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The establishment of an autotetraploid fish lineage produced by female allotetraploid hybrids × male homodiploid hybrids derived from *Cyprinus carpio* (♀) × *Megalobrama amblycephala* (♂)

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ABSTRACT

Distant hybridization leads to obvious changes in genotypes and phenotypes, giving rise to species with novel capabilities and adaptabilities. The establishment of autotetraploid lineages is difficult but useful in genetics and breeding. Here, we established a new autotetraploid fish lineage (4n = 200, F_1-F_4) via hybridization of an allotetraploid hybrid fish (4n = 148) (♀) × a crucian carp-like homodiploid fish (2n = 100) (♂) derived from common carp (*Cyprinus carpio*, 2n = 100) (♀) × blunt snout bream (*Megalobrama amblycephala*, 2n = 48) (♂). The results at the chromosome and DNA content levels indicated that the individuals of the autotetraploid fish had 200 chromosomes originating from common carp. The mean erythrocyte nuclear and spermatozoa volumes of these individuals were two times larger than those of common carp. Furthermore, the autotetraploid individuals harboured more DNA types in the 5S rDNA and *Hox* genes, including the recombination type, maternal-specific type, and paternal-specific type. The establishment of the autotetraploid fish lineage was caused by the fertilization of autotriploid eggs produced by the female allotetraploid fish (4n = 148) and haploid sperm produced by the male crucian carp-like homodiploid fish (2n = 100). The formation of the autotriploid eggs was associated with the mechanism of genomic doubling by premeiotic endoreduplication, endomitosis, or fusion of the oogonia of the female allotetraploid fish. The transmission of DNA variation between successive generations (F_1-F_3) suggested that the autotetraploid fish lineage is undergoing rapid diploidization to maintain tetraploid stability. The establishment of this new autotetraploid fish lineage provides new germplasm resources for fish genetic breeding and for studies on species evolution.

1. Introduction

As one of the most important economic fishes in the Cyprinidae family, common carp (*Cyprinus carpio*) was mainly cultured in Europe and Asia, with a culture history of several thousand years (Feng et al., 2018). Due to its successful artificial propagation and high economic value, the annual production capacity of *C. carpio* reached more than 4 million metric tons (Bostock et al., 2010; Fisheries, 2006). Because of artificial selection and breeding efforts over the past few centuries, many domesticated lineages with distinct phenotypes or economic traits adapted to local environments were established to meet consumer demands. The largest *C. carpio* producer was China, where there were abundant domesticated lineages and populations as well as many hybrid lineages (Dong et al., 2015; Xu et al., 2014). However, the escape, artificial propagation, and the release of hybrids into natural waters had the potential risk to affect the genetic profiles of the wild populations. The production of the sterile common carp was one of the best ways to avoid the above potential risk.

Recently, an increasing number of studies showed that triploid animals were almost always sterile due to the unequal disjunction of homologous chromosomes during meiosis, which generated aneuploid gametes or reduces gonadal development (Benfey, 1999; Tiwary et al., 2004). Examples included triploid molluscs (Maldonado-Amparo et al., 2004), triploid crustaceans (Xiang et al., 2006), and triploid fishes (Li

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et al., 2019; Tao et al., 2018; Tiwary et al., 2004). Physical and chemical treatments successfully applied to induce triploids in many fish species (Chourrout, 1984; Haffray et al., 2007; Xu et al., 2008), but the survival of triploids was usually very low due to these methods damaging fertilized eggs. On the other hand, as a safe and efficient method, distant hybridization could produce sterile triploids at a large scale by crossing tetraploid and diploid fish (Chen et al., 2009; Liu, 2010; Liu et al., 2001; Luo et al., 2011; Wang et al., 2019). The keys to the production of sterile triploids by this method were the acquisition and population expansion of tetraploids. To date, there were some reports on the successful development of some tetraploid fish lineages (Liu et al., 2001; Liu et al., 2016; Liu et al., 2007; Qin et al., 2019; Qin et al., 2014b). However, there was no report on the formation of tetraploid common carp lineages. Therefore, the development of tetraploid common carp and the establishment of stable lineages are important goals for the development of the common carp aquaculture industry.

The common carp (abbreviated as 2nCOC), with 100 chromosomes, belongs to the Cyprininae subfamily, and the blunt snout bream (*Megalobrama amblycephala*) (abbreviated as 2nBSB), with 48 chromosomes, belongs to the Cultrinae subfamily. In an earlier study, we obtained a crucian carp-like homodiploid fish (2n = 100, abbreviated as 2nNCRC) and an allotetraploid hybrid (4n = 148, abbreviated as 4nCB) from 2nCOC (♀) × 2nBSB (♂) (Wang et al., 2019; Wang et al., 2017a). In the present study, we artificially establish an autotetraploid common carp lineage (4n = 200, 4nNC-F_1-F_4) via the hybridization of 4nCB (♀) × 2nNCRC (♂). This is the first new autotetraploid fish lineage produced by means of distant hybridization and mating between different-ploidy sister taxa, providing new tetraploid fish germplasm resources. In fish genetic breeding, 4nNC were used to produce new excellent triploid fishes with the advantages of sterility and a faster growth rate by crossing tetraploids with diploids (Liu et al., 2001; Qin et al., 2014b). In this study, we investigated important biological traits of 4nNC, including morphological traits, chromosome numbers, DNA contents, erythrocyte traits, gonadal development, and genetic variation in 5S rDNA and *Hox* genes. This study is important for fish genetic breeding and species evolution.

2. Materials and methods

2.1. Ethics statement

The guidelines established by the Administration of Affairs Concerning Animal Experimentation 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 neither rare nor near extinction (first- or second-class state protection level). Therefore, approval was not required for the experiments conducted in this study.

2.2. Animals and crossing procedure

The natural materials, including 2nCOC (2n = 100) and 2nBSB (2n = 48), and the hybrid materials, including 2nNCRC (2n = 100) and 4nCB (4n = 148) from 2nCOC (♀) × 2nBSB (♂), were fed in a pool with suitable illumination, water temperature, dissolved oxygen content, and adequate forage at the Center for Polyploidy Fish Genetics Breeding of Hunan Province located at Hunan Normal University, Changsha, Hunan, China. The protocols for crossing and culturing were described previously (Wang et al., 2017a). In an earlier study, we obtained 2nNCRC and 4nCB via 2nCOC (♀) × 2nBSB (♂) (Wang et al., 2019; Wang et al., 2017a). In the present study, we successfully obtained a new autotetraploid fish (4n = 200, 4nNC-F_1) derived from the hybridization of 4nCB (♀) × 2nNCRC (♂). The self-cross offspring of 4nNC-F_1 are denoted 4nNC-F_2, the self-cross offspring of 4nNC-F_2 are denoted 4nNC-F_3, and the subsequent self-cross offspring are denoted 4nNC-F_4. The 4nNC (F_1-F_4) fish were cultured in ponds under the same conditions at the Center for Polyploidy Fish Genetics Breeding of Hunan Province, Hunan Normal University, Changsha, Hunan, China. All fishes were deeply anaesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) prior to dissection.

2.3. Preparation of chromosome spreads and measurement of DNA content

To determine ploidy, chromosome counts of the kidney tissues of 2nCOC, 2nBSB, and 4nNC-F_1-F_4 were carried out. The preparations were made according to the method described by Liu et al. (Liu et al., 2007), with minor modifications. After being cultured for 2-3 d at 20–22 °C, these fish samples were injected one to three times with concanavalin at a dose of 6–15 μg/g body weight and an interval of 12–24 h. Two to 3 h prior to dissection, each sample was injected with colchicine at a dose of 4–6 μg/g body weight. The kidney tissue was ground in 0.9% NaCl, subjected to hypotonic treatment with 0.075 M KCl at 37 °C for 40–60 min, and then fixed two to three times in 3:1 methanol: acetic acid. The cells were added dropwise to cold, wet slides and stained with 4% Giemsa for 40–60 min. The chromosome shapes and numbers were analysed under a light microscope. For each type of fish, 200 metaphase spreads (20 metaphase spreads for each sample) of chromosomes were analysed. The preparations were examined under an oil lens at a magnification of 330 × .

The DNA contents of erythrocytes from 2nCOC, 2nBSB, 2nNCRC, 4nCB, and 4nNC-F_1-F_4 were measured using a flow cytometer (Cell Counter Analyser, Partec, Germany). We collected 1–2 ml of blood from the caudal vein of each fish sample using a syringe containing 200–300 units of sodium heparin. The blood samples were processed following the method described by Liu et al. (Liu et al., 2007) and investigated under the same conditions. The ratios of polyploid hybrid DNA content to the sum of 2nCOC and 2nBSB DNA contents were calculated, and a chi-square test with Yates's correction was used to test for deviation from the expected ratio values.

2.4. Morphological traits

At one year of age, 20 2nCOC, 20 2nBSB, 20 2nNCRC, 20 4nCB, and 60 4nNC-F_1-F_3 individuals were randomly selected for morphological examination following the methods described in a previous study (Wang et al., 2017a). SPSS Statistics 17.0 (IBM Corp., NY, USA) was used to analyse covariance in the measurable and countable morphological traits among these fish samples.

2.5. Appearance of erythrocytes and measurement of nuclear volume

The erythrocytes of 2nCOC and 4nNC-F_1 were dehydrated in alcohol, added dropwise onto slides, desiccated, subjected to atomized gilding and analysed with a JSM-6360LV scanning electron microscope (SEM, JEOL, Japan). The blood smears from each fish sample were prepared in accordance with the methods described by Liu et al. and Lu et al. (Liu et al., 2007; Lu et al., 2009). The 20 erythrocytes from each fish sample were observed under oil immersion by using an ocular micrometer. The erythrocyte nuclear volume of each fish sample was calculated by (4/3) πab^2, where a is the major semi-axis and b is the minor semi-axis of a perfect ellipsoid. SPSS software was used to analyse the covariance of the erythrocyte nuclei.

