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Source: Biology of Reproduction, 91(4)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.114.122283

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BIOLOGY OF REPRODUCTION (2014) **91**(4):93, 1–11
Published online before print 27 August 2014.
DOI 10.1095/biolreprod.114.122283

The Autotetraploid Fish Derived from Hybridization of *Carassius auratus red var.* (Female) × *Megalobrama amblycephala* (Male)[1]

Qinbo Qin,[3] Yude Wang,[3] Juan Wang, Jing Dai, Jun Xiao, Fangzhou Hu, Kaikun Luo, Min Tao, Chun Zhang, Yun Liu, and Shaojun Liu[2]

Key Laboratory of Protein Chemistry and Developmental Biology of the State Education Ministry of China, College of Life Sciences, Hunan Normal University, Changsha, People's Republic of China

ABSTRACT

The establishment of the tetraploid organism is difficult but useful in genetics and breeding. In the present study, we have artificially established an autotetraploid fish line (F_2–F_8) derived from the distant hybridization of *Carassius auratus red var.* (RR, 2n = 100) (female) × *Megalobrama amblycephala* (BB, 2n = 48) (male). The autotetraploid line (F_2–F_8) possess four sets of chromosomes from red crucian carp (RRRR, 4n = 200) and produce diploid ova and diploid sperm, which maintains the formation of the autotetraploid line. The F_2 of the autotetraploid fish result from the fertilization of the autodiploidy diploid eggs and diploid sperm from the females and males of F_1 hybrids (RRBB, 4n = 148), which exhibit abnormal chromosome behavior during meiosis as revealed by gynogenesis and backcrossing. This is the first report concerning the establishment of an autotetraploid fish line derived from distant hybridization. The autotetraploid fish line provides an important gamete source for the production of triploids and tetraploids. The autotetraploid fish line also provides an ideal system to investigate the poorly understood mechanisms that drive diploidization in autotetraploids and to study the hybrid progenies' characteristics, including the appearance of new traits that promote a diversity of traits and facilitate adaptation.

allotetraploid, autotetraploid line, chromosome behavior, diploid gamete, distant hybridization

INTRODUCTION

Polyploids arise when a rare mitotic or meiotic catastrophe causes the formation of gametes that have more than one set of chromosomes [1]. There is a basic distinction between autopolyploids and allopolyploids. The autopolyploids (e.g., AAAA), have chromosome sets that come from the genome of one species while the allopolyploids (e.g., AABB) result from the combination of sets of chromosomes from two or more different taxa [2]. The allopolyploids contain two parental genomes that undergo bivalent pairing at meiosis because only the homologous chromosomes pair up [3, 4]. However, several studies have demonstrated that abnormal chromosome behavior, instead of bivalent pairing, is observed during mitosis and meiosis in polyploidy or diploid hybrid progeny of plants [5–8]. For example, complete separation of the parental genomes occurs during mitosis and meiosis in the intergeneric hybrids between *Orychophragmus violaceus* (2n = 24) and three cultivated *Brassica* tetraploids (*B. napus, B. carinata,* and *B. juncea*) [9, 10].

In fishes, tetraploids can be produced using hydrostatic pressure or cold thermal shock [11–14]. However, it is extremely difficult to obtain a fertile autotetraploid population and furthermore establish an autotetraploid line using these methods. Previously, we successfully obtained fertile allotetraploid hybrids (F_1, 4n = 148, RRBB) in the first generation of *Carassius auratus red var.* (RCC) (2n = 100, RR, ♀) × *Megalobrama amblycephala* (BSB) (2n = 48, BB, ♂) [15]. In the current study, the abnormal chromosome behavior during meiosis of F_1 hybrids leads to the formation of autodiploid sperm and autodiploid ova that fertilize each other and finally result in the formation of autotetraploids in F_2. Importantly, the females and males of the autotetraploids, including the F_2 and the following generations, are able to produce, respectively, diploid eggs and diploid spermatozoa and they can be fertilized to form the next generation of the autotetraploid fish. Until now, the F_3–F_8 of the autotetraploid population is formed in succession. This is the first report of the production of autotetraploid fish by successive generations of hybridization. The autotetraploid fish line is useful in both genetics and breeding. In genetics, the autotetraploid fish line provides an ideal system that can potentially lead to the creation of new traits, facilitating adaptation and promoting a diversity of traits. The autotetraploid fish line also build a good platform to facilitate studies of the poorly understood mechanisms that drive diploidization in autotetraploids, which potentially would provide insight into the genes involved in stabilization of meiosis. In breeding, the autotetraploid fish line can be used to produce triploids by crossing the tetraploids with diploids. Triploids potentially have the advantage of sterility and faster growth rate [16].

[1]This research was supported by National Natural Science Foundation of China (Grant Nos. 31201987, 31430088, and 31210103918), Doctoral Fund of Ministry of Education of China (Grant No. 20124306120006), Natural Science Foundation of Hunan Province (Grant No. 14JJ6008), Educational Commission of Hunan Province (Grant No. 12B084), Training Program of the Major Research Plan of the National Natural Science Foundation of China (Grant No. 91331105), the National Key Basic Research Program of China (Grant No. 2012CB722305), the National High Technology Research and Development Program of China (Grant No. 2011AA100403), Cooperative innovation center of engineering and new products for developmental biology (20134486), and the construct program of the key discipline in Hunan province and China.
[2]Correspondence: Shaojun Liu, Key Laboratory of Protein Chemistry and Developmental Biology of the State Education Ministry of China, College of Life Science, Hunan Normal University, Changsha 410081, P.R. China. E-mail: lsj@hunnu.edu.cn.
[3]These authors contributed equally to this work.

Received: 13 June 2014.
First decision: 14 July 2014.
Accepted: 18 August 2014.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

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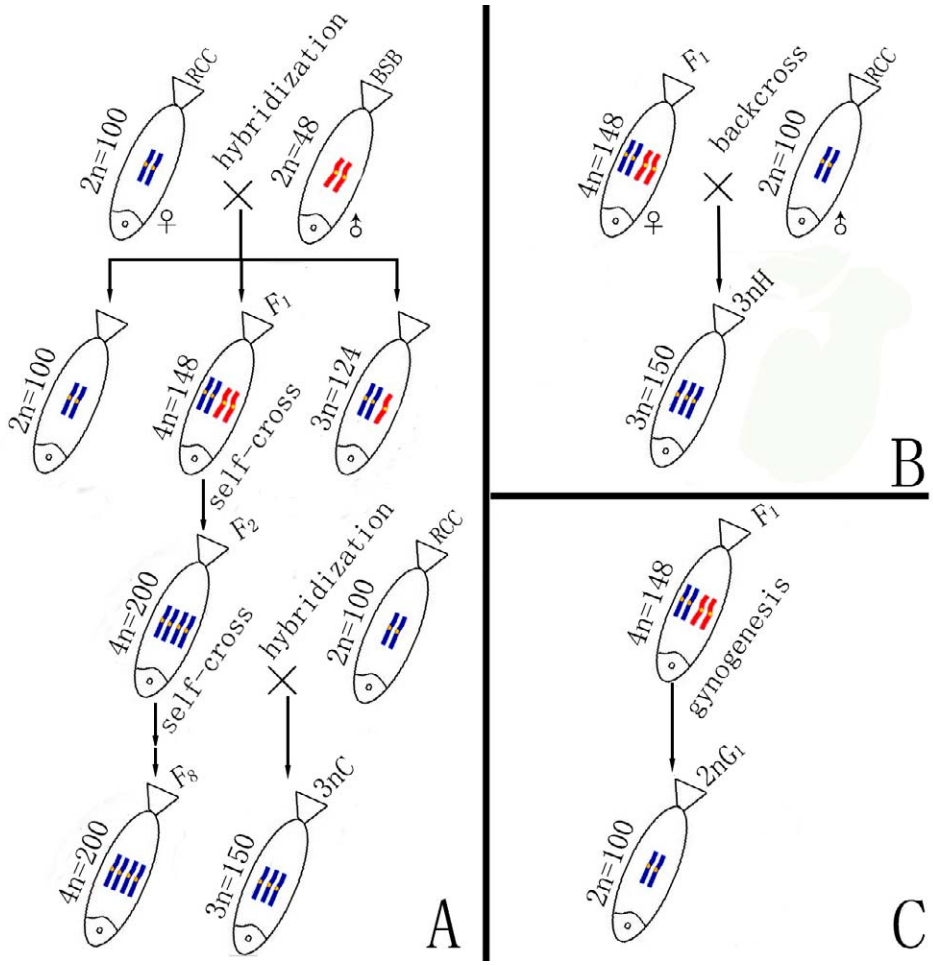

