in the species of interest that will contribute to their superior value to aquaculture, for instance, thermal and hypoxia tolerance (Chevassus 1983). Because of the extensive occurrence and diversity of the heritable variation between distant parents, the hybrid offspring can frequently outperform their parents. The ultimate goal of fish breeders using the distant hybridization is to obtain new fish germplasm resources and new varieties for production applications. In addition, distant hybridization plays a principal role in the origin of new species and appears to facilitate speciation and adaptive radiation in both animals and plants. Polyploid animals with many desired traits can be obtained using the distant hybridization (Liu 2010). For example, using a distant hybridization between female *Megalobrama amblycephala* and male *Xenocypris davidi Bleeker*, the sterile triploid hybrids were obtained with good performance, ultrafast growth, and strong resistance to diseases (Hu et al. 2012). The hybrid progenies from red crucian carp (♀) × improved female allotetraploid hybrids (4nIAT) (Duan et al. 2007) of red crucian carp (♀) × common carp (♂) and common carp (♀) × 4nIAT (♂) were obtained with a significant growth advantage in comparison to parental species (Liu et al. 2004). In addition, distant hybridization can lead to different ploidy of fishes with genetic variation among siblings, including fertile tetraploid hybrids, sterile triploid hybrids, fertile diploid hybrids, fertile diploid gynogenetic fish, and their derived progenies(Liu 2010; Liu et al. 2007; Qin et al. 2014; Wang et al. 2015; Xiao et al. 2014). However, the evidence showing that distant hybridization can lead to a new homoploid lineage is lacking. Formation of homodiploid hybrids is an important mode of speciation derived from interspecific hybridization that does not alter the chromosome number (Arnold 1997). A homodiploid lineage has two sets of chromosomes from the maternal parent into which some DNA fragments from the paternal parent are recombined. This lineage differs from an allodiploid lineage, which has two sets of chromosomes, one each from the maternal and paternal parents. However, there have been few reports on the formation of homoploids in animals; for example, the formation of "crucian" carp-like homodiploid fish (Wang et al. 2017). Red "crucian" carp-like homodiploid fish, as well as goldfish-like homodiploid fish (Wang et al. 2018). In conclusion, our knowledge of the role of cyprinid hybridization in the formation of new animal species is only fragmentary.

Koi carp is a variant of the common carp, which belongs to the Cyprinidae; koi carp exhibit fast growth and have bright body coloration. It is known as the "living jewel in the water" (Gouveia et al. 2003). Bighead carp (*Aristichthys nobilis*) is naturally distributed in the rivers and lakes from Heilongjiang province to the Pearl River in China (Beck et al. 1980). Bighead belongs to Cypriniformes, Cyprinidae, subfamily Hypophthalmichthyinae, and genus Hypophthalmichthys. Bighead carp is known locally as flower buds, black crickets, scaly scales, black scales, and is commonly referred as fat-headed fish or bighead fish. The fish is one of the most important large-scale freshwater cultured fish in China (Chen and Arratia 2010). Bighead carp has strong adaptability to the environment, is easy to breed, and has good disease resistance. Its head is rich in nutrients and provides a practical and delicious dish for people. Bighead carp is a typical omnivorous filter-feeding fish, and its meat quality is greatly affected by its culture conditions (Chen et al. 2007).

Because of its exceptional color, fast growth rate, strong resistance to disease, and high reproductive capacity (fecundity), inherent evolutionary tetraploidy (chromosome $2n = 100$), koi carp is often regarded as the best choice for a maternal parent that produces viable progeny with species possessing chromosome number $n = 48$. Reciprocal hybrid is not viable.

The aim of the present work was to combine these complementary production advantages of both species (koi carp and bighead carp), koi carp (♀) and bighead carp (♂) hybridization resulted in obtaining combination of four different types of parental genomes (The criterion of chosen was coloration plus ploidy karyotype.), high-back gray "crucian" carp-like homodiploid fish (2nGCC-L), high-back color "crucian" carp-like homodiploid fish (2nCCC-L), as well as sterile triploid hybrid (3nKB) and tetraploid hybrid (4nKB). The production of homodiploid fish and tetraploid fish plays an important role in enriching the germplasm resources of cyprinids, and sterile triploid hybrid has potential growth advantages, which is of great significance to the improvement of carp aquaculture.

Materials and Method

Ethic Statement

Fish treatments were carried out according to the regulations for protected wildlife and Administration of Affairs Concerning Animal Experimentation and approved by the Science and

Technology Bureau of China. The fish were deeply anesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) before dissection.

Origin of Experimental Broodstock, In Vitro Reproduction, and Progeny Rearing

This study was carried out at the State Key Laboratory for Developmental Biology of Freshwater Fishes in Hunan Normal University, Changsha, China. The KOC females ($n=10$) were obtained from State Laboratory for Developmental Biology of Freshwater Fishes, and BIC males ($n=10$) were obtained from Wuhu Aquatic Products, Ningxiang, Hunan, China. All KOC and BIC were tested for ploidy by flow cytometry. One KOC and one BSB were randomly selected to cross.

All progeny groups were produced by artificial spawning. Spawning was induced by injecting chorionic gonadotropin (Ningbo second hormone factory, Ningbo, Zhejiang, China) in doses of 600–800 units/kg body mass, luteinizing hormone releasing hormone (LHRH) A2 in doses of 3 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China) and domperidone in doses of 1–2 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China). The females were given a 10% priming injection and a 90% resolving injection, separated by 12 h. The males were given the full dosage in one injection (Human chorionic gonadotrophin (HCG) in doses of 1000–2000 units/kg body mass, LHRH A2 in doses of 5 μg/kg). Six hours after the females were given the resolving injection, eggs and sperm were collected by stripping, and the dry method of fertilization was used. Two minutes after fertilization, the eggs were transferred to Petri dishes (size = 12 cm diameter) and incubated until the hatching of larvae at 22–25 °C. Swim-up larvae were collected in Ceramic cylinders and then transferred to 15 m^3 outdoor tanks for rearing for 10 months. The water temperature in the cement pond was maintained at 22–25 ℃. The pH of the water was between 7.5 and 8, the oxygen content in the water was above 5 mg/L, and the ammonia nitrogen content was below 0.5 mg/L.

Flow Cytometry Analysis of Parental and Juvenile Fish

To measure the mean DNA content of erythrocytes in KOC, BIC, and their progenies, we collected samples from 10 KOC, 10 BSB, and 100 offspring at 10 months old. Approximately 0.2–0.5 mL of blood was collected from the caudal vein of each fish into syringes containing 250–350 units of sodium heparin. All samples were processed with the method described in Liu et al. (Liu et al. 2007) and measured by flow cytometry (Partec). The X^2 test with Yate's correction was used to test for deviation from the expected ratio of the ratios of the DNA content of the offspring to the sum of that from KOC and BIC.