2.6. Gonadal structure and spermatozoa phenotype

The gonads of 4nCB, 2nNCRC, and 4nNC-F_3 were fixed in Bouin's solution for the preparation of tissue sections. The paraffin-embedded sections were cut and stained with haematoxylin and eosin. The gonadal structures of 4nCB, 2nNCRC, and 4nNC-F_3 were observed by a light microscope and photographed with a Pixera Pro 600 ES (Pixera Corporation, Santa Clara, CA, USA).

Semen samples of 2nCOC and 4nNC-F_3 were collected with a clean

sucker and then transferred into a 2.5% glutaraldehyde solution. The semen samples were centrifuged at 2000 × *g* for 1 min, fixed in a 4% glutaraldehyde solution overnight, and fixed in a 1% osmic acid solution for 2 h. The spermatozoa were dehydrated in alcohol, dropped onto slides, and desiccated. Finally, the spermatozoa were subjected to atomized gilding and analysed with an X-650 (Hitachi) SEM (Nikon, Japan).

2.7. DNA extraction, PCR amplification, cloning and sequencing of genomic DNA (5S rDNA and Hox genes)

Total genomic DNA from the peripheral blood cells of 2nCOC, 2nBSB, 2nNCRC, 4nCB, and 4nNC-F_1-F_3 was extracted by routine approaches (Sambrook and Russell David, 1989). A set of primers (*5S* P1, 5′-GCTATGCCCGATCTCGTCTGA-3'; *5S* P2R, 5′-CAGGTTGGTATGGCC GTAAGC-3′) was designed and synthesized to amplify the *5S* rRNA genes and their NTS regions directly from genomic DNA according to Qin et al. (Qin et al., 2010). The PCRs and the thermal programme steps followed our previous method (Wang et al., 2017a). Several combinations of degenerate PCR primers (Luo et al., 2007; Wang et al., 2017b) were used to amplify up to ten *Hox* gene sequences (*HoxA4a*, *HoxA9a*, *HoxA2b*, *HoxB1a*, *HoxB5b*, *HoxB6b*, *HoxC4a*, *HoxC6b*, *HoxD4a*, and *HoxD10a*) in 2nCOC, 2nBSB, 2nNCRC, 4nCB, and 4nNC-F_1-F_3. The PCRs were performed in a volume of 50 μL using Taq DNA polymerase (TaKaRa, Dalian, China). The thermal cycling programme steps followed our previous method (Wang et al., 2017b). These PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China). The plasmids were transformed into *Escherichia coli* DH5a, purified, and then sequenced with vector-specific primers using the primer-walking method on an ABI 3730XL automatic sequencer (ABI PRISM 3730, Applied Biosystems, CA, USA).

2.8. Sequence comparison and analysis

The sequence homology and variation among the fragments amplified from 2nCOC, 2nBSB, 2nNCRC, 4nCB, and 4nNC-F_1-F_3 individuals were analysed using BioEdit (Hall, 1999), ClustalW (Thompson et al., 1994), and DNAStar 5.0 software (DNAStar Inc.). To increase the probability of detecting duplicated paralogs and circumventing errors from PCR of 5S rDNA and *Hox* genes, we sequenced 20-40 clones of each gene from 2nCOC, 2nBSB, 2nNCRC, 4nCB, and 4nNC-F_1-F_3. The obtained sequences were screened for 5S rDNA and *Hox* gene fragments using the ClustalW (http://www.ebi.ac.uk/) (Thompson et al., 1994), BLAST (http://www.ncbi.nlm.nih.gov), and MEGA 4.0 (Tamura et al., 2007) programs to determine identity.

3. Results

3.1. Formation of the new autotetraploid fish lineage

Individuals of 4nCB (4n = 148) and 2nNCRC (2n = 100) were obtained in the first generation of 2nCOC (2n = 100) (♀) × 2nBSB (2n = 48) (♂) (Wang et al., 2017a). In the present study, a new autotetraploid fish (4n = 200, 4nNC-F_1) was produced in the first generation of 4nCB (4n = 148) (♀) × 2nNCRC (2n = 100) (♂); subsequently, a new autotetraploid fish lineage (F_1-F_4, 4n = 200) was formed through successive self-crosses (Fig. 1). The establishment of the new autotetraploid fish lineage from F_1 to F_4 indicated that both males and females in F_1-F_3 were fertile.

3.2. Measurement of DNA content and examination of chromosome number

With the sum of the DNA contents of 2nCOC and 2nBSB as the controls, the distribution of the DNA content of 2nNCRC, 4nCB, and 4nNC-F_1-F_4 was presented in Table 1 and Supplementary Fig. S1. The mean DNA content of 2nNCRC was equal to that of 2nCOC ($P > 0.05$), suggesting that 2nNCRC had two sets of 2nCOC-derived chromosomes. The mean DNA content of 4nCB was equal to the sum of that of 2nCOC and 2nBSB ($P > 0.05$), suggesting that 4nCB had two sets of chromosomes from 2nCOC and two sets of chromosomes from 2nBSB (Wang et al., 2017a). The mean DNA content of 4nNC-F_1-F_4 was double that of 2nCOC ($P > 0.05$), suggesting that 4nNC-F_1-F_4 had four sets of 2nCOC-derived chromosomes (Table 1, Supplementary Fig. S1). The above results indicate that compared with 2nCOC and 2nBSB, 4nNC-F_1-F_4 is an autotetraploid common carp lineage.

In our previous research, ploidy analyses of chromosome number revealed that 2nNCRC had 100 chromosomes, consistent with of the number in 2nCOC, and that 4nCB had 148 chromosomes, consistent with the sum of chromosomes in 2nCOC and 2nBSB (Wang et al., 2017a). In this study, chromosomes were counted in 200 metaphase spreads for each sample of 2nCOC, 2nBSB, and 4nNC-F_1-F_4 (Table 2). Among the 2nCOC samples, 92.5% of the chromosomal metaphases had 100 chromosomes (Table 2, Fig. 2 A), indicating that they were diploids with 100 chromosomes, and no larger submetacentric chromosomes were observed in 2nCOC, the same as the patterns described in our previous study (Liu et al., 2001). We examined 2nBSB samples and found that they had 48 chromosomes (90.0% of chromosomal metaphases) (Table 2, Fig. 2 B), indicating that they were diploids with 48 chromosomes. Moreover, a large pair of submetacentric chromosomes was observed in 2nBSB, as described in previous studies (Hu et al., 2018; Liu et al., 2007; Qin et al., 2014b). Of the 4nNC-F_1-F_4 samples that we examined, 86.0% ~ 89.0% of chromosomal metaphases had 200 chromosomes and lacked the large submetacentric chromosomes from 2nBSB (Table 2, Fig. 2 C-F), indicating that they were tetraploid with four sets of 2nCOC-derived chromosomes. The above chromosomal spread results provided direct evidence to prove that 4nNC-F_1-F_4 individuals were autotetraploids with 200 chromosomes derived from 2nCOC.

3.3. Morphological traits

As shown in Fig. 1, there were obvious differences in morphological traits between 4nNC (F_1-F_3) (Fig. 1 E-G) or 2nCOC (Fig. 1 A) and 2nBSB (Fig. 1 B), 4nCB (Fig. 1 C) and 2nNCRC (Fig. 1 D). Tables 3 and 4 showed the values for the countable and measurable traits in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC (F_1-F_3). For the countable traits (Table 3), between 4nNC (F_1-F_3) and 2nCOC, except for the numbers of lateral scales and lower lateral scales, the traits were significantly different ($P < 0.01$). All of the countable traits except for the number of abdominal fins differed significantly ($P < 0.01$) between 4nNC (F_1-F_3) and 2nBSB. Between 4nNC (F_1-F_3) and 2nNCRC, except for the number of lateral scales, none of the countable traits were significantly different ($P > 0.01$). Moreover, none of the countable traits were significantly different ($P > 0.01$) between 4nNC (F_1-F_3) and 4nCB.

For the measurable traits (Table 4), between 4nNC (F_1-F_3) and 2nCOC, except for BW/BL, the traits were significantly different ($P < 0.01$). All of the measurable traits except for BL/WL, BW/BL, HW/HL, and TW/TL differed significantly ($P < 0.01$) between 4nNC (F_1-F_3) and 2nBSB. Moreover, none of the measurable traits were significantly different ($P > 0.01$) between 4nNC (F_1-F_3) and 2nNCRC, and none of the measurable traits except for HW/BW were significantly different ($P > 0.01$) between 4nNC (F_1-F_3) and 4nCB.