FIG. 1. Crossing procedure and formation of the different ploidy hybrids. The chromosomes of *Carassius auratus red var.* (RCC) and *Megalobrama amblycephala* (BSB) are marked by the blue and red color, respectively. **A**) In the first generation of *Carassius auratus red var.* (RCC) ($2n = 100$, ♀) × *Megalobrama amblycephala* (BSB) ($2n = 48$, BB, ♂), the allotetraploid fish (F_1, $4n = 148$), natural autodiploid gynogenetic fish ($2n = 100$), and allotriploid fish ($3n = 124$) are produced. The autotetraploid fish ($4n = 200$) is produced in the second generation of RCC (♀) × BSB (♂) by self-crossing of F_1, and the autotetraploid line (F_2–F_8, $4n = 200$) is formed in succession. The autotriploid (3nC, $3n = 150$) is obtained in the cross of RCC (♀) × F_2 (♂). **B**) The autotriploid (3nH, $3n = 150$) is formed in the backcross of F_1 (♀) × RCC (♂). **C**) The autodiploid gynogenetic fish ($2nG_1$, $2n = 100$) is produced by artificial gynogenesis from the eggs of the F_1 that are activated with UV-treated sterilized sperm of BSB without treatment for doubling the chromosomes.

MATERIALS AND METHODS

Ethics

All the samples were cultured in ponds at the Protection Station of Polyploidy Fish, Hunan Normal University, and fed with artificial feed. Fish treatments were carried out according to the Care and Use of Agricultural Animals in Agricultural Research and Teaching, approved by the Science and Technology Bureau of China. Approval from the Department of Wildlife Administration is not required for the experiments conducted in this paper. Fish was deeply anesthetized with 100 mg/L MS-222 (Sigma-Aldrich) before dissection.

Animals and Crosses

During the reproductive seasons (April to June) in 2004–2006, the first generation (F_1, RRBB, $4n = 148$)) of *Carassius auratus red var*. (RCC) ($2n = 100$, ♀) × *Megalobrama amblycephala* (BSB) ($2n = 48$, BB, ♂) was produced. During the reproductive seasons (April to June) of 2006 and 2007, the second generation (F_2) of RCC (♀) × BSB (♂) was produced by self-crossing of F_1. Gynogenetic offspring ($2nG_1$) was obtained by artificial gynogenesis from the eggs of the F_1 that were activated with ultraviolet (UV)-treated sterilized sperm of BSB without treatment for doubling the chromosomes. During the reproductive season of 2008, the third generation (F_3) of RCC (♀) × BSB (♂) was produced by self-crossing of F_2. The backcross progenies (3nH) of F_1 (♀) × RCC (♂) were produced, and the filial generation (3nC) of RCC (♀) × F_2 (♂) was obtained. During the reproductive seasons (April to June) of 2009–2013, the eight generation (F_8) was obtained, and the F_2–F_8 autotetraploid fish were formed in succession.

Measurement of DNA Content

To measure the DNA content of erythrocytes of RCC, BSB, $2nG_1$, 3nH, F_1, and F_2, 1–2 ml of red blood cells was collected from the caudal vein of the above fish into syringes containing ~200–400 units of sodium heparin. The blood samples were treated with the nuclei extraction and 4′,6-diamidino-2-phenylindole DNA-staining solution, cystain DNA 1 step (Partec). Then all the samples were filtered. A flow cytometer (cell counter analyzer; Partec) was used to measure the DNA content. Under the same conditions, the DNA content of each sample was measured. To calculate the probabilities of the ratios of the DNA content of the polyploid hybrids to the sum of RCC and BSB, the chi-square test with Yates correction was used for testing deviation from the expected ratio values.

Preparation of Chromosome Spreads

To determine ploidy, chromosome preparation was carried out on the kidney tissues of RCC, BSB, $2nG_1$, 3nH, F_1, and F_2–F_8 at 1 yr of age, according to the procedures reported by Liu et al. [16]. For each type of fish, 200 metaphase spreads (20 metaphase spreads in each sample) of chromosomes were analyzed. Preparations were examined under an oil lens at a magnification of 3330×. Good-quality metaphase spreads were photographed and used for analysis of karyotypes. Lengths of entire chromosomes, long and short arms,

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TABLE 1. Mean DNA content in BSB, RCC, $2nG_1$, 3nH, F_1, and F_2.

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BSB	78.65		
RCC	106.97		
$2nG_1$	107.48	$2nG_1$/ RCC = 1.00[a]	1
F_1	177.71	F_1/(RCC+BSB) = 0.96[a]	1
F_2	210.24	F_2/2RCC = 0.98[a]	1
3nH	150.26	3nH/1.5RCC = 0.93[a]	1

[a] The observed ratio was not significantly different ($P > 0.01$) from the expected ratio.

were measured. Chromosomes were classified on the basis of their long-arm to short-arm ratios according to the reported standards [17]. Values of 1.0–1.7 were classified as metacentric (m), 1.7–3.0 as submetacentric (sm), 3.1–7.0 as subtelocentric (st), and 7.1 as telocentric (t) chromosomes.

Spermatozoa Phenotype and Gonadal Structure

The semen of RCC and F_2 was collected with a clean sucker and transferred into a 2.5% glutaraldehyde solution. The semen was centrifuged at 2000 rpm for 1 min, fixed in 4% glutaraldehyde solution overnight, and then fixed in a 1% osmic acid solution for 2 h. The spermatozoa were dehydrated in alcohol, dropped onto slides, and desiccated. Finally, they were subjected to atomized gilding and were observed with an X-650 (Hitachi) SEM scanning electron microscope.

The gonads of F_2 were fixed in Bouin solution for preparation of tissue sections. The paraffin-embedded sections were cut and stained with hematoxylin and eosin. Gonadal structure was observed by a light microscope and photographed with a Pixera Pro 600ES.

Morphological Traits

At 1 yr of age, 20 RCC, 20 BSB, 20 $2nG_1$, 20 3nH, 20 F_1, and 140 F_2–F_8 were morphologically examined. Measurable traits, including whole length, body length and width, head length and width, and tail length and width, were observed. The average ratios of whole length to body length, body length to body width, body length to head length, head length to head width, tail length to tail width, and body width to head width were calculated for each group. Countable traits, including the number of dorsal fins, abdominal fins, anal fins, lateral scales, and upper and lower lateral scales, were also observed.

Fluorescence In Situ Hybridization

The probes for fluorescence in situ hybridization (FISH) for the 5S gene were constructed for RCC and amplified by PCR using the primers 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5′-CAGGTTGGTATG GCCGTAAGC-3′ [18]. The FISH probes were produced by Dig-11-dUTP labeling (using a nick translation kit; Roche) of purified PCR products. FISH was performed according to the method described by He et al. [19]. For each type of fish, 200 metaphase spreads (20 metaphase spreads in each sample) of chromosomes were analyzed.