Morphological Traits

After 10 months of culture, 10 KOC (body weight: 108.2 ± 1.6 g), 10 BIC (106.6 ± 1.6 g), 10 2nGCC-L (94.0 ± 1.5 g), 10 2nCCC-L (91.9 ± 1.5 g), and 10 3nKB (154.2 ± 1.9 g) were caught at random for examination of morphological characters as described by Hu et al. (2012) (Supplementary Table 1). Measurable traits consisted of total length (TL), body length (BL), body height (BH), head length (HL), caudal peduncle length (CPL), caudal peduncle height (CPH), and the average ratios BL/WL, BH/BL, HL/BL, and CPL/CPH. Their quantitative traits were also measured, such as the number of scales in lateral line (Lateral line scale number refers to the number of scales from the tail to the edge of the gill cover.), the number of rays of pelvic fins, the number of rays of anal fin, and the number of rays in dorsal including hard and soft rays. All quantitative data were analyzed for statistical differences by SPSS22.0 software (Corp 2013). One-way analysis of variance was used to evaluate significance.

We fed 10 KOC, 10 BIC, 10 2nGCC-L, 10 2nCCC-L, and 10 3nKB in an open pond to observe their feeding habits. By feeding with aquatic plants (*Eichhornia crassipes Solms*) and feed in the pond, we observed the fishes' food intake directly and found that both the grass and feed were ingested.

Chromosome Spread and Karyotype Analysis

Head kidney samples from fish of different ploidies were used for chromosome preparation. The preparation of chromosome was performed according to previous reports by Liu et al. (2004). For each fish treatment sample, 10 individuals were used for the experiments and more than 30 metaphase spreads of chromosomes were analyzed. Preparations were examined under an oil immersion lens at a magnification of 100 × and high quality photos of metaphase spreads were obtained. Chromosomes were classified on the basis of their long-arm to short-arm ratios according to the reported standards (Levan 1964). The software (Adobe Photoshop5.0) was used for karyotype analysis.

Measurement of the Nucleus Volume of Erythrocytes

Nucleus size of erythrocytes was used to further confirm of ploidy. One to two microliters of blood was sampled from KOC, BIC, 2nGCC-L, 2nCCC-L, 3nKB, and 4nKB. Blood smears were fixed with ethanol and stained with Giemsa solution. We observed 30 erythrocytes from each fish under an oil immersion at 330 × and used an ocular micrometer to measure the nucleon diameters. The cell nucleus volume

was calculated an $4/3\pi ab^2$ (where a was the major semi-axis and b was the minor semi-axis of a perfect ellipsoid). The volume might be slightly overestimated due to cell flattening. To calculate the ratios of nuclear volume of the hybrid offspring to that of KOC plus BIC, the X^2 test, and Yate's correction were used for testing deviation from expected ratio values. The appearance of the erythrocytes nuclei was also observed under light microscopy.

Microsatellite Analysis

Total genomic DNA was isolated from blood collected from the caudal vein using a standard phenol–chloroform procedure (Manniatis 1989). DNA concentration and quality were assessed using agarose gel electrophoresis.

One pair of primers (MFW1-F: 5′-TGGACCTGCTGT AATCTCTGT-3′, MFW1-R:5′-ATCGGGTAACATCTCCTC AC-3′) were synthesized to match the flanking regions including the repeated (CA)n dinucleotide microsatellites according to the published sequences (Crooijmans et al. 1997). One reaction consisted of 20 ng DNA template, 0.2 μM forward and 0.2 μM reverse primers, 0.25 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 × colorless GoTaq Flexi Buffer, and diH_2O for a volume of 10 μL. To determine allelic variation at each locus, PCR products were separated on an 8% polyacrylamide gel. PCR products were sized by pBR322 DNA/Msp ladder.

5S rDNA Analysis

One pair of primers (5S forward primer: GCTATGCCC GATCTCGTCTGA and 5S reverse primer: CAGGTT GGTATGGCCGTAAGC) was designed and synthesized to amplify the 5S rDNA repeats directly from 10 KOC, 10 BIC, 10 2nGCC-L, 10 2nCCC-L, and 10 3nKB by PCR. The PCRs and sequencing were performed as described by Qin et al. 2014. Sequences were analyzed using BioEdit software (version 7.0).

Results

Fertilization, Hatching, and Juvenile Survival Rates

The hybrids of KOC (♀) × BIC (♂) had high fertilization (80.6%) and hatching (65.7%) rates, but the survival rate (8 days post fertilization) decreased, and the fish were ± 5 mm in length (35.6%). The KOC progeny had a 95.6% fertilization rate, 85.36% hatching rate, and 80.7% survival rate at the age of 8 months, and the progeny of BIC had a 95.6% fertilization rate, 90.3% hatching rate, and 85.7% survival rate. At the age of 5 months, the survival rates of KOC (♀) × BIC (♂) was 20.5%. At the age of 10 months, the survival rates of KOC (♀) × BIC (♂) also was 20.3%. At the age of 5 and 10 months, the corresponding mean body weight data were in the Supplementary Table 1.

Formation of 2nCCC-L, 2nGCC-L, 3nKB, and 4nKB Hybrids

The appearance of the progeny as a result of crossing procedure to produce the 2nCCC-L, 2nGCC-L, 3nKB, and 4nKB was shown in Fig. 1. In the first generation of KOC (♀) × BIC (♂), 16.7% 2nGCC-L, 26.7% 2nCCC-L, 55.6% 3nKB hybrids, and 1% 4nKB hybrids were found. The obvious characteristic of 2nGCC-L and 4nKB hybrids were no