3.4. Appearance of erythrocytes and measurement of nuclear volume

Fig. 3 showed the nuclear traits of erythrocytes in 2nCOC and 4nNC-F_1. The nuclei of erythrocytes in 4nNC-F_1 were larger than those in 2nCOC. This result was similar to previous results from our laboratory (Liu et al., 2001; Liu et al., 2007). In addition to the difference in nuclear size, there was a difference in nuclear appearance between 4nNC-F_1 and 2nCOC. For example, normal erythrocytes with one nucleus and no unusual erythrocytes with two nuclei were observed in 2nCOC

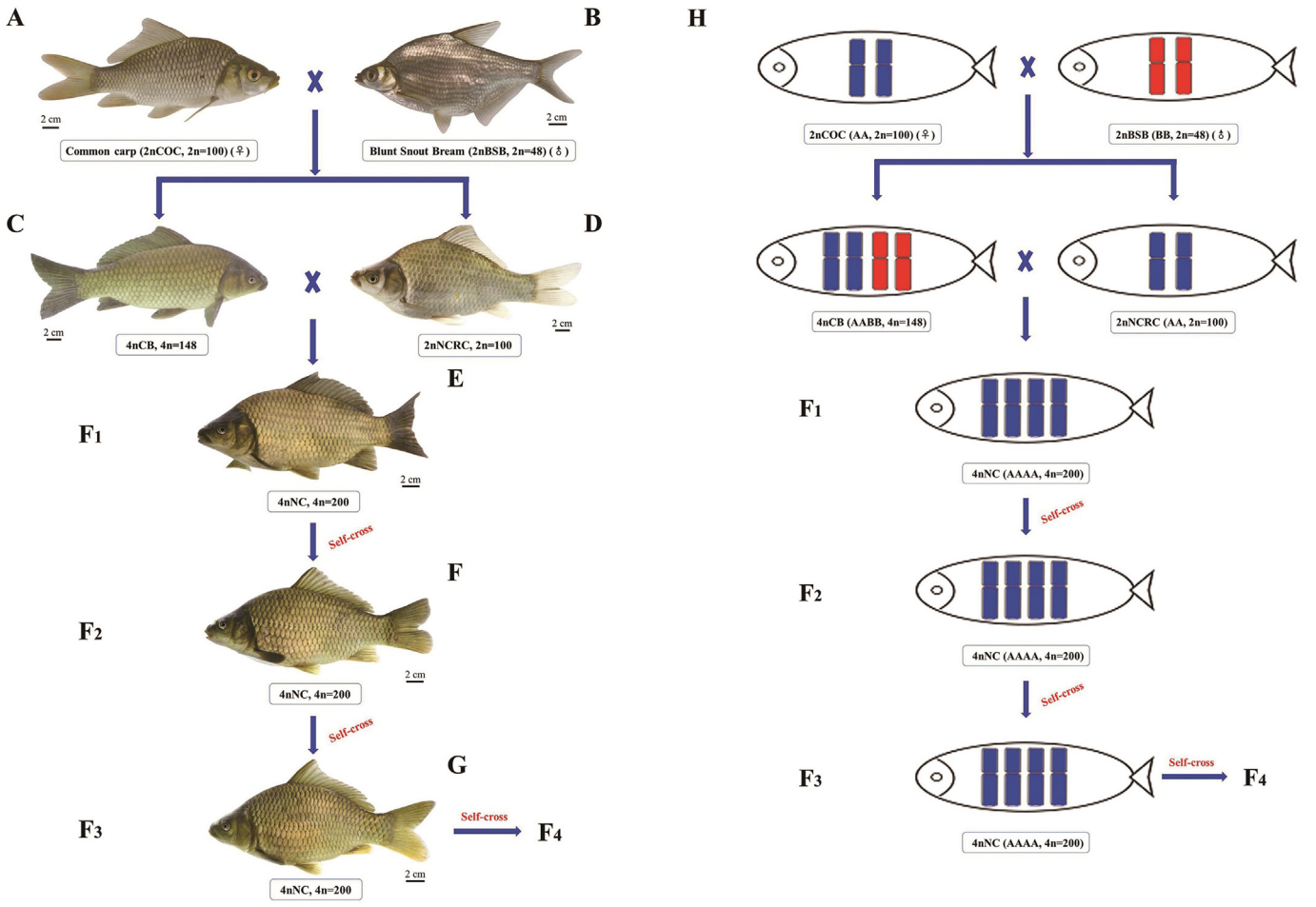

Fig. 1. The crossing procedure and appearance of 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3. (A) The appearance of 2nCOC. (B) The appearance of 2nBSB. (C) The appearance of 4nCB. (D) The appearance of 2nNCRC. (E) The appearance of 4nNC-F_1. (F) The appearance of 4nNC-F_2. (G) The appearance of 4nNC-F_3. (H) The formation of experimental fish. The chromosomes of common carp (2nCOC) and blunt snout bream (2nBSB) are marked by the blue and red color, respectively. Bar = 2 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The mean DNA content in 2nBSB, 2nCOC, 2nNCRC, 4nCB, and 4nNC-F_1-F_4.

Fish type	DNA content [a]	Ratio	
		Observed	Expected
2nBSB	67.29		
2nCOC	105.40		
2nNCRC	107.34	2nNCRC/2nCOC = 1.02[b]	1
4nCB	157.81	4nCB/(2nCOC + 2nBSB) = 0.91[b]	1
4nNC-F_1	209.03	4nNC-F_1/2nCOC = 1.98[b]	2
4nNC-F_2	200.26	4nNC-F_2/2nCOC = 1.90[b]	2
4nNC-F_3	196.55	4nNC-F_3/2nCOC = 1.86[b]	2
4nNC-F_4	198.45	4nNC-F_4/2nCOC = 1.88[b]	2

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

(Fig. 3 A). However, unusual erythrocytes with two nuclei were found in 4nNC-F_1 (Fig. 3 B). Table 5 showed the results of the measurements of the mean erythrocyte nuclear volume in 2nCOC and 4nNC-F_1. The mean erythrocyte nuclear volume of 4nNC-F_1 was two times larger than that of 2nCOC and was not significantly different ($P > 0.05$) from a 2:1 ratio, indicating that 4nNC-F_1 was tetraploid.

Table 2
The chromosome numbers in 2nBSB, 2nCOC, and 4nNC-F_1-F_4.

Fish type	Distribution of chromosome number						
	No. in metaphase	< 48[a]	48	< 100[a]	100	< 200[a]	200
2nBSB	200	20	180				
2nCOC	200			15	185		
4nNC-F_1	200					28	172
4nNC-F_2	200					22	178
4nNC-F_3	200					27	173
4nNC-F_4	200					25	175

[a] The chromosome number is less than what they should be, owning to the loss of chromosomes in the procedure of chromosome preparation.

3.5. Fertility and size of gametes

Our previous analysis of reproductive traits revealed that 2nCOC, 2nBSB, and 4nCB reached sexual maturity at two years of age, whereas 2nNCRC was sexually mature at one year of age (Liu et al., 2001; Liu et al., 2007; Qin et al., 2014b; Wang et al., 2017a). The ovaries of 22-month-old 4nCB females developed well and contained oocytes in stages II, III, and IV (Fig. 4 A). During the second breeding season, 4nCB females produced a large number of mature eggs. The testes of 22-month-old 4nCB males contained many spermatids that developed into abnormal sperm (Fig. 4 B). The 4nCB male gonads were dysplastic, and

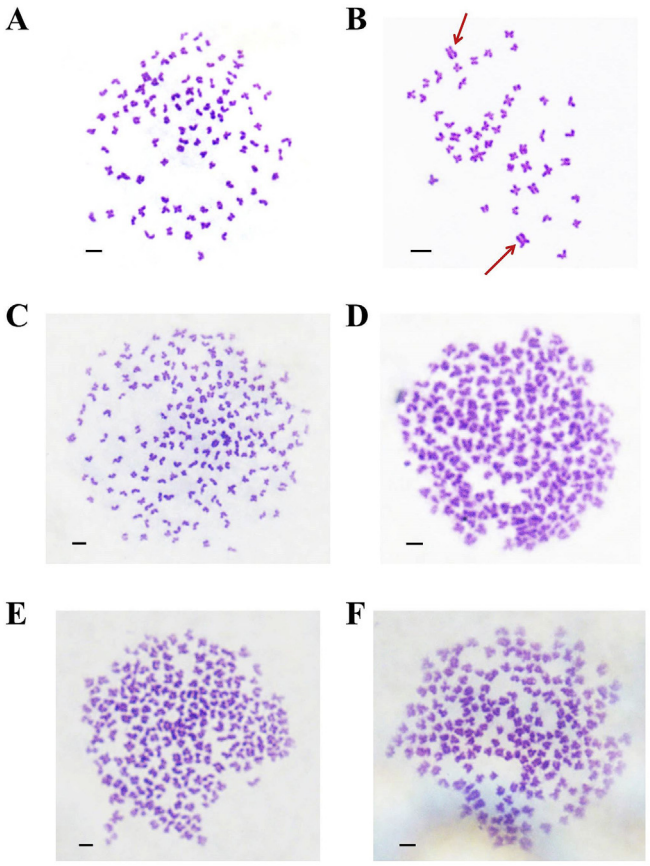

Fig. 2. The chromosome spreads at metaphase in 2nCOC, 2nBSB, and 4nNC-F_1-F_4. (A) The 100 chromosomes of 2nCOC, with no large submetacentric chromosomes. (B) The 48 chromosomes of 2nBSB, with a pair of the largest submetacentric chromosomes indicated (red solid arrows). (C) The 200 chromosomes of 4nNC-F_1, with no large submetacentric chromosomes. (D) The 200 chromosomes of 4nNC-F_2, with no large submetacentric chromosomes. (E) The 200 chromosomes of 4nNC-F_3, with no large submetacentric chromosomes. (F) The 200 chromosomes of 4nNC-F_4, with no large submetacentric chromosomes. Bar = 3 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

only a small amount of water-like semen was extruded during the second breeding season. The 4nCB males and females derived from self-crossing had no surviving progeny. The testes of 10-month-old 2nNCRC males contained many lobules with many mature spermatozoa (Fig. 4 D). During the breeding season, a large amount of white semen could be stripped from the 2nNCRC males. The same-species mating of 2nNCRC resulted in fertilization, hatching, and survival rates equal to 90.2, 85.6, and 75.1%, respectively. Furthermore, we successfully developed a new autotetraploid fish with a high fertilization rate (84.0%), a high hatching rate (72.5%), and a moderately high survival rate (58.2%) by artificial hybridization between female 4nCB and male 2nNCRC. The combination of 4nCB (♀) × 2nNCRC (♂) successfully avoided the problem of fertilization failure due to poor quality of male 4nCB semen. The ovaries of 10-month-old 4nNC-F_3 females developed well and contained oocytes in stages II, III, and IV (Fig. 4 E). The testes of 10-month-old 4nNC-F_3 males contained many lobules with a large number of mature spermatozoa (Fig. 4 F). During the breeding season, a large number of mature eggs and a large amount of white semen were produced by female and male 4nNC, respectively. We artificially established a new autotetraploid fish lineage (F_1-F_4) derived from the hybridization of 4nCB (♀) × 2nNCRC (♂).