RESULTS

Formation of Experimental Fish

The allotetraploid hybrids (F_1, 4n = 148) were obtained in the first generation of RCC (♀) × BSB (♂). Subsequently, the autotetraploids were produced in the second generation of RCC (♀) × BSB (♂) by self-crossing of F_1 and the autotetraploid line (F_2–F_8, 4n = 200) was formed in succession (Fig. 1A). The autotriploids (3nC, 3n = 150) were obtained in the cross of RCC (♀) × F_2 (♂) (Fig. 1A), and the autotriploids (3nH, 3n = 150) were formed in the backcross of F_1 (♀) × RCC (♂) (Fig. 1B). The autodiploid gynogenetic progenies ($2nG_1$, 2n = 100) were produced by artificial gynogenesis from the eggs of the F_1 that were activated with UV-treated sterilized sperm of BSB without treatment for doubling the chromosomes (Fig. 1C). The method of gynogenesis helped us to clarify the ploidy of the eggs because the diploid eggs were able to develop into living fish while the haploid eggs were not able to develop into the living fish. The formation of $2nG_1$ suggested that F_1 hybrids produced the diploid eggs. The establishment of the fish line from F_2 to F_8 indicated that both males and females in F_2–F_7 were fertile.

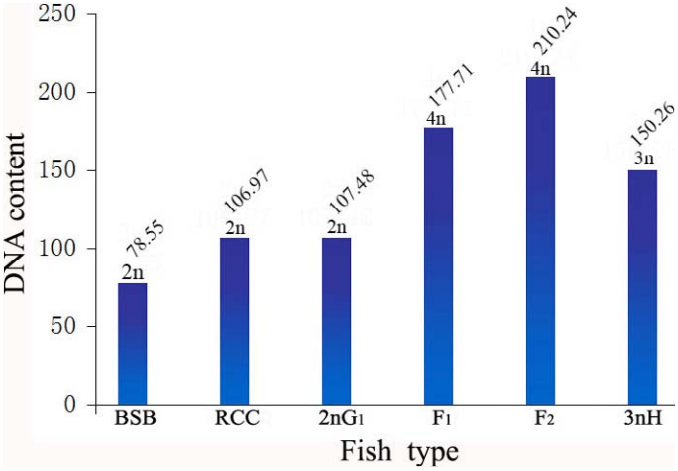

FIG. 2. Cytometric histograms of DNA fluorescence for BSB, RCC, $2nG_1$, 3nH, F_1, and F_2. The mean DNA content of diploid BSB (2n) is 78.65. The mean DNA content of diploid RCC (2n) is 106.97. The mean DNA content of $2nG_1$ is 107.48, which is equal to that of RCC ($P > 0.01$), suggesting that it has two sets of RCC-derived chromosomes (2n). The mean DNA content of F_1 is 177.71, which is equal to the sum of that of RCC and BSB ($P > 0.01$), suggesting that it has two sets of RCC-derived chromosomes and two sets of BSB-derived chromosomes (2n). The mean DNA content of F_2 is 210.24, which is equal to double the RCC content ($P > 0.01$), suggesting that F_2 has four sets of RCC-derived chromosomes (4n). The mean DNA content of 3nH is 150.26, which is equal to the sum of 1.5 × RCC ($P > 0.01$), suggesting that 3nH has three sets of RCC-derived chromosomes (3n).

Measurement of DNA Content

We used the sum of the DNA content of RCC and BSB as the controls. The distribution of DNA content of all the samples is illustrated in Table 1 and Fig. 2. The mean DNA content of $2nG_1$ hybrids was equal to that of RCC ($P > 0.01$), suggesting that $2nG_1$ had two sets of RCC-derived chromosomes (Fig. 2 and Table 1). The mean DNA content of F_1 hybrids was equal to the sum of that of RCC and BSB ($P > 0.01$), suggesting that F_1 had two sets of RCC-derived chromosomes and two sets of BSB-derived chromosomes (Fig. 2 and Table 1). The DNA content of F_2 was equal to double the RCC content ($P > 0.01$), suggesting that F_2 had four sets of RCC-derived chromosomes (Fig. 2 and Table 1). The mean DNA content of 3nH was equal to the sum of 1.5×RCC ($P > 0.01$), suggesting that 3nH had three sets of RCC-derived chromosomes (Fig. 2 and Table 1). The value of the DNA content was in direct proportion to the degree of chromosomal ploidy, that is, a high chromosomal ploidy was associated with increased DNA content. The above results indicate that compared with RCC and BSB, F_1 is an allotetraploid hybrid while F_2 is an autotetraploid fish.

Examination of Chromosome Number and Formation of Karyotype

Table 2 illustrates the distribution of chromosome numbers in RCC, BSB, $2nG_1$, 3nH, 3nC, F_1, and F_2–F_8. Among the RCC samples, 90% of the chromosomal metaphases had 100

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TABLE 2. Examination of chromosome number in RCC, BSB, $2nG_1$, 3nH, F_1, F_2–F_8, and 3nC.

		Distribution of chromosome number											
Fish type	No. of metaphase	<48	48	<100	100	<148	148	<150	150	<198	198	<200	200
RCC	200			20	180								
BSB	200	25	175										
$2nG_1$	200			30	170								
3nH	200							28	172				
F_1	200					34	166						
F_2–F_8	1400											236	1164
3nC	200							31	169				