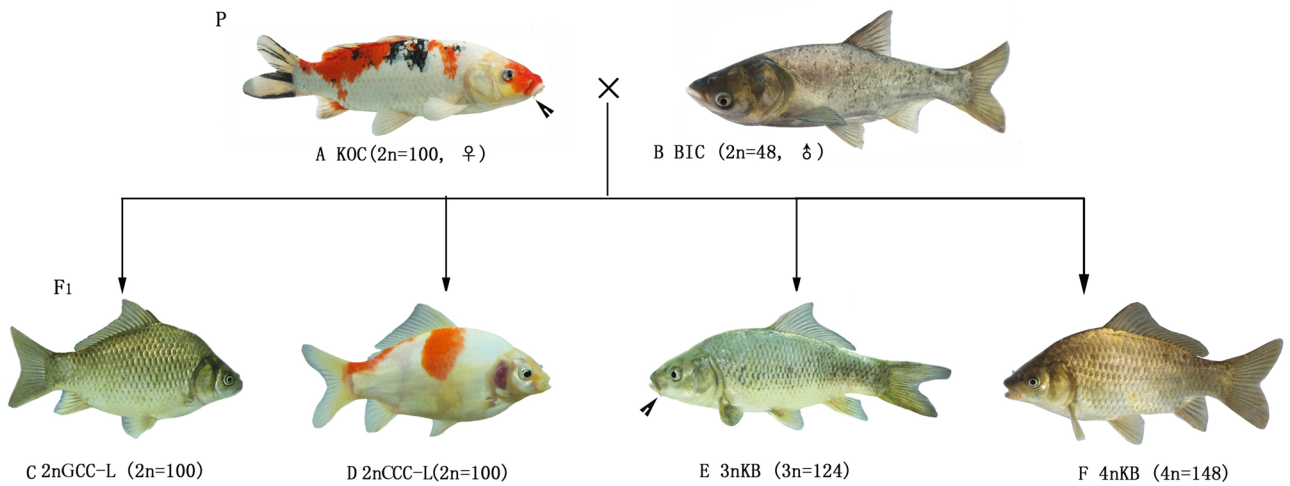

Fig. 1 Formation procedure and appearance of KOC, BIC, 2nGCC-L, 2nCCC-L, 3nKB, and 4nKB hybrids. (**A**): KOC; (**B**): BIC; (**C**): 2nGCC-L; (**D**): 2nCCC-L; (**E**): 3nKB; (**F**): 4nKB. Bar = 3 cm. Black arrow meant the two pairs of barbels in KOC. Black arrow meant the one pair of barbels in 3nKB

Table 1 The phenotype included several quantitative traits (the average ratios of body length to whole length (BL/WL), body height to body length (BH/BL), head length to body length (HL/BL), head height to head length (HH/HL), caudal peduncle height to caudal peduncle length (CPH/CPL), and head height to body height (HH/BH), and the countable traits (number of scales in lateral line, number of rays in dorsal fins, number of pelvic fins, number of rays anal fin in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB hybrids)

Phenotypes	Types of fish				
	KOC	BIC	2nGCC-L	2nCCC-L	3nKB
BL/WL	0.86 ± 0.01	0.65 ± 0.92	0.83 ± 0.02	0.77 ± 0.05	0.59 ± 0.03
BH/BL	0.38 ± 0.01	0.31 ± 0.01	0.49 ± 0.01	0.54 ± 0.08	0.48 ± 0.07
HL/BL	0.25 ± 0.02	0.43 ± 0.04	0.25 ± 0.06	0.30 ± 0.02	0.43 ± 0.04
HH/HL	0.96 ± 0.03	0.70 ± 0.05	0.59 ± 0.03	1.03 ± 0.09	0.81 ± 0.03
CPH/CPL	0.80 ± 0.09	0.78 ± 0.18	0.71 ± 0.07	0.81 ± 0.07	1.15 ± 0.07
No. of lateral scales	35.5 ± 0.71	104 ± 1.41	27.25 ± 2.87	29.25 ± 1.5	40.60 ± 0.89
No. of rays in dorsal fin	III + 20.5 ± 0.68	8	17.5 ± 0.6	16.25 ± 0.5	17.8 ± 1.10
No. of rays in abdominal fin	III + 11.5 ± 0.65	9	7.5 ± 1	8.25 ± 0.5	7.2 ± 0.43
No.of rays in anal fin	III + 9.5 ± 0.62	13	6.75 ± 0.5	7.5 ± 0.58	6.4 ± 0.55

barbels, the obvious characteristic of 2nCCC-L was white body color with color patches, and the obvious characteristic of 3nKB hybrids was one pair of barbels, compared with the parents (KOC and BIC).

Morphological Traits and Feeding Habits

Table 1 showed the examined measurable traits and countable traits in 2nGCC-L, 2nCCC-L, 3nKB, and their parents (KOC and BIC). In terms of the measurable traits, the HL/BL in 2nCCC-L, 2nGCC-L, and 3nKB were intermediate to those of KOC and BIC, but were different from those of KOC and BIC. The BL/WL in 2nCCC-L and 2nGCC-L were close to those of KOC and BIC. However, the BL/WL in 2nCCC-L and 2nGCC-L were lower than those of KOC and BIC. The BH/BL in 2nCCC-L, 2nGCC-L and 3nKB were higher than those in both KOC and BIC. The CPH/CPL in 2nGCC-L, 2nCCC-L and 3nKB were higher than those in either KOC or BIC. The HH/HL in 2nGCC-L, 2nCCC-L and 3nKB were lower, higher, or intermediate to those of KOC and BIC.

For the countable traits, except for the number of rays in dorsal fin and anal fin, 2nCCC-L, 2nGCC-L, and 3nKB were closer to KOC, the other traits including the number of lateral line scales, the number of upper lateral scales, and the number of lower lateral scales were similar to those of BIC and significantly different from those of KOC Table 1.

Feeding with aquatic plants and feed separately and long-term follow-up observation, it was found that KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB all ingested two kinds of food. In addition, we put together a feed preparation (feed and aquatic plants) and found that the fish ingested both diets. Therefore, we concluded that KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB were omnivorous fish.

DNA Contents

The distribution of the DNA content in KOC, BIC, and their offspring was shown in Supplementary Figure 1. We used the sum of the DNA content of KOC and BIC as the controls. The mean DNA content, of 2nGCC-L and 2nCCC-L were equal to the DNA content of KOC, suggesting that 2nGCC-L and 2nCCC-L were diploid. The mean DNA content of 3nKB was equal to the sum of that of KOC and half of BIC, indicating that 3nKB was triploid. The mean DNA content of 4nKB was equal to the sum of that of KOC and that of BIC, indicating that 4nKB was tetraploid hybrid (Supplementary Figure 1 and Supplementary Table 2).

Table 2 Examination of chromosome number in KOC, BIC, 2nCCC-L, 2nGCC-L, and 3nKB hybrids

Fish type	No. in metaphase	Distribution of chromosome number							
		< 48	48	< 100	100	< 124	124	< 148	148
KOC	200	10			190				
BIC	200	15	185						
2nCCC-L	200	2		18	180				
2nGCC-L	200	3		15	182				
3nKB	200	5		20			175		

Chromosomes Analysis

Chromosomes were counted in 200 metaphase spreads in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB. Of the individuals that were examined, 95.0% of chromosomal metaphases had 100 chromosomes in KOC with a karyotype formula of 22 m + 34sm + 22st + 22t, which was the same as that described in our previous study (Wang et al. 2018). Among the BIC individuals that were tested, 92.5% of chromosomal metaphases possessed 48 chromosomes with a karyotype formula of 30 m + 14sm + 2st + 2t, which was consistent with the formula described in our previous research (Kong et al. 2006). In the 2nGCC-L individuals under investigation, 90.0% of chromosomal metaphases had 100 chromosomes with a karyotype formula of 22 m + 34sm + 22t + 22st. In terms of 2nCCC-L individuals, 91.0% of chromosomal metaphases had 100 chromosomes with a karyotype formula of 22 m + 34sm + 22t + 22st. With regard to the 3nKB individuals, 87.5% of chromosomal metaphases had 124 chromosomes with a karyotype formula of 31 m + 45sm + 22st + 22t. The chromosomal spreads were examined directly to identify the chromosomal number, and the results suggested that 2nCCC-L, 2nGCC-L, and 3nKB were diploid, diploid, and triploid, respectively (Fig. 2 and Supplementary Figure 2, Table 2).