The spermatozoa of 10-month-old 4nNC-F_3 and 22-month-old 2nCOC were compared under a SEM. As shown in Fig. 5, the heads and tails of the sperm produced by 2nCOC and 4nNC-F_3 were well

Table 3
Comparison of the countable traits among 2nBSB, 2nCOC, 2nNCRC, 4nCB, and 4nNC-F_1-F_3.

Fish type	No. of lateral scales	No. of upper lateral scales	No. of lower lateral scales	No. of dorsal fins	No. of abdominal fins	No. of anal fins
2nCOC	36.35 ± 1.43(35-38)	5.37 ± 0.45(5–6)	5.30 ± 0.43(5 ~ 6)	III + 17.62 ± 0.89(III + 17 ~ 19)	8.58 ± 0.51 (8 ~ 9)	III + 6.37 ± 0.39(III + 6 ~ 7)
2nBSB	50.60 ± 1.20(49-52)	9.48 ± 0.55(9–10)	10.19 ± 0.97(9 ~ 11)	III + 8.50 ± 0.52(III + 8 ~ 9)	9.18 ± 0.69 (8 ~ 10)	III + 25.90 ± 0.88(III + 25 ~ 27)
2nNCRC	29.35 ± 0.38(29-30)	6.43 ± 0.52(6–7)	7.36 ± 0.56(7 ~ 8)	III + 18.34 ± 1.28(III + 17 ~ 20)	9.08 ± 1.69 (7 ~ 11)	III + 7.66 ± 1.25(III + 6 ~ 9)
4nCB	30.94 ± 0.86(30-32)	6.00 ± 0.00(6)	6.45 ± 0.42(6 ~ 7)	III + 17.94 ± 1.67(III + 16 ~ 20)	8.36 ± 1.29 (7 ~ 10)	III + 7.99 ± 1.67(III + 6 ~ 9)
4nNC-F_1-F_3	32.00 ± 1.00(31-33)	6.35 ± 0.39(6 ~ 7)	6.67 ± 0.46(6 ~ 7)	III + 17.67 ± 1.62(III + 16 ~ 19)	8.00 ± 1.00(7 ~ 9)	III + 7.00 ± 0.00(III + 7)

Note: the countable trait data for 2nCOC, 2nBSB, 2nNCRC, and 4nCB are from previous works (refer to Wang et al., 2017a, *Scientific Reports, 7, 4189*).

Table 4
Comparison of the measurable traits among 2nBSB, 2nCOC, 2nNCRC, 4nCB, and 4nNC-F_1-F_3.

Fish type	BL/WL	BW/BL	HL/BL	HW/HL	TW/TL	HW/BW
2nCOC	0.83 ± 0.07	0.34 ± 0.01	0.24 ± 0.02	0.81 ± 0.07	0.86 ± 0.11	0.60 ± 0.01
2nBSB	0.84 ± 0.04	0.41 ± 0.04	0.20 ± 0.04	0.88 ± 0.03	0.93 ± 0.04	0.49 ± 0.04
2nNCRC	0.84 ± 0.02	0.41 ± 0.02	0.26 ± 0.01	0.88 ± 0.02	0.88 ± 0.03	0.56 ± 0.01
4nCB	0.83 ± 0.02	0.38 ± 0.02	0.27 ± 0.01	0.88 ± 0.02	0.89 ± 0.01	0.62 ± 0.01
4nNC-F_1-F_3	0.85 ± 0.02	0.39 ± 0.01	0.26 ± 0.02	0.85 ± 0.05	0.99 ± 0.10	0.57 ± 0.03

Note: BL/WL (body length to whole length), BW/BL (body width to body length), HL/BL (head length to body length), HW/HL (head width to head length), TW/TL (tail width to tail length), HW/BW (head width to body width); the measurable trait data for 2nCOC, 2nBSB, 2nNCRC, and 4nCB are from previous works (refer to Wang et al., 2017a, *Scientific Reports, 7, 4189*).

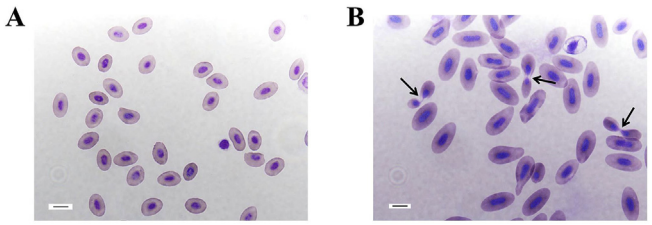

Fig. 3. Erythrocytes of 2nCOC and 4nNC-F_1. (A) Normal erythrocytes with one nucleus in 2nCOC. (B) Normal erythrocytes with one nucleus and unusual erythrocytes with two nuclei (arrows) in 4nNC-F_1. Bar in A–B, 10 μm.

developed. Moreover, the size of the head of 4nNC-F_3 sperm (Fig. 5 B, 5 D) was larger than that of 2nCOC sperm (Fig. 5 A, 5 C). The mean diameter of 2nCOC haploid sperm was 1.97 μm, whereas the mean diameter of 4nNC-F_3 sperm was 2.54 μm. The volume of spermatozoa of 4nNC-F_3 (8.58 μm^3) was two times larger than that of 2nCOC (4.00 μm^3) and was not significantly different ($P > 0.05$) from a 2:1 ratio, indicating that 4nNC-F_3 produced diploid spermatozoa.

The 4nNC-F_3 males and females exhibited normal gonadal development (Figs. 4 and 5) and produced mature sperm and eggs, which fused to form 4nNC-F_4. Currently, 4nNC is a stable lineage with stable inheritance from one generation to the next (F_1-F_4).

3.6. Genetic variation analysis of 5S rDNA sequences

In higher eukaryotes, 5S rDNA has traditionally been used as a genomic DNA marker for tracing evolutionary events (Pinhal et al., 2008; Ubeda-Manzanaro et al., 2010); thus, we analysed the 5S rDNA in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3. In our previous study (Wang et al., 2017a), one monomeric 5S rDNA class (designated class I: 203 bp or 406 bp, where the 406-bp DNA fragments were dimeric 5S rDNA tandem arrays consisting of two class I 203-bp sequences) was detected in 2nCOC. Two monomeric 5S rDNA classes (class II: 188 bp or 376 bp, class II-V_1: 374 bp, where the 376-bp DNA fragments were dimeric 5S rDNA tandem arrays consisting of two class II 188-bp sequences) were detected in 2nBSB. 2nNCRC contained five monomeric 5S rDNA classes: class I-V_2 (205 bp), class I-V_3 (196 bp), class II-V_1 (374, 398, 406, or 410 bp), class III (339, 340, or 341 bp), and class IV (478, 480, or 493 bp). 4nCB contained seven monomeric 5S rDNA classes: class I (203 or 406 bp), class I-V_1 (203 bp), class I-V_2 (205 bp), class I-V_3 (196 bp), class II-V_1 (386 bp), class III (340 bp), and class IV

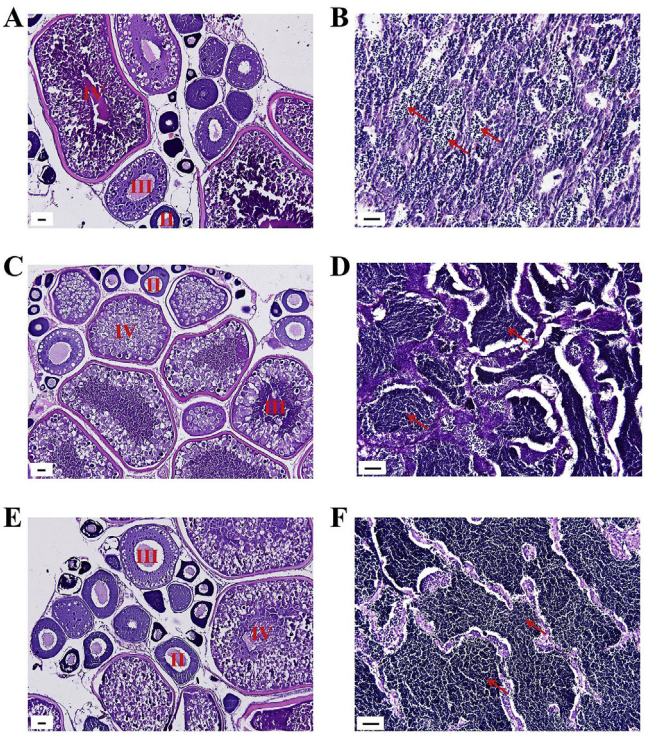

Fig. 4. The gonadal structure of 4nCB, 2nNCRC, and 4nNC-F_3. (A) The ovary of a 22-month-old 4nCB that has developed well and contains oocytes in stages II, III, and IV. (B) The testis of a 22-month-old 4nCB with many spermatids developing into abnormal sperm (red arrow). (C) The ovary of a 10-month-old 2nNCRC that has developed well and contains oocytes in stages II, III, and IV. (D) The testis of a 10-month-old 2nNCRC that contains many lobules in which there are many mature spermatozoa (red arrow). (E) The ovary of a 10-month-old 4nNC-F_3 that has developed well and contains oocytes in stages II, III, and IV. (F) The testis of a 10-month-old 4nNC-F_3 that contains many lobules in which there are many mature spermatozoa (red arrow). Bar in A–F, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(480, 493, or 507 bp). In addition, a portion of the 406-bp DNA fragments in 4nCB underwent recombination (Fig. 6).