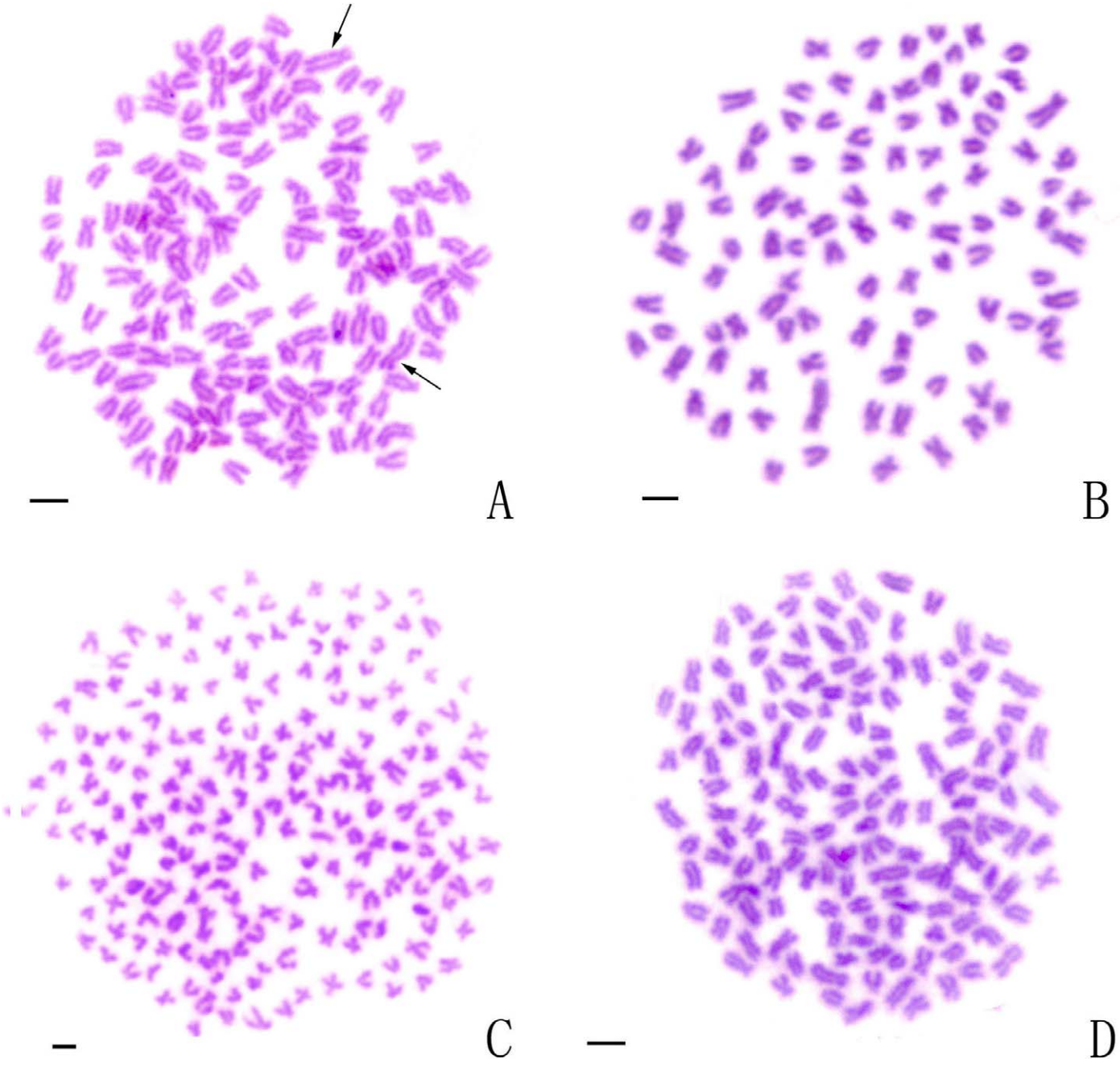

FIG. 3. Chromosome spreads at metaphase in F_1, $2nG_1$, F_2–F_8, and 3nH. **A**) The 148 chromosomes of F_1 in which a pair of the largest submetacentric chromosomes (arrows) is indicated. Bar = 3 μm. **B**) The 100 chromosomes of $2nG_1$ in which the largest submetacentric chromosome is not found. Bar = 3 μm. **C**) The 200 chromosomes of F_2–F_8 in which the largest submetacentric chromosome is not found. Bar = 3 μm. **D**) The 150 chromosomes of 3nH in which the largest submetacentric chromosomes is not found. Bar = 3 μm.

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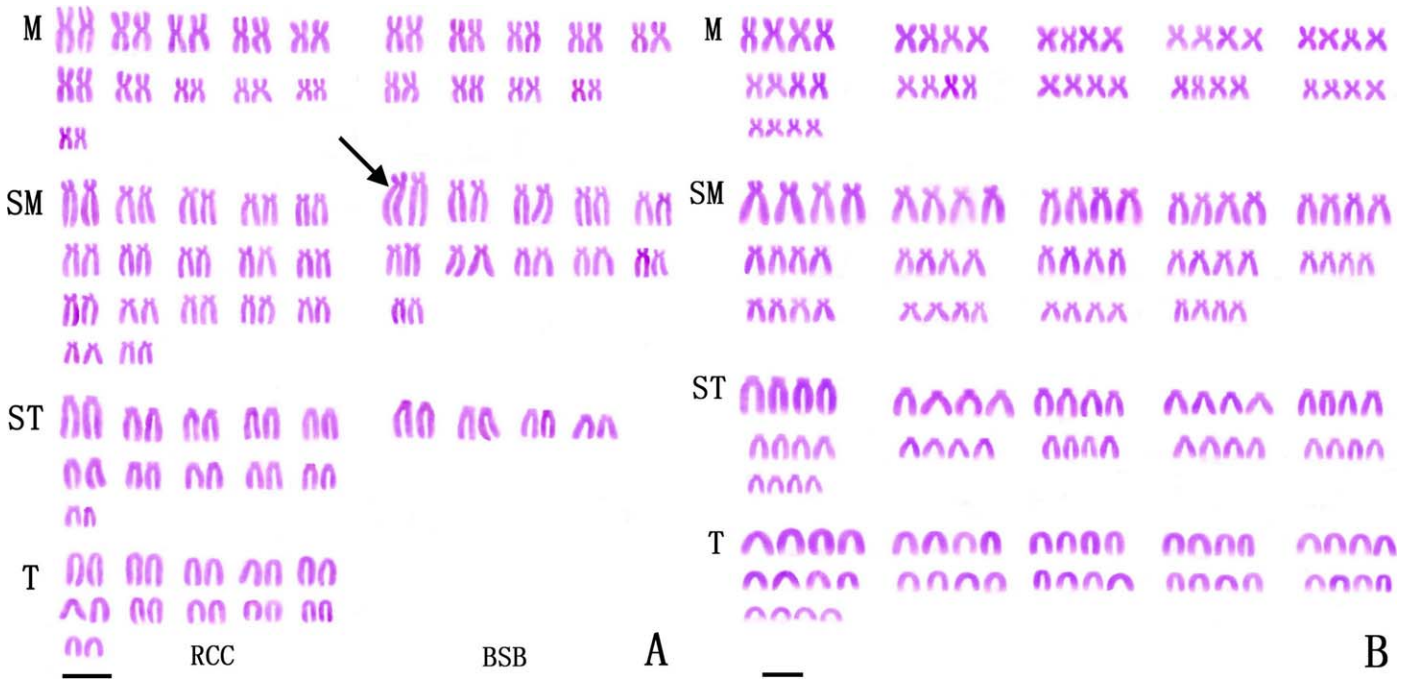

FIG. 4. Karyotypes of F_1 and F_2–F_8. **A**) The karyotype of F_1 is 40m+56sm+30st+22t, which consists of two sets of chromosomes from RCC and two sets of chromosomes from BSB. The arrows indicate a pair of the largest submetacentric chromosomes. Bar = 3 μm. **B**) The karyotype of F_2–F_8 is 44m+68sm+44st+44t, which consists of four sets of chromosomes from RCC. Bar = 3 μm.

chromosomes (Table 2), indicating that they were diploids with 100 chromosomes with a karyotype of 22m+34sm+22st+22t, the same as that described in our previous study [16]. Among the BSB samples, 87.5% of the chromosomal metaphases possessed 48 chromosomes (Table 2), indicating that they were diploids with 48 chromosomes with a karyotype of 18m+22sm+8st [15]. A large pair of submetacentric chromosomes was observed in BSB, which was used as a chromosomal marker to identify this species. Among the chromosomes of RCC, there was no evidence for a special larger submetacentric chromosome. Among the F_1 samples, 83% of the chromosomal metaphases had 148 chromosomes with a karyotype of 40m+56sm+30st+22t in which the pair of the largest submetacentric chromosomes from BSB were observed, indicating that they were a tetraploid possessing two sets of BSB-derived chromosomes and two sets of RCC-derived chromosomes (Figs. 3A and 4A, and Table 2). Among the $2nG_1$ samples, 85% of the chromosomal metaphases had 100 chromosomes with a karyotype of 22m+34sm+22st+22t in which none of the large submetacentric chromosomes from BSB was observed, indicating that they were diploids and possessed of two sets of chromosomes from RCC (Fig. 3B and Table 2). Among the F_2–F_8 samples, 83.1% of the chromosomal metaphases had 200 chromosomes with a karyotype of 44m+68sm+44st+44t in which the large submetacentric chromosomes from BSB were absent, indicating that they were tetraploid with four sets of RCC-derived chromosomes (Figs. 3C and 4B, and Table 2). Among the 3nH samples, 86% of the chromosomal metaphases had 150 chromosomes with a karyotype of 33m+51sm+33st+33t in which the large submetacentric chromosome from BSB was absent, indicating that they were triploid and possessed three sets of RCC-derived chromosomes (Fig. 3D and Table 2). Among the 3nC samples, 84.5% of the chromosomal metaphases had 150 chromosomes (Table 2), the same karyotype as 3nH. Examining the chromosomal spreads could directly identify the chromosomal number. The results provided direct evidence to prove that F_1 hybrids were allotetraploid hybrids with 148 chromosomes derived from RCC and BSB and F_2–F_8 were autotetraploids with 200 chromosomes derived from RCC.