Measurement of the Nuclear Volume of Erythrocytes

The results of the measurements of mean erythrocyte nuclear volume for KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB were shown in Table 3 and Fig. 3A–F. With the increase in the ploidy level, the mean erythrocyte nuclear volume increased regularly among 2nGCC-L, 2nCCC-L, and 3nKB. The mean erythrocyte nuclear volume ratio of 2nGCC-L to KOC was not significantly different ($P > 0.05$) from a ratio of 1:1, suggesting that the 2nGCC-L were diploids. There was no obvious difference ($P > 0.05$) between the mean erythrocyte nuclear volume ratio of 2nCCC-L to KOC and a ratio of 1:1, indicating that the 2nCCC-L were diploids. There was no prominent difference between the mean erythrocyte nuclear volume ratio of the 3nKB to KOC plus half of BIC and a ratio of 1:1, showing that the 3nKB were triploids.

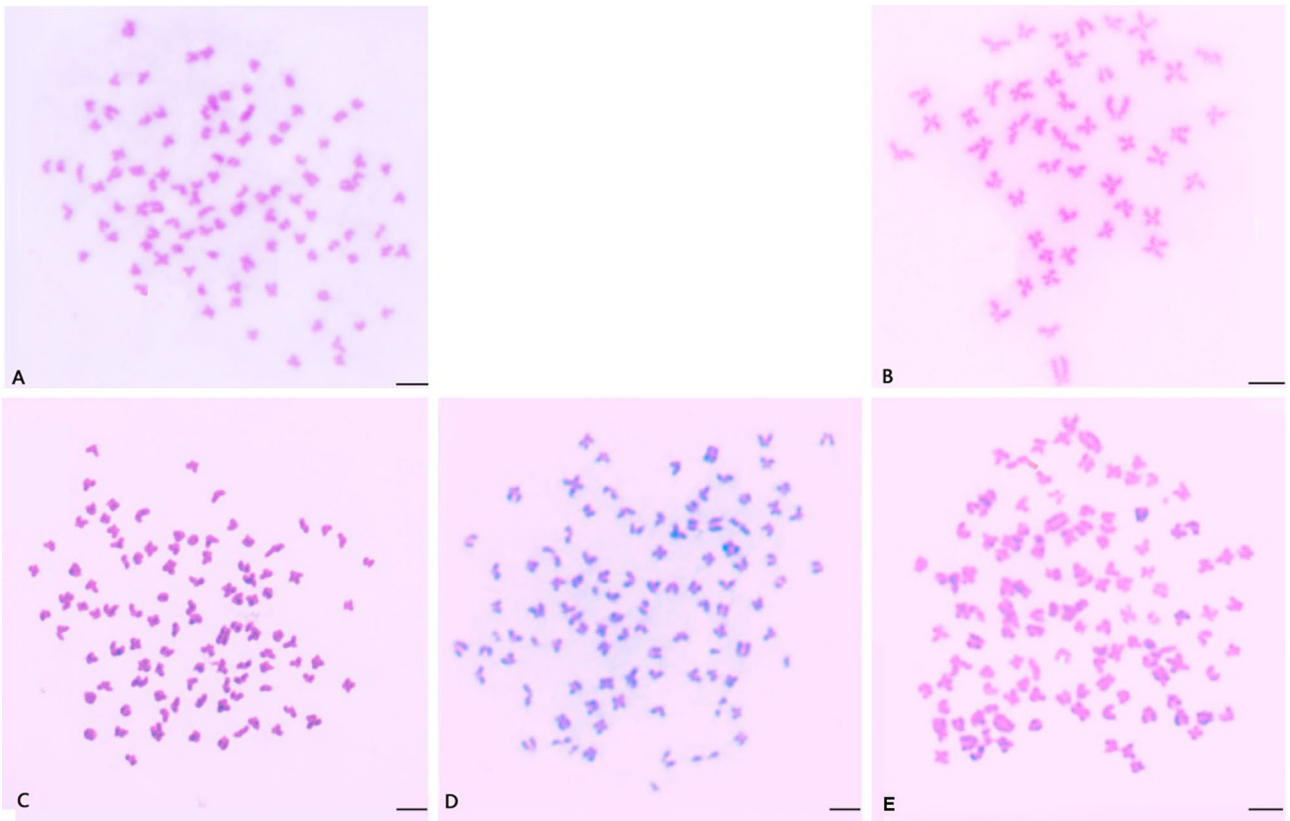

Fig. 2 Chromosome spreads at metaphase in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB**. A**: KOC; **B**: BIC; **C**: 2nGCC-L; **D**: 2nCCC-L; **E**: 3nKB. **A** The 100 chromosomes of KOC. **B** The 48 chromosomes of BIC. **C** The 100 chromosomes of 2nGCC-L. **D** The 100 chromosomes of 2nCCC-L. **E** The 124 chromosomes of 3nKB. Bar = 3 μm

Table 3 Mean erythrocyte nuclear volume measurements for KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB

Fish type	Major axis (μm)	Minor axis (μm)	Volume (μm^3)	Volume ratio	
				Observed	Expected
KOC	5.15 ± 0.52	3.25 ± 0.23	29.25 ± 8.22		
BIC	4.22 ± 0.39	2.33 ± 0.26	12.02 ± 2.73		
2nGCC-L	5.44 ± 0.34	3.36 ± 0.23	32.31 ± 5.02	2nGCC-L/KOC = 1.10[a]	1
2nCCC-L	5.34 ± 0.30	3.14 ± 0.19	27.68 ± 3.28	2nCCC-L/KOC = 0.95[a]	1

[a]The observed ratio was not significantly different ($P > 0.05$) from the expected ratio.

Nuclear Traits of Erythrocytes

Figure 3 showed the nuclear traits of erythrocytes in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB. The size of the nuclei of erythrocytes in 2nGCC-L and 2nCCC-L was equal to that in KOC; however, the size of the nuclei of erythrocytes in 3nKB was larger than in KOC or BIC. With the increase in ploidy level, the size of the nuclei of erythrocytes increased regularly among 2nGCC-L, 2nCCC-L, and 3nKB. Except for the difference in nuclear size, there was also a difference in nuclear appearance between the polyploid hybrids and their parents. For example, only the normal erythrocytes with one nucleu were observed in KOC, BIC, 2nGCC-L, and 2nCCC-L. However, unusual erythrocytes with two nuclei were found in 3nKB and 4nKB at a proportion of 16.7% and 12.35%, respectively (Fig. 3).