In this study, a total of 240 clones were produced, including 80 clones from 4nNC-F_1 (~200-bp band and ~400-bp band), 80 clones

Table 5
Mean erythrocyte nuclear volume measurements for 2nCOC and 4nNC-F_1.

Fish type	Major axis (μm)	Minor axis (μm)	Volume (μm^3)	Volume ratio	
				Observed	Expected
2nCOC	6.37 ± 0.58	4.28 ± 0.54	60.59 ± 13.82		
4nNC-F_1	9.70 ± 1.09	4.78 ± 0.43	114.43 ± 12.77	2.00[a]	2

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

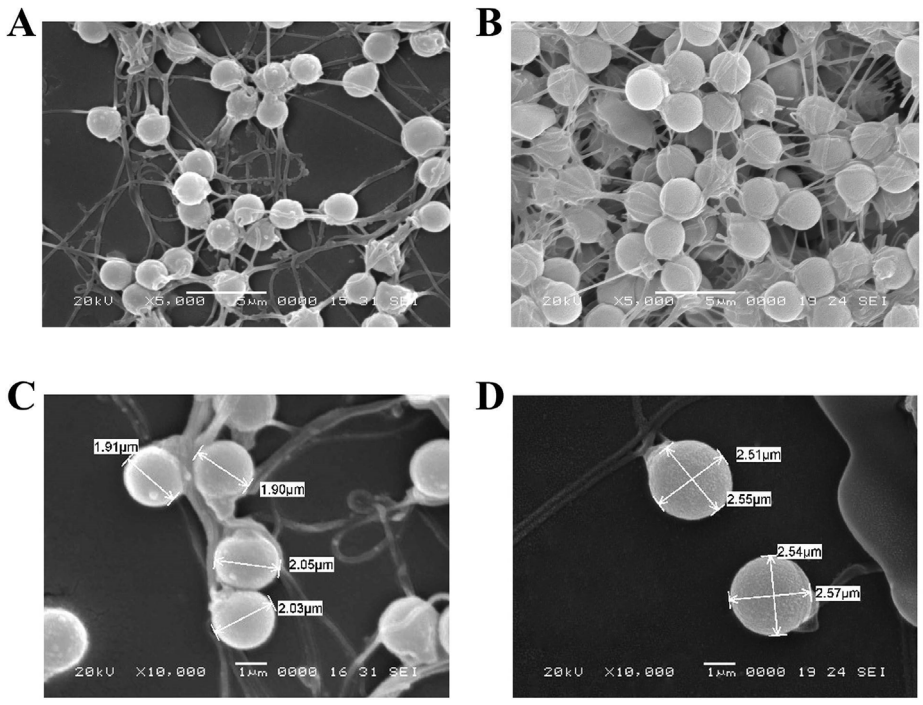

Fig. 5. The spermatozoa of 2nCOC and 4nNC-F_3. (A) The spermatozoa of 2nCOC. Bar = 5 μm. (B) The spermatozoa of 4nNC-F_3. Bar = 5 μm. (C) The spermatozoa of 2nCOC. Bar = 1 μm. (D) The spermatozoa of 4nNC-F_3. Bar = 1 μm.

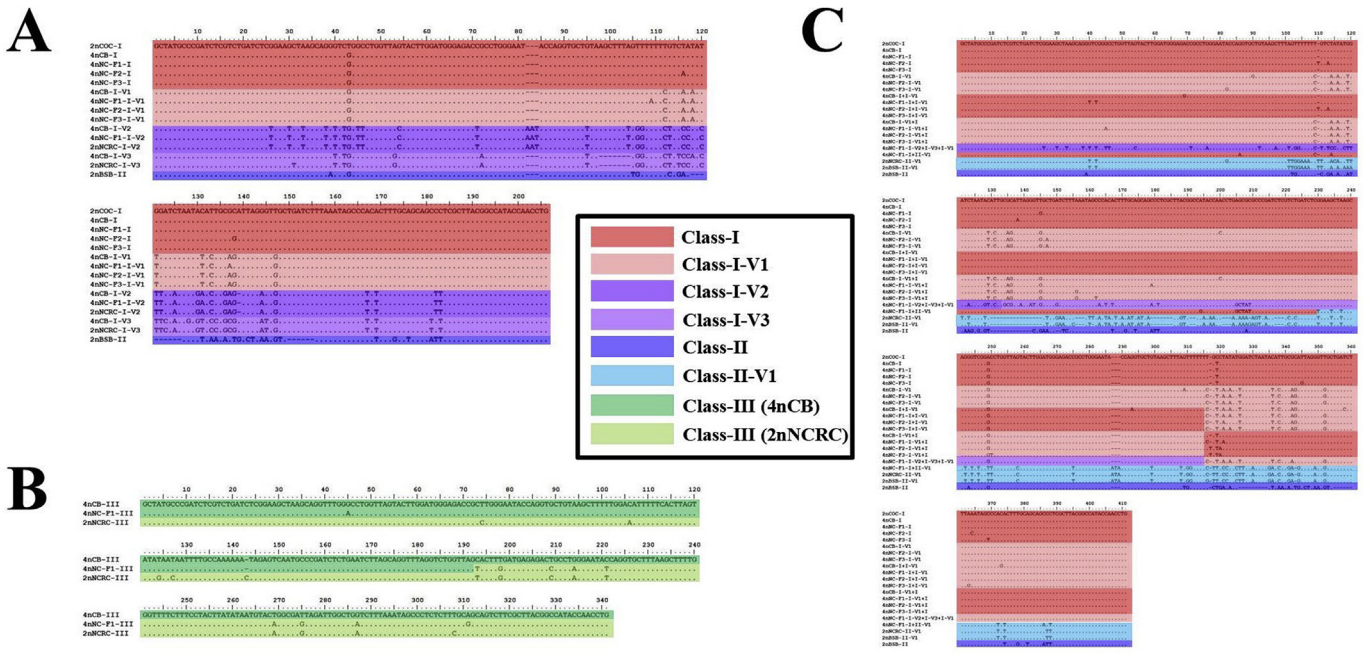

Fig. 6. Nucleotide sequence alignment of sequenced 5S rDNA fragments in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3. (A) Nucleotide sequence alignment of 5S rDNA fragments (~200 bp). (B) Nucleotide sequence alignment of 5S rDNA fragments (~340 bp) among 4nNC-F_1, 4nCB and 2nNCRC. (C) Nucleotide sequence alignment of 5S rDNA fragments (~400 bp). The dots indicate sequence identity, and the hyphens represent insertions/deletions. The 5S rDNA nucleotide sequence for each fragment is marked by a separate colour, as shown in the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

from 4nNC-F_2 (~200-bp band and ~400-bp band), and 80 clones from 4nNC-F_3 (~200-bp band and ~400-bp band). Sequencing analysis indicated that 4nNC-F_1 had three monomeric 5S rDNA classes (Fig. 6): class I (203 or 406 bp), class I-V_1 (203 bp), and class I-V_2 (205 bp). Moreover, 4nNC-F_1 showed five recombinant 5S rDNA classes: class III (340 bp, where the first half was consistent with 4nCB and the last half was consistent with 2nNCRC) (Fig. 6 B), class I + I-V_1 (406 bp, where the first part was consistent with class I and the last part was consistent with class I-V_1) (Fig. 6 C), class I-V_1 + I (406 bp) (Fig. 6 C), class I + II-V_1 (408 bp) (Fig. 6 C), and class I-V_2 + I-V_3 + I-V_1 (406 bp, where the first part was consistent with class I-V_2, the middle part was consistent with class I-V_3, and the last part was consistent with class I-V_1) (Fig. 6 C). In addition to class IV, 4nNC-F_1 inherited all 5S rDNA fragments of the parents, retained the entire fragments of the original parent 2nCOC,

Table 6
PCR amplification bands (non-recombinant bands) in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3.