Fluorescence In Situ Hybridization

The 5S gene probe (GenBank Accession No. GQ485557) was hybridized to the metaphase chromosomes of RCC, BSB, F_1, F_2–F_8, $2nG_1$, and 3nH, and the results of FISH are shown in Table 3. Hybridization of the probe yielded eight 5S gene loci in 93% of the chromosomal metaphases of RCC (Fig. 5A and

TABLE 3. Examination of hybridizing signals by FISH in RCC, BSB, $2nG_1$, 3nH, F_1, and F_2–F_8.

			Distribution of chromosome loci number					
Fish type	No. of Fish	No. of metaphase	<8	8	<12	12	<16	16
RCC	10	200	14	186				
BSB	10	200	0	0				
$2nG_1$	10	200	16	184				
3nH	10	200			23	177		
F_1	10	200	22	178				
F_2–F_8	70	1400					252	1148

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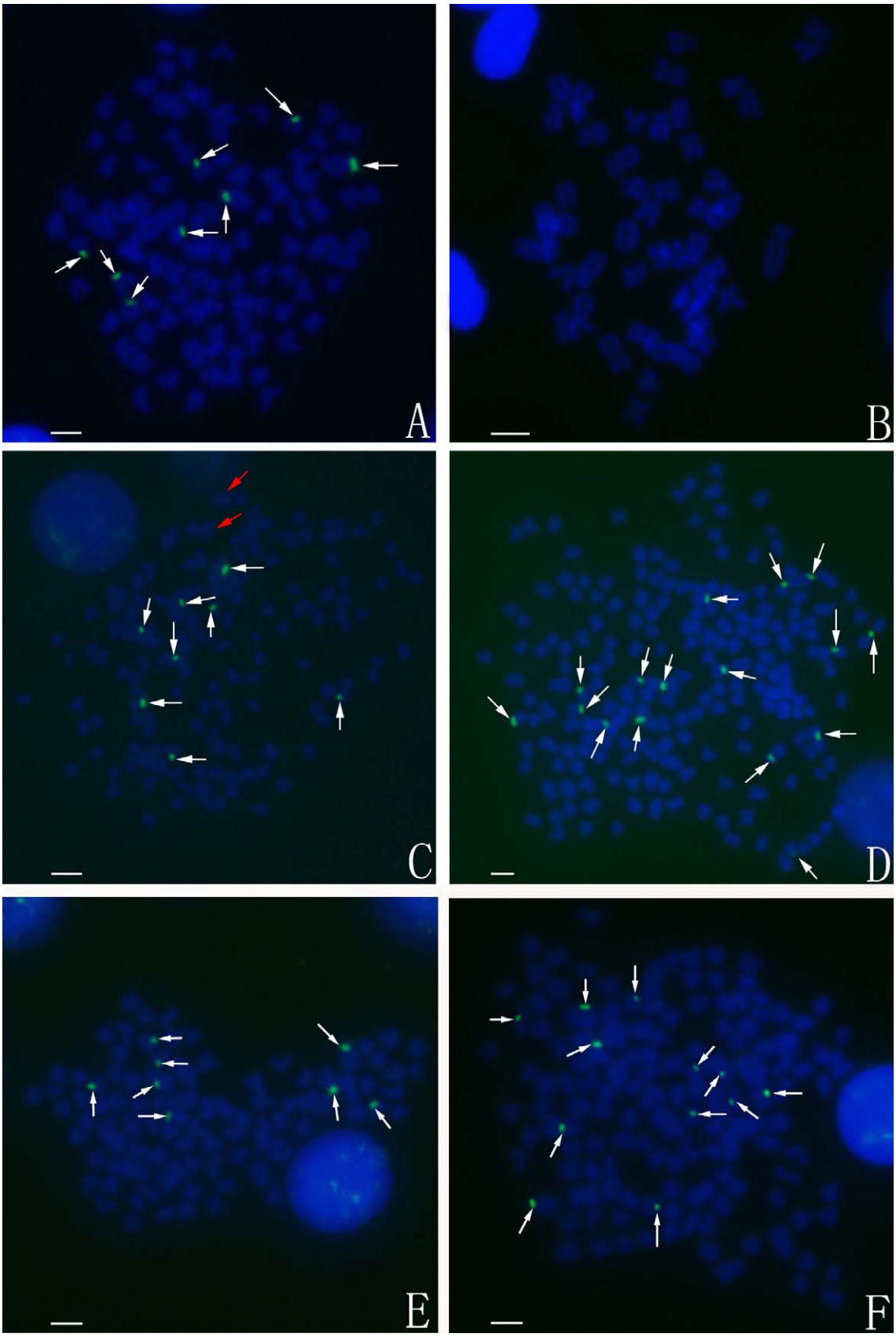

FIG. 5. Examination of hybridizing signals by FISH in RCC, BSB, $2nG_1$, 3nH, F_1, and F_2. **A**) There are eight 5S gene loci (white arrows) in RCC. Bar = 3 μm. **B**) No 5S gene locus is found in BSB. Bar = 3 μm. **C**) There are eight 5S gene loci (white arrows) in F_1, and red arrows indicate a pair of the largest submetacentric chromosome from BSB. Bar = 3 μm. **D**) There are 16 5S gene loci (white arrows) in F_2. Bar = 3 μm. **E**) There are eight 5S gene loci (white arrows) in $2nG_1$. Bar = 3 μm. **F**) There are 12 5S gene loci (white arrows) in 3nH. Bar = 3 μm.

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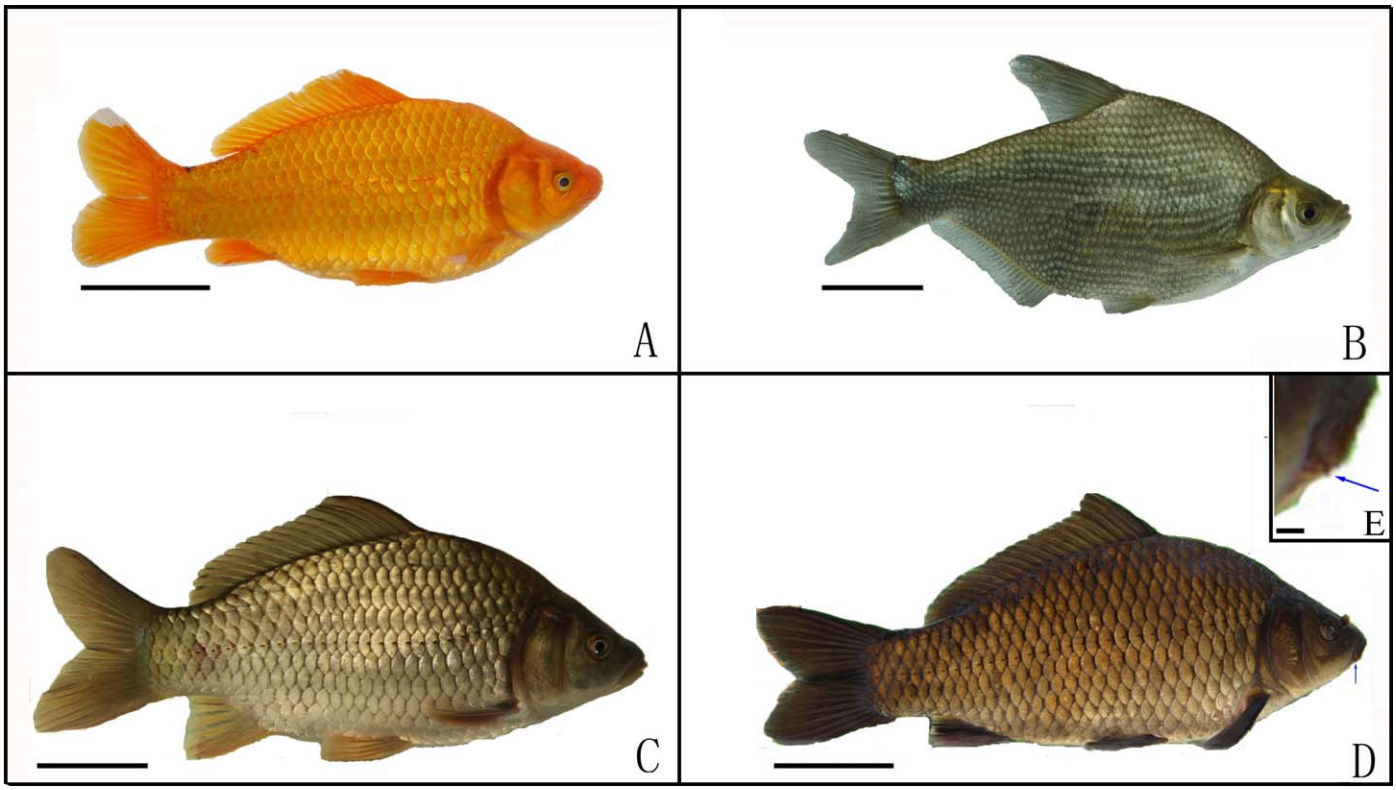