Microsatellite DNA Markers

One pair of primers (MFW1) for microsatellite DNA was able to distinguish KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB. Figure 4 showed the photograms of microsatellite DNA produced by the primer MFW1 in four BIC, four 2nGCC-L, four 2nCCC-L, four 3nKB, and four KOC. The results indicated that KOC and BIC could be detected with this pair of primers because they had quite different microsatellite DNA patterns. In 2nGCC-L and 2nCCC-L, similar DNA fragments to those in BIC were observed, suggesting that 2nGCC-L and 2nCCC-L had inherited DNA fragments from BIC (green arrows). In addition, there were similar DNA fragments among 2nGCC-L, 2nCCC-L, and KOC, showing that 2nGCC-L and 2nCCC-L had also inherited DNA fragments from KOC (red arrows). The existence of similar DNA fragments in 3nKB and BIC implied that 3nKB had inherited DNA fragments from BIC (yellow arrows). On the other hand, in 3nKB, there also were similar DNA fragments to those in KOC and BIC, showing that 3nKB had inherited DNA fragments from KOC and BIC. Interestingly, the new DNA fragments were only found in 2nGCC-L and 2nCCC-L, not in either KOC or BIC, indicating that DNA structural variations occurred in 2nGCC-L and 2nCCC-L (blue arrows). DNA fragments similar to those of the parents (KOC and BIC) and new DNA fragments were observed in both males and females of 2nGCC-L, 2nCCC-L, and 3nKB, confirming that these DNA fragments were not related to sex determination (Fig. 4).

5S rDNA Analysis

PCR amplification of the 5S rDNA was performed in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB. The KOC′ 5S rDNA bands of about 200 bp and 400 bp and the BIC′ 5S rDNA bands of about 180 bp and 360 bp were obtained by amplification, and the 2nGCC-L and 2nCCC-L all both yielded about 200 bp, 340 bp, and 480 bp bands. The 3nKB yielded about 180 bp, 200 bp, and 400 bp sequence bands, while about 180 bp was amplified from the BIC and 200 bp and 400 bp from the KOC. Through sequence analysis, we found that the 400 bp band of KOC was a dimer of 200 bp, while the 360 bp band of BIC was a dimer of 180 bp (Fig. 5).

The 5S rDNA structure was consisted of a 120 bp coding sequence (CDS) and several bases of a non-coding region that led to the diversity of different fragments. Based on PCR sequencing, there was a band of 202 bp in KOC, which was composed of 120 bp coding sequence and 82 bp non-coding sequence. BIC had a 176 bp band which was composed of 120 bp coding sequence and 56 bp non-coding sequence. Both 2nGCC-L and 2nCCC-L had bands of 201 bp, 338 bp, and 475 bp. The 201 bp band consisted of 120 bp coding sequence and 81 bp non-coding sequence, and 338 bp band was the combination of 120 bp coding sequence and 218 bp non-coding sequence. The 475 bp consisted of 120 bp coding sequence and 355 bp non-coding sequences. The 3nKB had bands of 178 bp, 202 bp, and 404 bp. The 178 bp band was composed of a 120 bp coding sequence and 58 bp non-coding sequence; the 202 bp band was formed of 120 bp coding sequence and 82 bp non-coding sequence. The 404 bp was a dimer of 202 bp. The sequence similarity analysis showed that the similarity of the 178 bp sequences from 3nKB and BIC was 98.80%, and the similarity of 202 bp sequences from 3nKB and KOC was 82.10%. The 201 bp

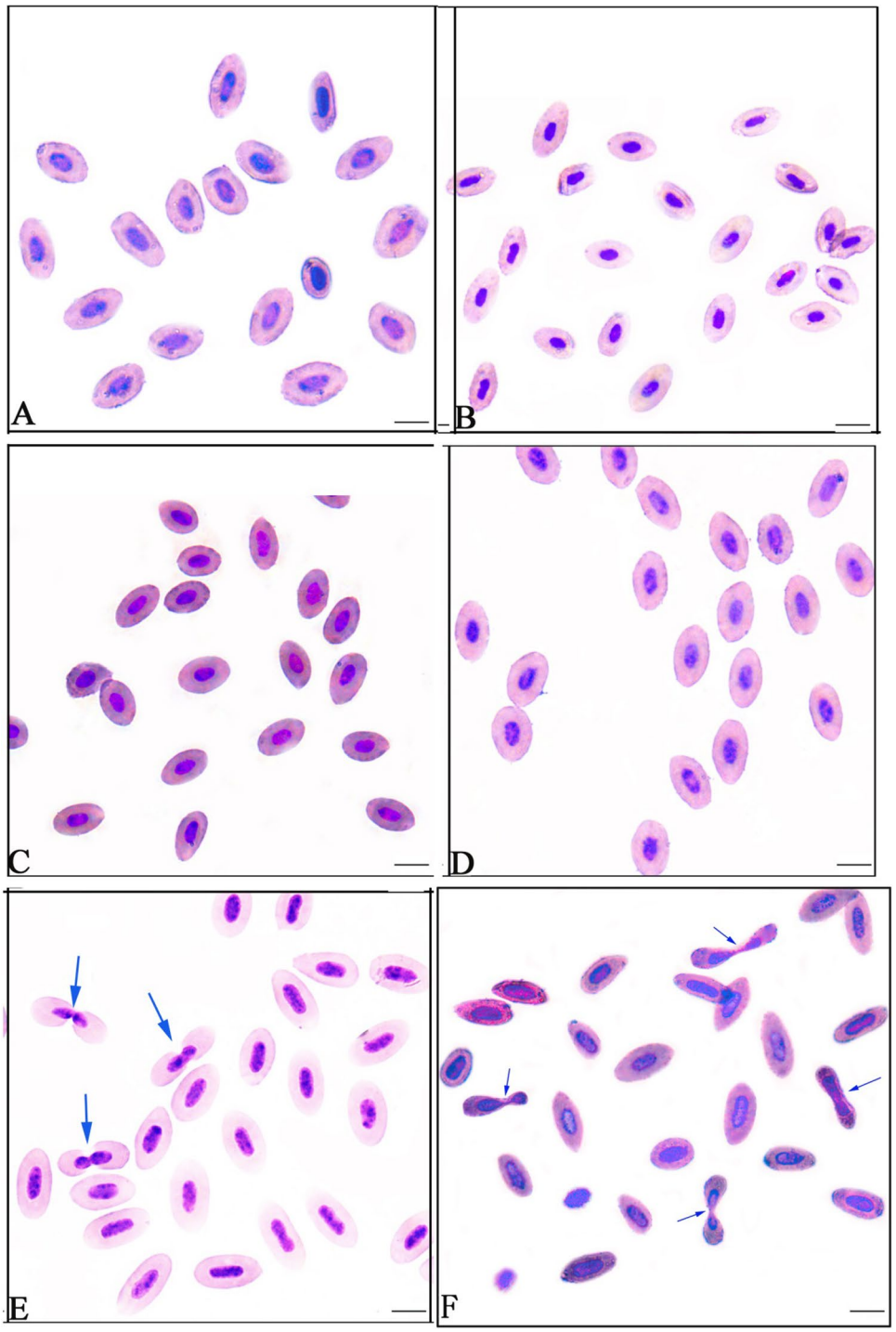

Fig. 3 Erythrocytes of KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB hybrids in peripheral blood. **A**. Mature erythrocytes of KOC, 100×. **B**. Mature erythrocytes of BIC, 100×. **C**. Mature erythrocytes of 2nGCC-L, 100×. **D**. Mature erythrocytes of 2nCCC-L, 100×. **E**. Mature erythrocytes of 3nKB. **F**. Mature erythrocyte of 4nKB. Bar = 3 μm. The arrows indicated there were two nuclei in deformed mature erythrocytes

bands of 2nGCC-L and 2nCCC-L were both from the KOC and the similarity of the 201 bp sequences from 2nGCC-L and 2nCCC-L compared to that of KOC was 99.50% and 94.50%, respectively. In addition, the 338 bp and 475 bp bands were due to variation fragments, which made the appearance of 2nGCC-L and 2nCCC-L more like that of "crucian" carp (Fig. 6).