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxA4a	2nCOC	HoxA4ai	1177	89-500	501-970	971-1177
		HoxA4aii	1182	89-500	501-975	976-1182
	2nBSB	HoxA4a	1188	89-500	501-981	982-1188
	4nCB	HoxA4ai	1177	89-500	501-970	971-1177
		HoxA4aii	1182	89-500	501-975	976-1182
		HoxA4aiii	1184	89-500	501-977	978-1184
		HoxA4a-1	1181	89-500	501-974	975-1181
	2nNCRC	HoxA4ai	1177	89-500	501-970	971-1177
		HoxA4aii	1182	89-500	501-975	976-1182
		HoxA4aiii	1184	89-500	501-977	978-1184
		HoxA4a-1	1181	89-500	501-974	975-1181
	4nNC-F_1	HoxA4aii	1182	89-500	501-975	976-1182
		HoxA4aiii	1184	89-500	501-977	978-1184
		HoxA4a-1	1181	89-500	501-974	975-1181
	4nNC-F_2	HoxA4ai	1177	89-500	501-970	971-1177
		HoxA4aii	1182	89-500	501-975	976-1182
		HoxA4aiii	1184	89-500	501-977	978-1184
		HoxA4a-1	1181	89-500	501-974	975-1181
	4nNC-F_3	HoxA4ai	1177	89-500	501-970	971-1177
		HoxA4aii	1182	89-500	501-975	976-1182
		HoxA4aiii	1184	89-500	501-977	978-1184
		HoxA4a-1	1181	89-500	501-974	975-1181
HoxA9a	2nCOC	HoxA9ai	817	1-381	382-620	621-817
		HoxA9aii	891	1-381	382-694	695-891
	2nBSB	HoxA9a	879	1-381	382-682	683-879
	4nCB	HoxA9ai	817	1-381	382-620	621-817
		HoxA9aiii	867	1-381	382-670	671-867
		HoxA9a-1	863	1-381	382-666	667-863
	2nNCRC	HoxA9ai	817	1-381	382-620	621-817
		HoxA9aiii	867	1-381	382-670	671-867
	4nNC-F_1	HoxA9ai	817	1-381	382-620	621-817
		HoxA9aiii	867	1-381	382-670	671-867
		HoxA9a-1	863	1-381	382-666	667-863
	4nNC-F_2	HoxA9ai	817	1-381	382-620	621-817
	4nNC-F_3	HoxA9ai	817	1-381	382-620	621-817
		HoxA9aiii	867	1-381	382-670	671-867
HoxA2b	2nCOC	HoxA2bi	1490	1-314	315-905	906-1490
		HoxA2bii	1475	1-314	315-890	891-1475
	2nBSB	HoxA2b	1479	1-311	312-894	895-1479
	4nCB	HoxA2bi	1493	1-314	315-908	909-1493
		HoxA2biii	1486	1-314	315-901	902-1486
		HoxA2b-1	1448	1-314	315-863	864-1448
		HoxA2b-3	1486	1-314	315-901	902-1486
	2nNCRC	HoxA2bii	1475	1-314	315-890	891-1475
		HoxA2biii	1486	1-314	315-901	902-1486
		HoxA2b-1	1448	1-314	315-863	864-1448
		HoxA2b-2	1450	1-314	315-865	866-1450
		HoxA2b-3	1486	1-314	315-901	902-1486
	4nNC-F_1	HoxA2bi	1493	1-314	315-908	909-1493
		HoxA2bii	1475	1-314	315-890	891-1475
		HoxA2biii	1486	1-314	315-901	902-1486
		HoxA2b-1	1448	1-314	315-863	864-1448
		HoxA2b-2	1450	1-314	315-865	866-1450
		HoxA2b-3	1486	1-314	315-901	902-1486
	4nNC-F_2	HoxA2bi	1493	1-314	315-908	909-1493
		HoxA2bii	1475	1-314	315-890	891-1475
		HoxA2biii	1486	1-314	315-901	902-1486
		HoxA2b-1	1447	1-314	315-862	863-1447
	4nNC-F_3	HoxA2bi	1493	1-314	315-908	909-1493
		HoxA2bii	1475	1-314	315-890	891-1475
		HoxA2biii	1486	1-314	315-901	902-1486
		HoxA2b-1	1447	1-314	315-862	863-1447

(*continued on next page*)

Table 6 (*continued*)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxB1a	2nCOC	HoxB1ai[a]	1510	-	-	-
		HoxB1aii	1526	1-462	463-1250	1251-1526
	2nBSB	HoxB1a	1522	1-459	460-1246	1247-1522
	4nCB	HoxB1ai[a]	1510	-	-	-
		HoxB1aiii	1469	1-450	451-1193	1194-1469
	2nNCRC	HoxB1aii	1529	1-462	463-1253	1254-1529
		HoxB1aiii	1469	1-450	451-1193	1194-1469
	4nNC-F_1	HoxB1ai[a]	1510	-	-	-
		HoxB1aiii	1487	1-450	451-1211	1212-1487
	4nNC-F_2	HoxB1ai[a]	1510	-	-	-
		HoxB1aii	1529	1-462	463-1253	1254-1529
		HoxB1aiii	1487	1-450	451-1211	1212-1487
	4nNC-F_3	HoxB1ai[a]	1510	-	-	-
		HoxB1aii	1529	1-462	463-1253	1254-1529
HoxB5b	2nCOC	HoxB5bi	1191	1-561	562-985	986-1191
		HoxB5bii	1190	1-564	565-984	985-1190
	2nBSB	HoxB5b	1227	1-564	565-1021	1022-1227
	4nCB	HoxB5bi	1190	1-561	562-984	985-1190
		HoxB5biii	1196	1-561	562-990	991-1196
	2nNCRC	HoxB5bi	1190	1-561	562-984	985-1190
		HoxB5biii	1196	1-561	562-990	991-1196
		HoxB5b-1	1186	1-564	565-980	981-1186
		HoxB5b-2	1187	1-564	565-981	982-1187
	4nNC-F_1	HoxB5bi	1190	1-561	562-984	985-1190
		HoxB5biii	1196	1-561	562-990	991-1196
	4nNC-F_2	HoxB5bi	1190	1-561	562-984	985-1190
		HoxB5biii	1195	1-561	562-989	990-1195
	4nNC-F_3	HoxB5bi	1190	1-561	562-984	985-1190
		HoxB5biii	1195	1-561	562-989	990-1195
HoxB6b	2nCOC	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	671	1-170	171-532	533-671
	2nBSB	HoxB6b	770	1-170	171-631	632-770
	4nCB	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	673	1-170	171-534	535-673
		HoxB6biii	807	1-170	171-668	669-807
		HoxB6b-1	683	1-170	171-544	545-683
		HoxB6b-3	819	1-170	171-680	681-819
	2nNCRC	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	673	1-170	171-534	535-673
		HoxB6biii	807	1-170	171-668	669-807
		HoxB6b-2	683	1-170	171-544	545-683
		HoxB6b-3	819	1-170	171-680	681-819
	4nNC-F_1	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	673	1-170	171-534	535-673
		HoxB6biii	807	1-170	171-668	669-807
		HoxB6b-3	819	1-170	171-680	681-819
	4nNC-F_2	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	673	1-170	171-534	535-673
		HoxB6biii	807	1-170	171-668	669-807
	4nNC-F_3	HoxB6bi	812	1-170	171-673	674-812
		HoxB6bii	673	1-170	171-534	535-673
		HoxB6biii	807	1-170	171-668	669-807
		HoxB6b-3	819	1-170	171-680	681-819
HoxC4a	2nCOC	HoxC4ai	1169	1-410	411-928	929-1169
		HoxC4aii	1176	1-410	411-935	936-1176
	2nBSB	HoxC4a	1125	1-410	411-933	934-1125
	4nCB	HoxC4ai	1169	1-410	411-928	929-1169
		HoxC4aiii	1173	1-410	411-932	933-1173
		HoxC4a-1	1179	1-410	411-938	939-1179
	2nNCRC	HoxC4ai	1169	1-410	411-928	929-1169
		HoxC4aii	1176	1-410	411-935	936-1176
		HoxC4aiii	1173	1-410	411-932	933-1173
		HoxC4a-1	1179	1-410	411-938	939-1179
	4nNC-F_1	HoxC4aiii	1173	1-410	411-932	933-1173
		HoxC4a-1	1179	1-410	411-938	939-1179
	4nNC-F_2	HoxC4ai	1169	1-410	411-928	929-1169
		HoxC4aii	1176	1-410	411-935	936-1176
		HoxC4aiii	1173	1-410	411-932	933-1173
	4nNC-F_3	HoxC4ai	1169	1-410	411-928	929-1169
		HoxC4aii	1176	1-410	411-935	936-1176
		HoxC4aiii	1173	1-410	411-932	933-1173
		HoxC4a-1	1179	1-410	411-938	939-1179

(*continued on next page*)

Table 6 (*continued*)

Genes	Species	Locus	Size (bp)	Exon 1 (bp)	Intron (bp)	Exon 2 (bp)
HoxC6b	2nCOC	HoxC6bi	942	2-392	393-763	764-942
	2nBSB	HoxC6b	922	2-392	393-737	738-922
	4nCB	HoxC6bi	943	2-392	393-764	765-943
		HoxC6bii	958	2-392	393-779	780-958
		HoxC6biii	958	2-392	393-779	780-958
	2nNCRC	HoxC6bi	943	2-392	393-764	765-943
		HoxC6bii	957	2-392	393-778	779-957
		HoxC6biii	958	2-392	393-779	780-958
	4nNC-F_1	HoxC6bi	943	2-392	393-764	765-943
		HoxC6bii	957	2-392	393-778	779-957
	4nNC-F_2	HoxC6bi	943	2-392	393-764	765-943
		HoxC6bii	957	2-392	393-778	779-957
	4nNC-F_3	HoxC6bi	943	2-392	393-764	765-943
		HoxC6bii	957	2-392	393-778	779-957
HoxD4a	2nCOC	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aii	944	1-315	316-719	720-944
	2nBSB	HoxD4a	911	1-306	307-686	687-911
	4nCB	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aii	944	1-315	316-719	720-944
		HoxD4aiii	952	1-315	316-727	728-952
		HoxD4a-1	954	1-315	316-729	730-954
		HoxD4a-2	960	1-315	316-735	736-960
	2nNCRC	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aii	944	1-315	316-719	720-944
		HoxD4aiii	952	1-315	316-727	728-952
		HoxD4a-2	960	1-315	316-735	736-960
	4nNC-F_1	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aiii	952	1-315	316-727	728-952
		HoxD4a-1	954	1-315	316-729	730-954
		HoxD4a-2	960	1-315	316-735	736-960
	4nNC-F_2	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aiii	952	1-315	316-727	728-952
		HoxD4a-2	959	1-315	316-734	735-959
	4nNC-F_3	HoxD4ai	942	1-315	316-717	718-942
		HoxD4aii	944	1-315	316-719	720-944
HoxD10a	2nCOC	HoxD10ai	1551	1-589	590-1321	1322-1551
		HoxD10aii	1546	1-592	593-1316	1317-1546
	2nBSB	HoxD10a-BSB	1574	1-592	593-1344	1345-1574
	4nCB	HoxD10ai	1553	1-589	590-1323	1324-1553
		HoxD10a-1	1481	1-592	593-1251	1252-1481
	2nNCRC	HoxD10ai	1554	1-589	590-1324	1325-1554
		HoxD10aiii	1495	1-592	593-1265	1266-1495
		HoxD10a-1	1484	1-592	593-1254	1255-1484
		HoxD10a-2	1507	1-586	587-1277	1278-1507
	4nNC-F_1	HoxD10ai	1553	1-589	590-1323	1324-1553
		HoxD10aiii	1505	1-592	593-1275	1276-1505
		HoxD10a-1	1480	1-592	593-1250	1251-1480
		HoxD10a-2	1507	1-586	587-1277	1278-1507
	4nNC-F_2	HoxD10ai	1554	1-589	590-1324	1325-1554
		HoxD10a-1	1481	1-592	593-1251	1252-1481
		HoxD10a-BSB	1573	1-592	593-1343	1344-1573
	4nNC-F_3	HoxD10ai	1553	1-589	590-1323	1324-1553
		HoxD10aiii	1500	1-592	593-1270	1271-1500
		HoxD10a-1	1481	1-592	593-1251	1252-1481
		HoxD10a-BSB	1574	1-592	593-1344	1345-1574