FIG. 6. The appearances of RCC, BSB, F_1, and F_2. **A**) The appearances of RCC. Bar = 6 cm. **B**) The appearances of BSB. Bar = 6 cm. **C**) The appearances of F_1. Bar = 6 cm. **D**) The appearances of F_2 in which the blue arrow indicates the barbel. Bar = 6 cm. **E**) The amplification of **D** in which the head of F_2 is amplified and the blue arrow indicates the barbel. Bar = 0.5 cm.

Table 3) but none in BSB (Fig. 5B and Table 3). Eight 5S loci were detected in 89% of the chromosomal metaphases of F_1, suggesting that they were derived from RCC and possessed two sets of RCC-derived chromosomes (Fig. 5C and Table 3). Sixteen 5S gene loci were detected in 82% of the chromosomal metaphases of F_2–F_8 (Fig. 5D and Table 3), which was twice that of RCC, suggesting that F_2–F_8 possessed four sets of RCC-derived chromosomes. Eight 5S loci were detected in 92% of the chromosomal metaphases of $2nG_1$ (Fig. 5E and Table 3), which were similar to those of RCC, indicating that $2nG_1$ possessed two sets of RCC-derived chromosomes. Twelve 5S gene loci were detected in 88.5% chromosomal metaphases of 3nH (Fig. 5F and Table 3), which was 1.5 times that of RCC, suggesting that 3nH had three sets of RCC-derived chromosomes. The method of FISH identified the origin of the chromosomes in the hybrids at the molecular level. The above results provided further evidence to prove that F_2–F_8 were autotetraploids with 200 chromosomes derived from RCC.

Morphological Traits

There were obvious differences in the morphological traits between F_1 (Fig. 6C) or F_2 (Fig. 6D) and RCC (Fig. 6A) and BSB (Fig. 6B). Regarding body color, RCC was red while F_1 and F_2 were gray. The body color of F_1 and F_2 was similar to that of BSB but was a little bit different from that of BSB (Fig. 6A–D). The most interesting difference was the presence of barbel in F_1 and F_2 but not in their parents (RCC and BSB) (Fig. 6D). Most of the morphological indices differed significantly between the F_1 and F_2 (Tables 4 and 5), suggesting that the variation in traits occurred in F_2.

Tables 4 and 5 show the values for the measurable and countable traits in RCC, BSB, F_1, and F_2. The ratios of the measurable traits all differed significantly between F_1 and BSB and between F_2 and BSB. Similarly, the ratios of the measurable traits differed significantly between F_1 and RCC with the exception of body length/body width and head length/head width, which were not significantly different ($P > 0.01$). The ratios of body length/body width, tail length/tail length, and body width/head width were significantly different between F_2 and RCC. The ratios of the measurable traits differed significantly between F_1 and F_2 with the exception of head length/head width, which were not significantly different ($P > 0.01$).

All of the countable traits differed significantly between F_1 and BSB and between F_2 and BSB. Similarly, with the exception of the number of abdominal fins and dorsal fins, all

TABLE 4. Comparison of the measurable traits between RCC, BSB, F_1, and F_2–F_8.

Fish type	Whole length/ body length	Body length/ body width	Body length/ head length	Head length/ head width	Tail length/ tail width	Body width/ head width
RCC	1.22 ± 0.02	2.18 ± 0.02	3.72 ± 0.03	1.07 ± 0.03	0.82 ± 0.03	1.84 ± 0.03
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04
F_1	1.18 ± 0.02	2.18 ± 0.02	3.83 ± 0.03	1.08 ± 0.04	0.75 ± 0.04	1.92 ± 0.02
F_2–F_8	1.23 ± 0.02	2.23 ± 0.08	3.73 ± 0.02	1.08 ± 0.02	0.84 ± 0.02	1.88 ± 0.06

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TABLE 5. Comparison of the countable traits between RCC, BSB, F_1, and F_2–F_8.[a]

Fish type	No. of lateral scales	No. of upper lateral scale	No. of lower lateral scale
RCC	29.20 ± 0.70 (28–30)	5.60 ± 0.50 (5–6)	5.70 ± 0.47 (5–6)
BSB	50.90 ± 0.91 (49–52)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)
F_1	31.65 ± 0.49 (31–32)	6.55 ± 0.51 (6–7)	6.45 ± 0.51 (6–7)
F_2–F_8	29.54 ± 1.03 (29–32)	5.36 ± 0.50 (5–6)	6.81 ± 0.75 (5–7)

[a] The numbers within the parentheses indicate the variation range.
[b] III means the first three fins are the hard fins.

other countable traits differed significantly between F_1 and RCC. The number of lateral scales, upper lateral scales, dorsal fins, and anal fins differed significantly between F_1 and F_2. In contrast, only the number of lower lateral scales differed significantly between F_2 and RCC. Comparing the measurable and countable traits between the hybrid progenies and their parents was useful to identify the similarities and differences between the hybrid progenies and their parents. The above results indicated that F_1 and F_2 were significantly different from RCC and BSB in appearance. Furthermore, F_1 was also morphologically different from F_2.