Discussion

Makeyeva (Makeyeva 1972) was the first who reported successful hybridization of common carp and bighead carp with 40–60% fertilization; however, deformities (notochord, caudal, and head regions) occurred in hatching larvae. In the follow-up studies Verigin and Makeeva

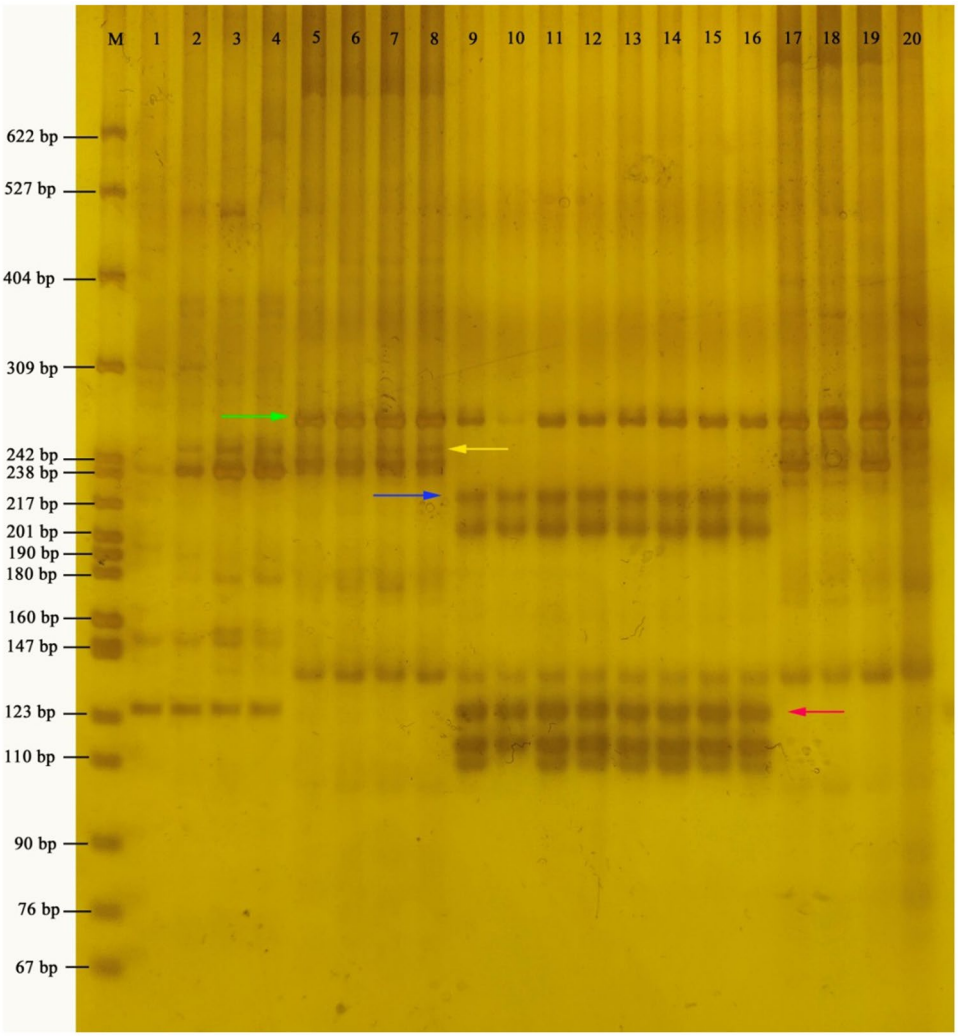

Fig. 4 Electrophotogram of microsatellite DNA patterns produced by the primer MFW1 in KOC, 3nKB, 2nGCC-L, 2nCCC-L, and BIC. Lanes 1–4 represented KOC. Lanes 5–8 represented 3nKB. Lanes 9–12 represented 2nGCC-L. Lanes 13–16 represented 2nCCC-L. Lanes 17–20 represented BIC. Green arrow indicated the DNA bands derived from BIC. 2nGCC-L, 2nCCC-L, and 3nKB hybrids commonly had this kind of band, respectively, but KOC did not have it. Yellow arrow indicated the DNA bands derived from KOC. The 3nKB had this kind of band, but the BIC, 2nGCC-L and 2nCCC-L did not have it. Blue arrow showed that 2nGCC-L and 2nCCC-L commonly only had this kind of band. Red arrow indicated the DNA band derived from KOC. 2nGCC-L and 2nCCC-L commonly had this kind of band, respectively, but 3nKB and BIC did not have it. M represented the pBR322 DNA/Mspl Marker

(Makeyeva 1972) were able to improve survival rates of hybrids by increasing water temperature to 26–28 °C in comparison to 18–20 °C used in common carp embryogenesis (Makeyeva 1972). Hybrids had two rudimentary barbels, frequently one pair, and morphometric characteristics, number of soft rays in dorsal fin (12–17) was distinctly different than in parental species, bighead carp (7) or common carp (18–21). The most comprehensive hybridization studies thus far on bighead carp (♂) × common carp (♀), as well as silver carp and grass carp hybrids with common carp, were carried out in Hungary by Bacos et al. (Opuszynski 1989). Hybrids of common carp and bighead reached 65% fertilization rate and viability, and growth was good during the first two years when raised in ponds. Interestingly, hybrids of common carp and silver carp reached maturity as four years old, and all fish were either females or hermaphrodites. All attempts of produce backcrosses were unsuccessful, and authors concluded that hybrids were sterile. No analysis of ploidy was performed on those hybrids to the best of our knowledge.

