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

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

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**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\(_2\text{-}F_8\)) derived from the distant hybridization of *Carassius auratus red var.* (RR, 2\(n = 100\)) (female) × *Megalobrama amblycephala* (BB, 2\(n = 48\)) (male). The autotetraploid line (F\(_2\text{-}F_8\)) possess four sets of chromosomes from red crucian carp (RRRR, 4\(n = 200\)) 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 autodiploid diploid eggs and diploid sperm from the females and males of F\(_1\) hybrids (RRBB, 4\(n = 148\)), 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.

**INTRODUCTION**

Polyplpoids 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 autoploids 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* (2\(n = 24\)) 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\(_1\), 4\(n = 148\), RRBB) in the first generation of *Carassius auratus red var.* (RCC) (2\(n = 100\), RR, \(?), \times \) *Megalobrama amblycephala* (BSB) (2\(n = 48\), BB, \(\delta\)) [15]. In the current study, the abnormal chromosome behavior during meiosis of F\(_2\) hybrids leads to the formation of autoploid sperm and autodiploid ova that fertilize each other and finally result in the formation of autotetraploids in F\(_3\). Importantly, the females and males of the autotetraploids, including the F\(_3\) 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].

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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 (F1, RRBB, 4n = 148) of Carassius auratus red var. (RCC) (2n = 100, R × Megalobrama amblycephala (BSB) (2n = 48, BB, δ) is produced. The allotetraploid fish (F1, 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 (R × BSB (δ)) by self-crossing of F1, and the autotetraploid line (F1 × F1, 4n = 200) is formed in succession. The autotriploid (3nC, 3n = 150) is obtained in the cross of RCC (R × F1 (δ)). The autotriploid (3nH, 3n = 150) is formed in the backcross of F1 (R × RCC (δ)). The autotriploid gynogenetic fish (2nG1, 2n = 100) is produced by artificial gynogenesis from the eggs of the F1 that are activated with UV-treated sterilized sperm of BSB without treatment for doubling the chromosomes.

Measurement of DNA Content

To measure the DNA content of erythrocytes of RCC, BSB, 2nG1, 3nH, F1, and F2, 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 40,6-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, 2nG1, 3nH, F1, and F2 at 1yr 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 3330X. Good-quality metaphase spreads were photographed and used for analysis of karyotypes. Lengths of entire chromosomes, long and short arms,

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, R × Megalobrama amblycephala (BSB) (2n = 48, BB, δ), the allotetraploid fish (F1, 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 (R × BSB (δ)) by self-crossing of F1, and the autotetraploid line (F1 × F1, 4n = 200) is formed in succession. The autotriploid (3nC, 3n = 150) is obtained in the cross of RCC (R × F1 (δ)). B) The autotriploid (3nH, 3n = 150) is formed in the backcross of F1 (R × RCC (δ)). C) The autotriploid gynogenetic fish (2nG1, 2n = 100) is produced by artificial gynogenesis from the eggs of the F1 that are activated with UV-treated sterilized sperm of BSB without treatment for doubling the chromosomes.
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), 1.7–7.0 as submetacentric (sm), 3.1–7.0 as subteloctentric (st), and 7.1 as telocentric (t) chromosomes.

**Spermatozoa Phenotype and Gonadal Structure**

The semen of RCC and F₂ 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 F₂ 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 2nG₁, 20 3nH, 20 F₁, and 140 F₂–F₈ 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, head length to head width, tail length to tail width, and body length 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'-GCTATGCGCATCTCGCTGTA-3' and 5'-CAGGTTGTTATGGCGCTAAAGC-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 autotetraploid hybrids (F₂, 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 F₁ and the autotetraploid line (F₂–F₈, 4n = 200) was formed in succession (Fig. 1A). The autotetraploids (3nC, 3n = 150) were obtained in the cross of RCC (♀) × F₂ (♂) (Fig. 1A), and the autotetraploids (3nH, 3n = 150) were formed in the backcross of F₁ (♀) × RCC (♂) (Fig. 1B). The autodiploid gynogenetic progenies (2nG₁, 2n = 100) were produced by artificial gynogenesis from the eggs of the F₁ 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 2nG₁ suggested that F₁ hybrids produced the diploid eggs. The establishment of the fish line from F₂ to F₈ indicated that both males and females in F₂–F₈ were fertile.