Fertility and Size of Gametes

The ovaries of 10-mo-old F_1 partially developed. Many oogonia proliferated massively with a few having developed into oocytes of phase II (Fig. 7A). In the testes of 10-mo-old

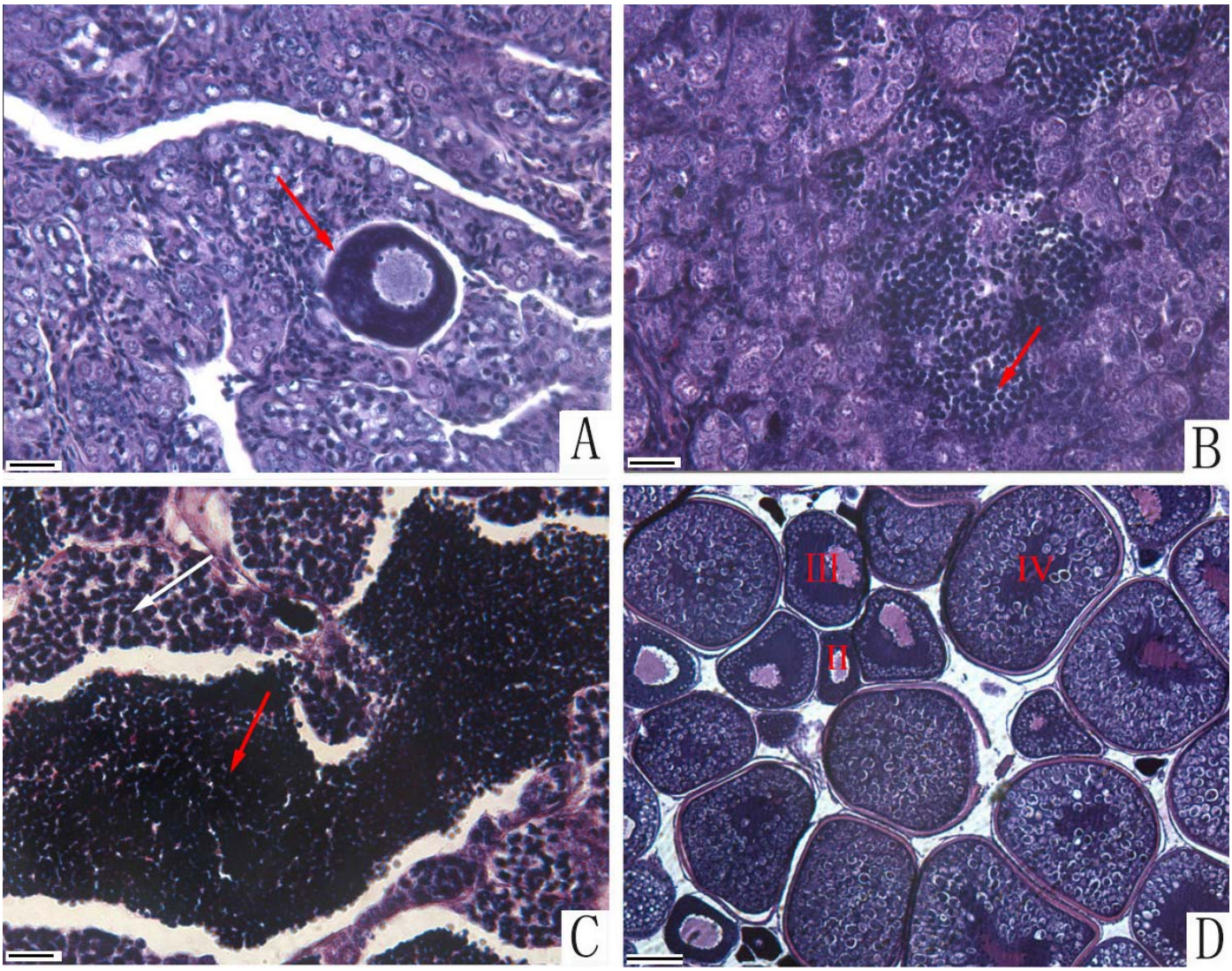

FIG. 7. The gonadal structure of F_1 and F_2. **A**) The ovary of 10-mo-old F_1 with many oogonia proliferating massively and a few developing into oocytes of phase II (red arrow). Bar = 20 μm. **B**) The testis of 10-mo-old F_1 with some spermatids developing into sperm (red arrow). Bar = 20 μm. **C**) The testis of 10-mo-old F_2 that contains many lobules in which there are many mature spermatozoa (red arrow) and spermatids (white arrow). Bar = 20 μm. **D**) The ovary of 10-mo-old F_2 that has developed well and contains stages II, III, and IV oocytes. Bar = 20 μm.

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TABLE 5. *Extended.*

Fish type	No. of dorsal fins[b]	No. of abdominal fins	No. of anal fins[b]
RCC	III+18.65 ± 0.49 (III+18–19)	8.55 ± 0.51 (8–9)	III+5.65 ± 0.49 (III+5–6)
BSB	III+8.65 ± 0.49 (III+8–9)	9.10 ± 0.55 (8–10)	III+25.85 ± 0.59 (III+25–27)
F_1	III+18.70 ± 0.98 (III+17–20)	8.60 ± 0.50 (8–9)	III+6.40 ± 0.68 (III+5–7)
F_2–F_8	III+18.27 ± 0.46 (III+18–19)	8.63 ± 0.50 (8–9)	III+5.45 ± 0.52 (III+5–6)

F_1, some spermatids developed into sperm (Fig. 7B), but no semen could be squeezed out of these testes. Relatively few mature eggs and waterlike semen were collected from 2-yr-old females and males of F_1, respectively. Thus, only a small number of F_2 were produced by self-crossing. The testes of 10-mo-old F_2–F_8 contained many lobules in which there were a large number of mature spermatozoa and spermatids (Fig. 7C). The ovaries of 10-mo-old F_2–F_8 developed well and contained stages II, III, and IV oocytes (Fig. 7D). Furthermore, large numbers of eggs or white sperm were stripped from 1-yr-old females and males of F_2–F_8, respectively. Observing the gonadal development was important to help identify whether the hybrid progenies were fertile or not. The results showed that some males and females of F_1 and all of F_2–F_8, had normal gonadal development, which resulted in their being fertile and being capable of producing offspring.

The spermatozoa of RCC and F_2 were compared under a scanning electron microscope. The size of the head of red crucian carp sperm (Fig. 8, A and C) was smaller than that of F_2–F_8 (Fig. 8, B and D). The diameter of red crucian carp haploid sperm was ~1.90 μm, whereas the diameter of diploid sperm of F_2–F_8 was ~2.40 μm. Observing the sizes of mature gametes was helpful for us to identify the ploidy of the gametes because the size of the diploid gametes was evidently larger than that of the haploid gametes. The above results suggest that F_2–F_8 was able to generate diploid spermatozoa.