Long-term biological research on the evolutionary aspects of cyprinid hybridization have shown that hybridization is an important way to increase genetic diversity, and that hybridization leads to polyploidy. These mechanisms have played a very important role in the evolution of fishes and amphibians, including triploid, tetraploid, and pentaploid, as well as homodiploid (Wang et al. 2017, 2018; Zhang et al. 2014b). The results of distant hybridization of *Ctenopharyngodon idellus* (grass carp, $2n = 48$, ♀) × *Megalobrama amblycephala* (blunt snout bream, $2n = 48$, ♂) showed that distant hybridization can lead to formation of hybrid diploid and hybrid triploids (He et al. 2013). In this example, the diploid individuals and triploid individuals were phenotypically different from grass carp. In terms of growth rate, the triploid individuals showed an obvious growth advantage. Allen and Stanley (1983) performed hybridization of grass carp (♀) and bighead carp (♂) and gynogens, diploids, triploids, and "possible tetraploids" (tri-tetraploid mosaics) were obtained. The authors claimed that they identified pure species of triploid bighead and grass carp in the progeny of

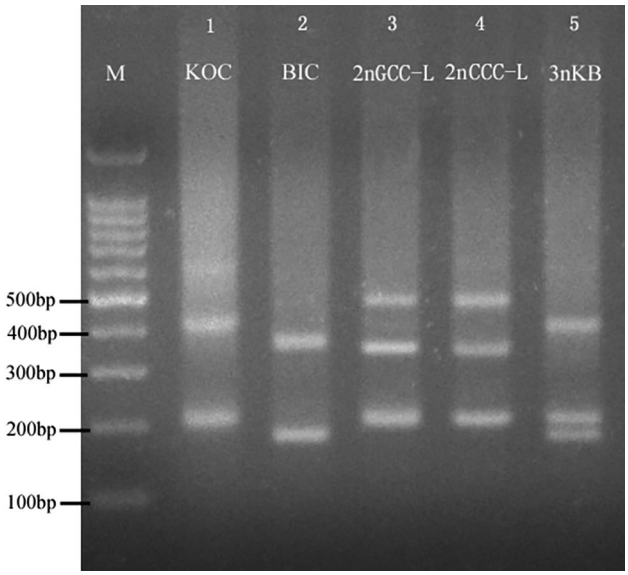

Fig. 5 DNA (5S rDNA) bands amplified by the primer pair 5SF-5SR in KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB. Lane 1, two DNA fragments (approximately 200 and 400 bp) were found in KOC. Lane 2, two DNA fragments (approximately 180 and 360 bp) were found in BIC. Lane 3, three DNA fragments (approximately 200, 340, and 500 bp) were found in 2nGCC-L. Lane 4, three DNA fragments (approximately 200, 340, and 500 bp) were found in 2nCCC-L. Lane 5, three DNA fragments (approximately 180, 200, and 400 bp) were found in 3nKB. M represented DNA ladder markers (100 bp increments)

original crosses; however, no proof of that conclusion was provided. Our laboratory also took successfully the grass carp (♀) × *Erythroculter ilishaeformis* (topmouth culter, $2n = 48$,♂) hybridization to obtain gynogenetic grass carp and, due to sterility, triploid hybrids of grass carp × topmouth culter also showed an obvious growth advantage (Wu et al. 2019). In addition, the "crucian" carp-like homodiploid fish of common carp (♀) × blunt snout bream (♂) and homodiploid fish (red "crucian" carp-like homodiploid fish and goldfish-like homodiploid fish) of koi carp (♀) × blunt snout bream (♂) had obvious reproductive advantage (Wang et al. 2017, 2018).

In the present study, 2nGCC-L, 2nCCC-L, and 3nKB were obtained by distant hybridization. One tetraploid hybrid was also obtained. However, it requires further work, with larger number of offspring to induce tetraploidy. Hybridization was not only a catalyst for speciation, but also played a leading role in the explosion of life (Mallet 2007). If the somatic chromosomes of the offspring of hybrid organisms were doubled, they could become allopolyploids (Mallet 2007). In addition, hybrid diploids had abnormal chromosomal pairing during meiosis, which was largely solved by producing non-meiotic gametes (Otto 2007). The formation and fusion of non-meiotic gametes in organisms accelerated the rate of polyploidy in hybrid lineage. In this study, the formation of hybrid triploid was probably due to abnormal chromosome pairing during meiosis of the hybrid, and the formation of hybrid triploid was largely due to the production of non-meiotic gametes. The occurrence of hybrid tetraploid was probably due to the formation of somatic cell doubling of the hybrid.

An appearance of 2nGCC-L and 2nCCC-L was observed and obvious differences were recorded in comparison to *C. auratus*: their heads were rounder and they had higher backs. The 3nKB had one pair of barbels and could grow faster than the KOC. The offspring had obvious signs of hybridization characteristics. In terms of examined traits, 2nGCC-L, 2nCCC-L, and 3nKB were intermediate to those of KOC and BIC, but they were significantly different from those of KOC and BIC.

In terms of chromosome structure (chromatids) and karyotype, KOC had 100 chromosomes with a karyotype of 22 m + 30sm + 24t + 24st, and BIC had 48 chromosomes with a karyotype of 30 m + 14sm + 2st + 2t. The 2nGCC-L and 2nCCC-L fish both had 100 chromosomes with a karyotype of 22 m + 30sm + 24t + 24st, while in the 3nKB 124 chromosomes with a karyotype of 31 m + 45sm + 22st + 22t were found. We determined that the chromosome composition of 3nKB consisted of one set of KOC and half of BIC. These results confirmed that 2nGCC-L and 2nCCC-L were homodiploids; however, 3nKB was triploid hybrid (Supplementary Table 2 and Fig. 2).

The presence of unusual erythrocytes with two nuclei could be used as a cytological marker to distinguish polyploid hybrids from their diploid parents (Lu et al. 2009). With the increase in the ploidy level, the percentage of unusual erythrocytes with two nuclei increased, suggesting the possibility of the significance of unusual erythrocytes in polyploid hybrids. In our study, 17.6% of unusual erythrocytes with two nuclei was found in the 3nKB hybrids of KOC (♀) × BIC(♂). It was concluded that the higher the DNA content, the easier it was for the cell nuclei to divide. The large volume and relatively small surface area of erythrocytes in polyploid fish made them inconducive in oxygen transport and blood circulation. Erythrocytes in triploids can enhance the ability to carry and transport oxygen through morphological variation in the circulatory system. At the same time, the deformed cell nuclei of polyploid fish resulted in an increase in the surface area of the nucleus (Fig. 3).