[a] Denotes a pseudogene.

and retained partial fragments of the original parent 2nBSB. These results suggested a clear source of hybridization for 4nNC-F_1, clearly reflecting instability in the newly established autotetraploid genomes. 4nNC-F_2-F_3 had two monomeric 5S rDNA classes (Fig. 6): class I (203 or 406 bp) and class I-V_1 (203 or 406 bp). Moreover, 4nNC-F_2-F_3 had two recombinant 5S rDNA classes: class I + I-V_1 (406 or 407 bp) (Fig. 6 C) and class I-V_1 + I (407 bp) (Fig. 6 C). Compared to the 5S rDNA fragments of 4nNC-F_1, those of 4nNC-F_2-F_3 were rapidly purified by purifying selection during genomic DNA replication. 4nNC-F_2-F_3 retained only the entire fragments of the original parent 2nCOC, suggesting that 4nNC is undergoing rapid diploidization.

3.7. Genetic variation analysis of Hox gene sequences

The organization of the *Hox* clusters in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3 was shown in Table 6 and Supplementary Table S1. Fig. 7 and Supplementary Figs. S2–S3 visually depicted the genetic variation in the *Hox* gene clusters of the new autotetraploid fish lineage. Based on the cluster organization of these *Hox* genes, 4nNC-F_1 had greater genetic variation; for example, the *HoxA2b* of 4nNC-F_1 combined all *Hox* gene cluster types (in addition to recombinant clusters) of both parents (Fig. 7 B, Table 6 and Supplementary Table S1), some *Hox* gene (such as *HoxA9a*, *HoxB1a*, and *HoxB5b*) cluster types were completely inherited from the female parent 4nCB (Supplementary Figs. S2–S3, Table 6 and Supplementary Table S1), and some *Hox* gene (such as *HoxD10a*) cluster types, except for the partial

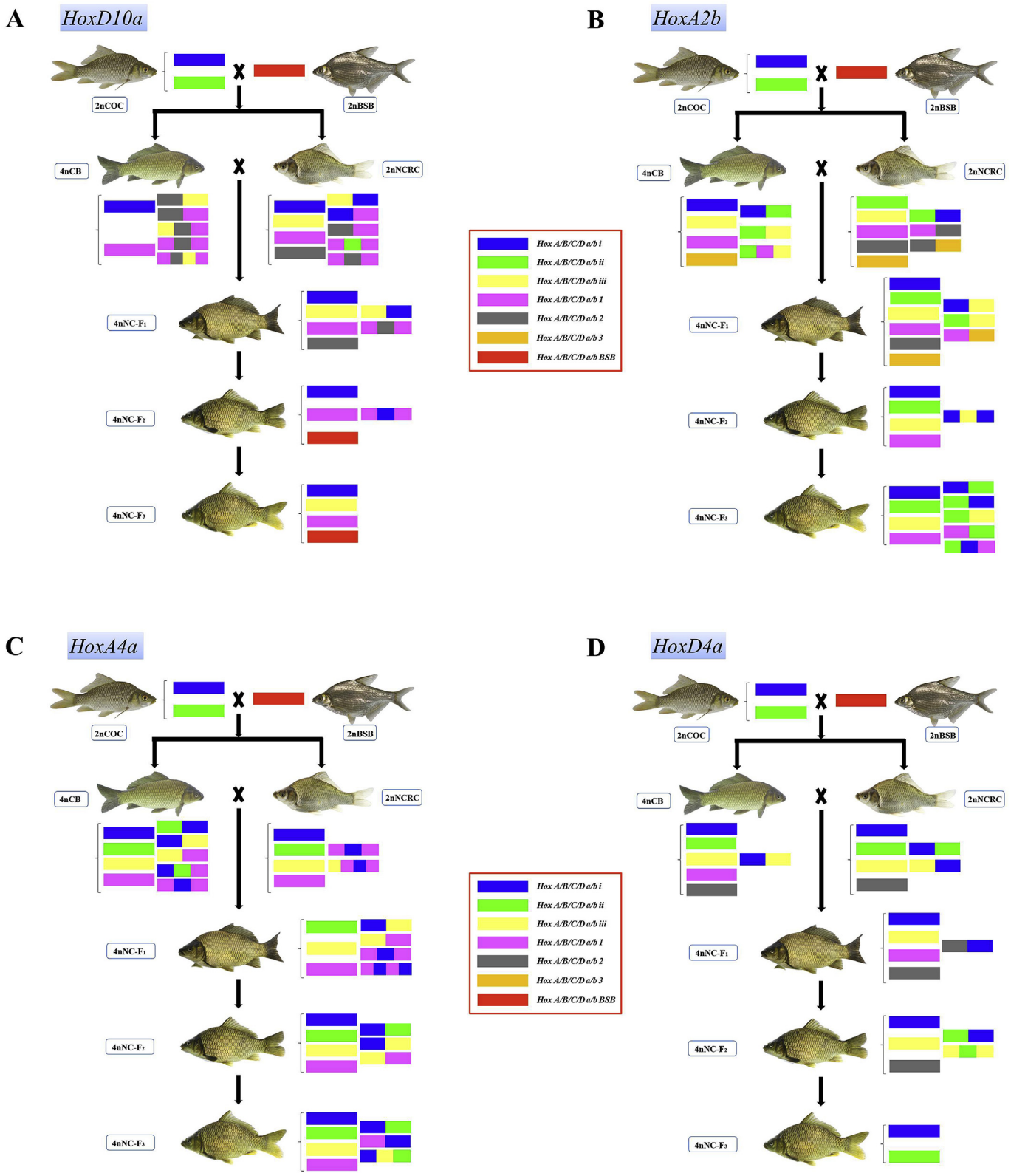

Fig. 7. Variable sequence types (including haplotypes and recombinant clusters) of different *Hox* genes in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3. A: Variable sequence types (including haplotypes and recombinant clusters) of *HoxD10a* in these species. B: Variable sequence types (including haplotypes and recombinant clusters) of *HoxA2b* in these species. C: Variable sequence types (including haplotypes and recombinant clusters) of *HoxA4a* in these species. D: Variable sequence types (including haplotypes and recombinant clusters) of *HoxD4a* in these species.

recombinant clusters, were completely inherited from the male parent 2nNCRC (Fig. 7 A, Table 6 and Supplementary Table S1). Moreover, 4nNC-F_1 presented a richer variety of recombinant clusters, such as *HoxA4a* and *HoxA2b* (Fig. 7, Table 6 and Supplementary Table S1). These results suggested a clear source of hybridization for 4nNC-F_1, clearly reflecting instability in the newly established autotetraploid genomes. Most of the *Hox* gene clusters (including a few recombinant clusters) in 4nNC-F_1 were stably inherited by 4nNC-F_2-F_3. Interestingly, we detected a specific cluster in *HoxD10a* derived from the original paternal parent 2nBSB in 4nNC-F_2-F_3 (Fig. 7 A, Table 6), suggesting that although 4nNC possessed only four sets of 2nCOC-derived chromosomes, the genetic material of its original paternal parent 2nBSB was still preserved. In addition, for these *Hox* genes, the transmission of some *Hox* gene clusters, such as *HoxD4a*, *HoxB5b*, *HoxA9a*, and *HoxC6b*, between successive generations (F_1-F_3) of 4nNC showed rapid diploidization (Fig. 7, Supplementary Figs. S2–S3, Table 6 and Supplementary Table S1). Among these *Hox* gene clusters, we found that all copies of *HoxB1ai* in 2nCOC, 4nCB, and 4nNC-F_1-F_3 were pseudogenes containing a stop codon that prematurely terminates the expression of a full-length functional product (Table 6). Moreover, we also found pseudogenes in the recombinant clusters (Supplementary Table S1), for example, *HoxB1aiii* + *HoxB1ai* in 4nNC-F_1, *HoxB1ai* + *HoxB1aiii* in 4nNC-F_2, *HoxB1ai* + *HoxB1aii* and *HoxB1ai* + *HoxB1aiii* in 4nNC-F_3, *HoxD10a-2* + *HoxD10aiii* in 4nCB, and *HoxD10a-1*+*HoxD10a-2*+*HoxD10a-1* in 4nNC-F_1. These results revealed that the *Hox* gene family in cyprinid fishes has undergone rapid evolution, with some genes gradually becoming pseudogenes.

Additionally, we analysed the percent nucleotide identity and percent amino acid identity between duplicated *Hox* coding regions in 2nCOC, 2nBSB, 4nCB, 2nNCRC, and 4nNC-F_1-F_3 (Additional file 1- Supplementary Table S2). The identities of the orthologous *Hox* genes between 4nNC-F_1-F_3 and 2nCOC were much higher than those between 4nNC-F_1-F_3 and 2nBSB, except for the gene clusters inherited from 2nBSB. The identities of the orthologous *Hox* genes between 4nNC-F_1-F_3 and 4nCB were similar to those between 4nNC-F_1-F_3 and 2nNCRC. For some *Hox* genes, such as *HoxA4a*, *HoxA2b*, *HoxB5b*, and *HoxC4a*, both the nucleotide and amino acid sequences of 4nNC-F_1-F_3 had a high degree of similarity with 2nCOC, 2nBSB, 4nCB, or 2nNCRC. For some *Hox* genes, such as *HoxB6b*, *HoxC6b*, *HoxD4a* and *HoxD10a*, although the nucleotide sequences between 4nNC-F_1-F_3 and 2nCOC, 2nBSB, 4nCB, or 2nNCRC had lower degrees of similarity, they had higher amino acid sequence similarities, which suggested that most mutations were synonymous (Additional file 1- Supplementary Table S2). All of the sequence information in this study is detailed in Additional file 1, Supplementary Table S3.