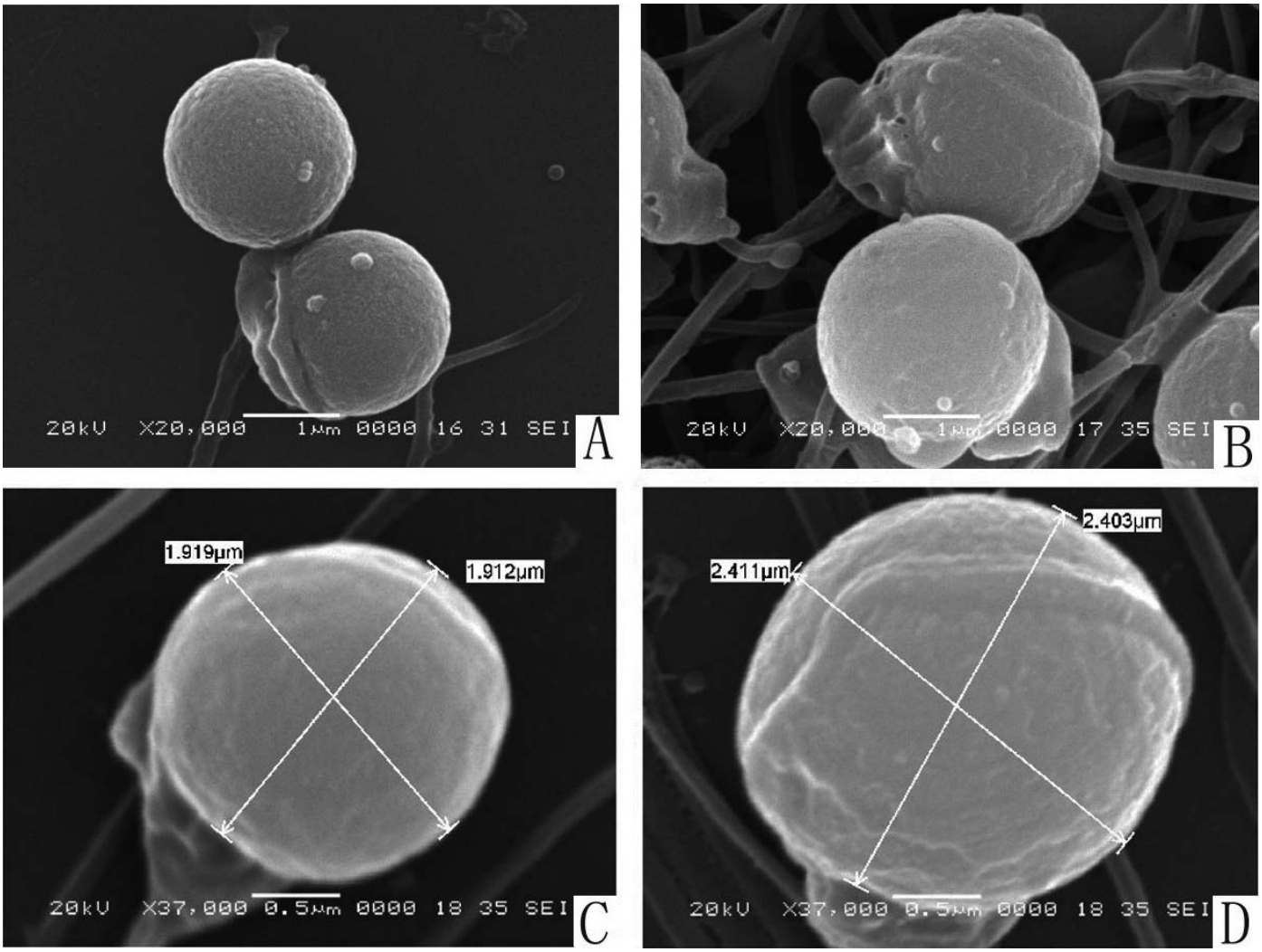

FIG. 8. The spermatozoa of RCC and F_2. **A**) The spermatozoa of RCC. Bar = 1 μm. **B**) The spermatozoa of F_2. Bar = 1 μm. **C**) The spermatozoa of RCC. Bar = 0.5 μm. **D**) The spermatozoa of F_2. Bar = 0.5 μm.

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DISCUSSION

The Establishment of Autotetraploid Line

Hybridization between two different species with differentiated genomes is one of the primary mechanisms for the origin of species leading to the formation of allopolyploids [20, 21]. In our previous study, the F_2 hybrids of *Carassius auratus red var.*(RCC) (RR, 2n = 100) (♀) × *Cyprinus carpio L.*(CC) (CC, 2n = 100) (♂) were diploid hybrids with 100 chromosomes (RC, 2n = 100). Interestingly, the males and females of diploid F_2 hybrids (RC, 2n = 100) were able to generate unreduced diploid spermatozoa and diploid eggs (RC, 2n = 100) by endoreduplication, endomitosis, or the fusion of germ cells, respectively, which were fertilized to form allotetraploid hybrids in the F_3 [16, 22]. In the current study, we successfully obtain fertile F_1 (RRBB, 4n = 148) in the first generation of RCC (RR, 2n = 100) (♀) × BSB (BB, 2n = 48) (♂). We speculate that diploid hybrid embryos developed into surviving F_1 by inhibition of the first cleavage, which results in chromosome doubling [15]. Allopolyploids generally undergo bivalent pairing at meiosis because only the homologous chromosomes pair up [3]. It is important that a diploidlike pairing system prevents meiotic irregularities and improves the efficiency of gamete production in allopolyploid species [23, 24]. On the other hand, several studies have documented abnormal chromosome behavior during mitosis and meiosis in hybrid progeny (polyploidy or diploid) [5, 7, 8]. For example, complete separation of the parental genomes occurs during mitosis and meiosis in the intergeneric hybrids between *O. violaceus* (2n = 24) and three cultivated *Brassica* tetraploids (*B. napus, B. carinata,* and *B. juncea*), which leads to the production of gametes with a complete set of *Brassica* or *O. violaceus* chromosomes [9, 10].

In the present study, the diploid gynogenetic offspring ($2nG_1$) have two sets of RCC-derived chromosomes and the triploids (3nH) have three sets of RCC-derived chromosomes, indicating that F_1 produce eggs with two sets of RCC-derived chromosomes. Based on these results, we conclude that complete separation of the parental genomes during meiosis in the F_1 (4n = 148, RRBB) gives rise to production of diploid gametes (2n = 100, RR) with two sets of RCC-derived chromosomes. Consequently, the diploid sperm and eggs of the F_1 are fertilized to form the autotetraploid progenies (4n = 200, RRRR) with four sets of RCC-derived chromosomes in F_2. By self-crossing, F_2 produces F_3. Until now, the autotetraploidy is stably inherited from one generation to another, and the autotetraploid population (F_3–F_8) is formed in succession, which forms an autotetraploid line.

The Bisexual Fertility of the Auotetraploid Fish

The females and males of F_1 reach sexual maturity at 2 yr of age and only produce a small number of mature eggs and waterlike semen, respectively [15]. In contrast, the females and males of F_2 reach sexual maturity at 1 yr of age and produce a large number of mature eggs and white semen, respectively. Compared to F_1, the increased fertility of the F_2 facilitates the successful establishment of the autotetraploid fish lineage as evidence by the F_2–F_8 generations. The hybrid progeny of RCC (♀) × F_2 (♂) are triploids with 150 chromosomes, suggesting that F_2 is able to produce normal diploid sperm with 100 chromosomes. In addition, the diameter of the sperm of F_2–F_8 is about 2.40 μm, which is same as that of diploid sperm of allotetraploid hybrids (4n = 200, RRCC) of the RCC (♀) ×CC (♂) [16], providing further evidence that F_2–F_8 can produce diploid sperm. This autotetraploid lineage is able to produce normal diploid eggs and diploid spermatozoa, thereby maintaining the tetraploidy from one generation to the next (F_2–F_8). The univalent, trivalent, and quadrivalent pairing will inhibit the formation of diploid gametes during meiosis in autotetraploids or allotetraploids, whereas bivalent pairing is considered advantageous for maintaining genetic stability in tetraploids [25]. Our results also suggest that the coexistence of four sets of homologous chromosome does not result in disordered meiosis chromosome pairing and that diploidlike meiotic behavior still occurs during meiosis in F_2–F_8.

The Significance of the Autotetraploid Fish with Phenotypic Changes

With respect to the genetic composition, the autotetraploid fish are derived from the whole genome duplication of RCC and possess four sets of chromosomes derived from RCC. However, phenotypic changes occur in autotetraploid fish, including the presence of the gray body color and the barbel, which are absent in RCC. In addition, the ratios of body length/body width, tail length/tail length, and body width/head width are significantly different between F_2 and RCC. The countable traits, including the number of lower lateral scales, also differ significantly between F_2 and RCC, suggesting that these phenotypic variances likely occur during whole genome duplication in the autotetraploid fish. This is the first report of formation of autotetraploid fish with phenotypic variation by successive generations of hybridization. The autotetraploid fish line is important in both genetics and breeding. In genetics, the establishment of the autotetraploid fish line (F_2–F_8) provides an excellent mode to investigate the phenotypic changes and genotypic changes of autotetraploids that facilitate adaptation and promote biodiversity. Based on this good platform, we will be able to facilitate the study of the mechanisms that drive diploidization in autotetraploids, including the genes involved in stabilization of meiosis. In breeding, the autotetraploid fish line is useful in the production of the sterile triploids that potentially have the advantage of a faster growth rate.

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