Microsatellite markers had high polymorphism and rich information content. At present, microsatellite markers are widely used in gene linkage and genetic map construction, genetic diversity research, pedigree and development research, disease detection, variety identification, parent analysis, and individual and pure line test (Varshney et al. 2005). In the present study MFW1 primers were used to amplify a DNA fragment from female KOC, and this DNA

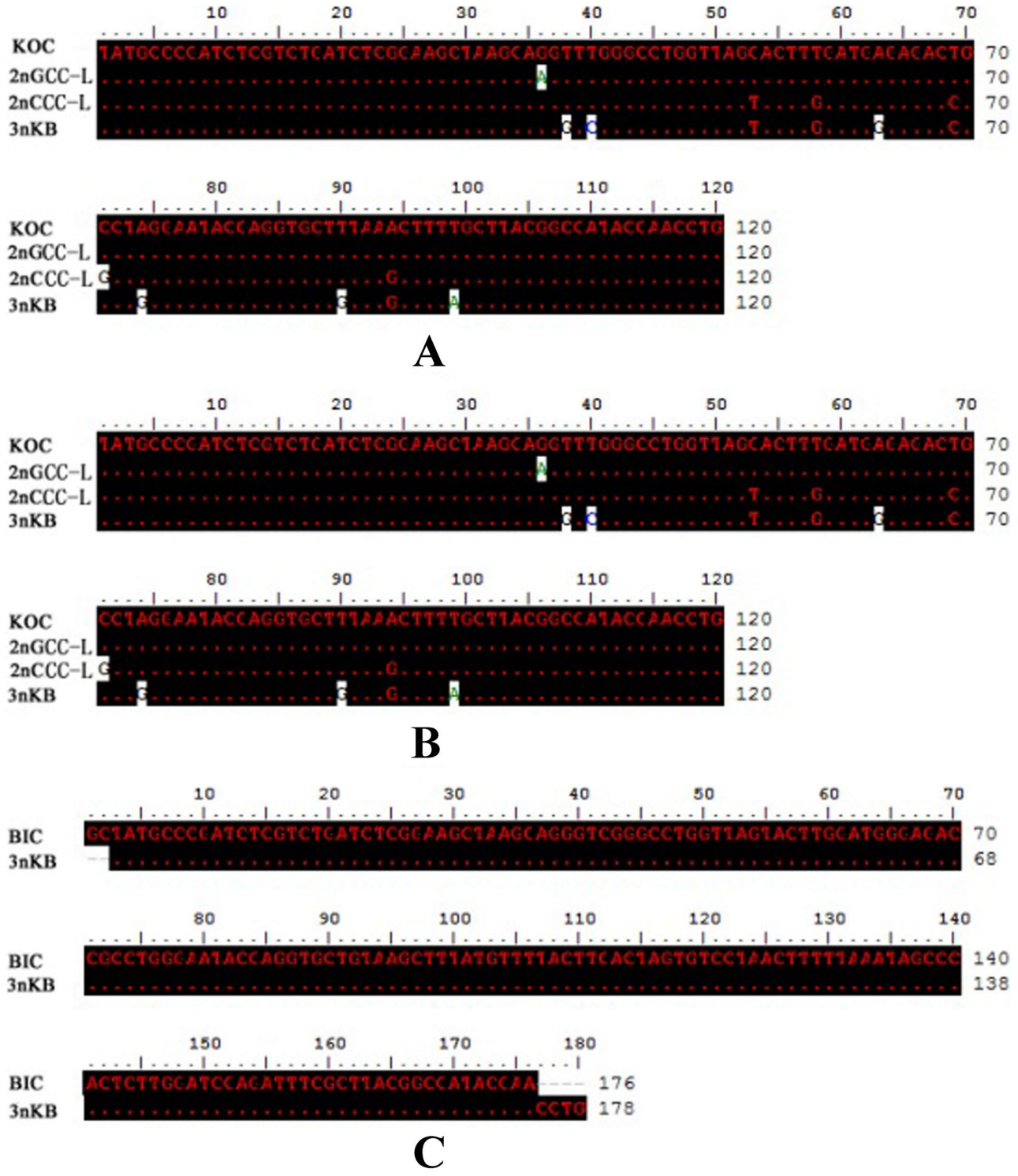

Fig. 6 Sequence comparison of 5S rDNA. (**A**) Sequence comparison of the 5S rDNA coding region in KOC, 2nGCC-L, 2nCCC-L, and 3nKB. (**B**) Sequence comparison of the 5S rDNA NTS region in KOC, 2nGCC-L, 2nCCC-L, and 3nKB. (**C**) Sequence comparison of the 5S rDNA in BIC and 3nKB

fragment was inherited by the offspring (2nGCC-L, 2nCCC-L and 3nKB). The DNA fragment from the male parent, BIC, was also inherited, whereas a DNA fragment that the parents did not have appeared in the hybrid generation. It was worth mentioning that there was a large number of male parental DNA inserted into the genomes of homodiploids

2nGCC-L and 2nCCC-L, resulting in a substantial variation in their performance, and a goldfish-like body shape (Fig. 4).

In terms of taxonomy, KOC and BIC belong to different subfamilies. KOC belongs to carp genus (Cyprinus sp.) (Subfamily Cyprininae) and BIC belongs to *Hypophthalmichthys* (Subfamily Xenocyprinae). Kongtian [35] suggested that in plants among distantly related individuals, the male parent impacts the hybrid offspring in a manner that often showed many genetic and biochemical differences from the maternal parent, such as the appearance and growth rate. The hybrid offspring of KOC (♀) × BIC (♂) expressed obvious differences compared with KOC and BIC. This might be associated with the kinship of distant hybrids.

The results of 5S rDNA amplification and sequencing of KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB showed that there was only one 5S rDNA structural unit in the genome of KOC, one 5S rDNA structural unit in the genome of BIC, three 5S rDNA structural units in 2nGCC-L, 2nCCC-L, and 3nKB. According to the sequence comparison of the common CDSs, remarkable base changes were found in the internal control regions Box A and Box C of the 5S rDNA coding region. Only individual base changes were found in the internal element (IE), which was evidence that the 5S rDNA coding region was highly conservative, but there were base differences. By comparing the NTS regions of KOC, BIC, 2nGCC-L, 2nCCC-L, and 3nKB, it was found that 3nKB was highly similar to KOC and BIC, which supports the notion of the genetic similarity between KOC and BIC (Figs. 5 and 6).

In summary, the establishment of the homodiploid 2nGCC-L and 2nCCC-L was novel in respect to genetics and breeding of different subfamily hybrids. The 3nKB and 4nKB had obvious growth advantages and were the hybrids with potential economic benefits to aquaculture industry (Chourrout et al. 1986; Zhang et al. 2014a). The ploidy and genetic composition of the offspring were confirmed by analyzing the DNA content, chromosome number, and 5S rDNA. The establishment of the homodiploid 2nGCC-L and 2nCCC-L provided an excellent model to investigate the phenotypic and genotypic changes in homodiploid fish that facilitate adaptation and promotes biodiversity. Based on this useful platform, we will be able to facilitate the study of the mechanisms that drive homologous diploidization, including the stabilization of meiosis at the gene level. In respect to commercial breeding, the homodiploid fish lineages will be useful for the production of polyploids that potentially have the advantage of a faster growth rate and disease resistance.

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Availability of Data and Material All data generated or analyzed during this study are included in this paper.

Declarations

Ethics Approval The fish treatments were reviewed and approved by the Institute of Experiment Animals, Hunan Province, China. All fish were deeply anesthetized using 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) before dissection.

Conflict of Interest The authors declare no competing interests.

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