**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 2nG₁ hybrids was equal to that of RCC (P > 0.01), suggesting that 2nG₁ had two sets of RCC-derived chromosomes (2n). The mean DNA content of F₁ was 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 F₂ was 210.24, which is equal to double the RCC content (P > 0.01), suggesting that F₂ 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).

**Examination of Chromosome Number and Formation of Karyotype**

Table 2 illustrates the distribution of chromosome numbers in RCC, BSB, 2nG₁, 3nH, F₁, and F₂–F₈. Among the RCC samples, 90% of the chromosomal metaphases had 100...
TABLE 2. Examination of chromosome number in RCC, BSB, 2nG1, 3nH, F1, F2–F8, and 3nC.

<table>
<thead>
<tr>
<th>Fish type</th>
<th>No. of metaphase</th>
<th>Distribution of chromosome number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC</td>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>BSB</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>2nG1</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>3nH</td>
<td>200</td>
<td>28</td>
</tr>
<tr>
<td>F1</td>
<td>200</td>
<td>34</td>
</tr>
<tr>
<td>F2–F8</td>
<td>1400</td>
<td>236</td>
</tr>
<tr>
<td>3nC</td>
<td>200</td>
<td>31</td>
</tr>
</tbody>
</table>

FIG. 3. Chromosome spreads at metaphase in F1, 2nG1, F2–F8, and 3nH. A) The 148 chromosomes of F1 in which a pair of the largest submetacentric chromosomes (arrows) is indicated. Bar = 3 μm. B) The 100 chromosomes of 2nG1 in which the largest submetacentric chromosome is not found. Bar = 3 μm. C) The 200 chromosomes of F2–F8 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.
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 F1 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 tetraploid possessing two sets of BSB-derived chromosomes and two sets of RCC-derived chromosomes (Figs. 3A and 4A, and Table 2). Among the 2nG1 samples, 85% of the chromosomal metaphases had 100 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, 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 F1 hybrids were allotetraploid hybrids with 148 chromosomes derived from RCC and BSB and F2–F8 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, F1, F2–F8, 2nG1, 3nH, 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>
<thead>
<tr>
<th>Fish type</th>
<th>No. of Fish</th>
<th>No. of metaphase</th>
<th>Distribution of chromosome loci number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC</td>
<td>10</td>
<td>200</td>
<td>&lt;8  14  186  8  12  16  12  16  16</td>
</tr>
<tr>
<td>BSB</td>
<td>10</td>
<td>200</td>
<td>0  0  16  184</td>
</tr>
<tr>
<td>2nG1</td>
<td>10</td>
<td>200</td>
<td>23  177</td>
</tr>
<tr>
<td>3nH</td>
<td>10</td>
<td>200</td>
<td>22  178</td>
</tr>
<tr>
<td>F1</td>
<td>10</td>
<td>200</td>
<td>252 1148</td>
</tr>
<tr>
<td>F2–F8</td>
<td>70</td>
<td>1400</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 4. Karyotypes of F1 and F2–F8. A) The karyotype of F1 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 F2–F8 is 44m + 68sm + 44st + 44t, which consists of four sets of chromosomes from RCC. Bar = 3 µm.

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FIG. 5. Examination of hybridizing signals by FISH in RCC, BSB, 2nG1, 3nH, F1, and F2. 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 F1, 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 F2. Bar = 3 μm. E) There are eight 5S gene loci (white arrows) in 2nG1. Bar = 3 μm. F) There are 12 5S gene loci (white arrows) in 3nH. Bar = 3 μm.
Table 3) but none in BSB (Fig. 5B and Table 3). Eight 5S loci were detected in 89% of the chromosomal metaphases of F1, 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 F2–F8 (Fig. 5D and Table 3), which was twice that of RCC, suggesting that F2–F8 possessed four sets of RCC-derived chromosomes. Eight 5S loci were detected in 92% of the chromosomal metaphases of 2nG1 (Fig. 5E and Table 3), which were similar to those of RCC, indicating that 2nG1 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 F2–F8 were autotetraploids with 200 chromosomes derived from RCC.