4. Discussion

Distant hybridization is defined as hybridization between species or higher taxa and is a useful strategy for producing hybrid offspring with altered genotypes and phenotypes or with different ploidy levels (Bullini, 1994; Liu, 2010; Mallet, 2007; Song et al., 2012; Wang et al., 2019). In plants, polyploidization caused by hybridization can result in obvious phenotypic changes as well as alterations of fertility, hybrid vigour, apomixis and flowering time (Chai et al., 2015; Chen, 2007). Moreover, abnormal chromosome behaviour during meiosis has been observed among the polyploid hybrids of many plants, which may lead to the production of gametes with complete paternal or maternal chromosomes, e.g., the intergeneric hybrids *Brassica juncea* × *Orychophragmus violaceus* and *Brassica carinata* × *Orychophragmus violaceus* (Li and Ge, 2007; Li and Heneen, 1999; Li et al., 1998) and the *Avena sativa L.* - *Zea mays L.* addition line (Riera-Lizarazu et al., 2000). Polyploid species are common in plants but rarely successful in animals, where polyploidization is restricted to insects, amphibians, reptiles, and teleost fishes (Bogart, 1980; Mable et al., 2011). Several hypotheses attempt to explain why polyploidy is much rarer in animals than in plants and usually involve a series of disorders in sex determination, physiology, and developmental processes (Mable, 2004; Wertheim et al., 2013) and genome shock or dramatic genomic restructuring (Wertheim et al., 2013). In fish genetic breeding, the establishment of tetraploid lineages is difficult but useful. Tetraploid lineages can be used to produce new excellent triploid fishes with the advantages of sterility and a faster growth rate by crossing tetraploids with diploids (Liu et al., 2001; Qin et al., 2014b). At present, only a few successful cases of tetraploid fish lineages have been reported (Liu et al., 2001; Liu et al., 2016; Liu et al., 2007; Qin et al., 2019; Qin et al., 2014b). In our previous study, we artificially established a genetically stable allotetraploid fish lineage (4n = 200, F_3-F_{28}) derived from red crucian carp (*Carassius auratus* red *var.*, 2n = 100, ♀) × common carp (2n = 100, ♂); this allotetraploid fish lineage had two sets of chromosomes from red crucian carp and two sets of chromosomes from common carp (Liu, 2010; Liu, 2014; Liu et al., 2001; Liu et al., 2016; Wang et al., 2015; Wang et al., 2019). In addition, we obtained allotetraploid hybrids (F_1, 4n = 148) derived from red crucian carp (2n = 100, ♀) × blunt snout bream (2n = 48, ♂). Unexpectedly, abnormal chromosome behaviour during meiosis, but not bivalent pairing, occurred in the allotetraploid hybrids (4n = 148) and led to the production of several types of gametes with different genetic compositions, including allotetraploid (4n = 148, AABB), autotriploid (3n = 150, AAA), autodiploid (2n = 100, AA), and haploid (n = 50, A) gametes (Qin et al., 2014a; Qin et al., 2015b; Qin et al., 2016). Interestingly, due to abnormal chromosome behaviour during meiosis in the allotetraploid hybrids (F_1, 4n = 148), the autotetraploids were successfully produced in the second generation of red crucian carp (♀) × blunt snout bream (♂) by self-crossing of F_1 (4n = 148), and the autotetraploid lineage (F_2-F_{14}, 4n = 200) was formed in succession; this autotetraploid fish lineage had four sets of chromosomes from red crucian carp (Liu et al., 2007; Qin et al., 2015a; Qin et al., 2015b; Qin et al., 2016; Qin et al., 2019; Qin et al., 2014b). However, the hatching rate of the key generations at the beginning of the formation of these two tetraploid lineages was extremely low, and some even had a hatching rate of one in ten thousand (Liu et al., 2001; Qin et al., 2014b). The formation of these tetraploid lineages was extremely difficult, which severely limited their population expansion and application. To date, there was no report on the formation of tetraploid common carp lineages. Because of fast domestication and over-fishing, however, germplasm resources of common carp are under threat of recession and mixture due to artificial breeding (Dong et al., 2015). To alleviate this problem, it is necessary to develop a new tetraploid common carp lineage with a high hatching rate and a high survival rate, which can be used to produce sterile triploid common carp with good traits at a large scale. In the present study, we artificially established an autotetraploid common carp lineage (4n = 200, 4nNC-F_1-F_4) produced by 4nCB (4n = 148, ♀) × 2nNCRC (2n = 100, ♂) derived from the distant hybridization of 2nCOC (2n = 100, ♀) × 2nBSB (2n = 48, ♂). The 4nCB (♀) × 2nNCRC (♂) cross showed high fertilization (84.0%) and hatching (72.5%) rates, and the offspring presented a moderately high survival rate (58.2%). This new autotetraploid fish produced by means of distant hybridization and mating between different-ploidy sister taxa had the advantages of high hatching rate and high survival rate. The females and males of 4nNC-F_1-F_3 reached sexual maturity at one year of age and produced a large number of mature eggs and a large amount of white semen, respectively, which could guarantee their population expansion and application. This autotetraploid common carp lineage had four sets of chromosomes from common carp. We look forward to using this autotetraploid common carp lineage to prepare sterile triploid common carp with good traits at a large scale, which will be of great value in the commercial production of common carp.

At the level of reproductive biology, abnormal chromosome behaviour during meiosis also occurred in female 4nCB (4n = 148), which led to the production of gametes with complete maternal chromosomes. The establishment of 4nNC (4n = 200, AAAA) was achieved by the fertilization of autotriploid eggs (3n = 150, AAA) produced by female

4nCB (4n = 148, AABB) and haploid sperm (n = 50, A) produced by male 2nNCRC (2n = 100, AA) (Fig. 1 H). The formation of autotriploid eggs is associated with the mechanism of genomic doubling by premeiotic endoreduplication, endomitosis, or fusion of the oogonia of female allotetraploid fish (Qin et al., 2014a; Qin et al., 2015b; Qin et al., 2016). The results at the chromosome and DNA content levels indicated that the individuals of 4nNC-F_1-F_4 had 200 chromosomes originating from common carp. The newly established autotetraploid common carp lineage was able to produce normal diploid eggs and diploid spermatozoa, thereby maintaining tetraploidy from one generation to the next (F_1-F_4). At the genetic level, 4nCB and 2nNCRC in the first generation of distant hybridization had very high genetic variation at the genomic DNA level (5S rDNA and *Hox* genes). 5S rDNA is used as a genomic DNA marker to trace recent evolutionary events, which are often species specific, and has successfully served as a marker in evolutionary studies (Campo et al., 2009; Ferreira et al., 2007). As encoded transcription factors, *Hox* genes are generally considered to be recent evolutionary novelties associated either with the emergence of this lineage or with important steps in its evolution (Duboule, 2007). These highly variable 5S rDNA and *Hox* genes provide a good starting point for the study of variation in genomic DNA. Importantly, the vast majority of types of 5S rDNA and *Hox* genes in 4nCB and 2nNCRC were stably inherited by 4nNC-F_1. At the same time, the 5S rDNA and *Hox* gene sequence results revealed that 4nNC-F_1 harboured more genetic variation, including the recombination type, maternal-specific type, and paternal-specific type. These results suggested a clear source of hybridization for 4nNC-F_1, clearly reflecting instability in the newly established autotetraploid genomes. Most of the gene types in 4nNC-F_1 were stably inherited by 4nNC-F_2-F_3. Interestingly, we detected a specific type derived from the original paternal parent 2nBSB in the 5S rDNA and *Hox* genes in 4nNC-F_1-F_3, suggesting that although 4nNC possesses only four sets of 2nCOC-derived chromosomes, the genetic material of its original paternal parent 2nBSB is still preserved. In addition, for these genes, the transmission of DNA variation between successive generations (F_1-F_3) suggests that 4nNC is undergoing rapid diploidization to maintain tetraploid stability. Related research shows that univalent, trivalent, and quadrivalent pairing will inhibit the formation of diploid gametes during meiosis in autotetraploids and allotetraploids, whereas bivalent pairing is considered advantageous for maintaining stability in tetraploids (DENİZ, 2002; Parisod et al., 2010; Paterson et al., 2004). Consequently, successful tetraploidy requires diploid-like behaviour (Comai, 2000; Soltis and Soltis, 2000; Soltis and Soltis, 2009).

We reported for the first time that new autotetraploid fish lineages (F_1-F_4) can be produced by means of distant hybridization and mating between different-ploidy sister taxa, providing tetraploid common carp germplasm resources. Distant hybridization leads to obvious changes in genotypes and phenotypes, giving rise to species with novel capabilities and adaptabilities (Sriswasdi et al., 2019). This manuscript constitutes the first report on the formation of a new autotetraploid common carp with phenotypic and genotypic variation through successive generations of hybridization. The genomic DNA results revealed that the autotetraploid common carp is undergoing rapid diploidization to maintain tetraploid stability. The newly established autotetraploid common carp lineage can be used to produce new excellent triploid fishes with the advantages of sterility and a faster growth rate by crossing tetraploids with diploids (Liu et al., 2001; Qin et al., 2014b). The establishment of the new autotetraploid fish lineage provides new germplasm resources for fish genetic breeding and for studies on species evolution.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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