Morphological Traits

There were obvious differences in the morphological traits between F1 (Fig. 6C) or F2 (Fig. 6D) and RCC (Fig. 6A) and BSB (Fig. 6B). Regarding body color, RCC was red while F1 and F2 were gray. The body color of F1 and F2 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 F1 and F2 but not in their parents (RCC and BSB) (Fig. 6D). Most of the morphological indices differed significantly between the F1 and F2 (Tables 4 and 5), suggesting that the variation in traits occurred in F2.

Tables 4 and 5 show the values for the measurable and countable traits in RCC, BSB, F1, and F2. The ratios of the measurable traits all differed significantly between F1 and BSB and between F2 and BSB. Similarly, the ratios of the measurable traits differed significantly between F1 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 F2 and RCC. The ratios of the measurable traits differed significantly between F1 and F2 with the exception of head length/head width, which were not significantly different (P > 0.01).

All of the countable traits differed significantly between F1 and BSB and between F2 and BSB. Similarly, with the exception of the number of abdominal fins and dorsal fins, all

<table>
<thead>
<tr>
<th>Fish type</th>
<th>Whole length/ body length</th>
<th>Body length/ body width</th>
<th>Body length/ head length</th>
<th>Head length/ head width</th>
<th>Tail length/ tail width</th>
<th>Body width/ head width</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC</td>
<td>1.22 ± 0.02</td>
<td>2.18 ± 0.02</td>
<td>3.72 ± 0.03</td>
<td>1.07 ± 0.03</td>
<td>0.82 ± 0.03</td>
<td>1.84 ± 0.03</td>
</tr>
<tr>
<td>BSB</td>
<td>1.19 ± 0.03</td>
<td>2.37 ± 0.03</td>
<td>4.75 ± 0.04</td>
<td>1.14 ± 0.03</td>
<td>1.08 ± 0.04</td>
<td>2.09 ± 0.04</td>
</tr>
<tr>
<td>F1</td>
<td>1.18 ± 0.02</td>
<td>2.18 ± 0.02</td>
<td>3.83 ± 0.03</td>
<td>1.08 ± 0.04</td>
<td>0.75 ± 0.04</td>
<td>1.92 ± 0.02</td>
</tr>
<tr>
<td>F2–F8</td>
<td>1.23 ± 0.02</td>
<td>2.23 ± 0.08</td>
<td>3.71 ± 0.02</td>
<td>1.08 ± 0.02</td>
<td>0.84 ± 0.02</td>
<td>1.88 ± 0.06</td>
</tr>
</tbody>
</table>

FIG. 6. The appearances of RCC, BSB, F1, and F2. A) The appearances of RCC. Bar = 6 cm. B) The appearances of BSB. Bar = 6 cm. C) The appearances of F1. Bar = 6 cm. D) The appearances of F2, in which the blue arrow indicates the barbel. Bar = 6 cm. E) The amplification of D in which the head of F2 is amplified and the blue arrow indicates the barbel. Bar = 0.5 cm.
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

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TABLE 5. Comparison of the countable traits between RCC, BSB, \( F_1 \), and \( F_2-F_8 \). a

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

a The numbers within the parentheses indicate the variation range.

b III means the first three fins are the hard fins.
F1, 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 F1, respectively. Thus, only a small number of F2 were produced by self-crossing. The testes of 10-mo-old F2–F8 contained many lobules in which there were a large number of mature spermatozoa and spermatids (Fig. 7C). The ovaries of 10-mo-old F2–F8 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 F2–F8, 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 F1 and all of F2–F8 had normal gonadal development, which resulted in their being fertile and being capable of producing offspring.

The spermatozoa of RCC and F2 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 F2–F8 (Fig. 8, B and D). The diameter of red crucian carp haploid sperm was \(1.90 \mu m\), whereas the diameter of diploid sperm of F2–F8 was \(2.40 \mu m\). 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 F2–F8 was able to generate diploid spermatozoa.

### Table 5. Extended.

<table>
<thead>
<tr>
<th>Fish type</th>
<th>No. of dorsal fins(^b)</th>
<th>No. of abdominal fins</th>
<th>No. of anal fins(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC</td>
<td>III+18.65 ± 0.49 (III+18–19)</td>
<td>8.55 ± 0.51 (8–9)</td>
<td>III+5.65 ± 0.49 (III+5–6)</td>
</tr>
<tr>
<td>BSB</td>
<td>III+8.65 ± 0.49 (III+8–9)</td>
<td>9.10 ± 0.55 (8–10)</td>
<td>III+25.85 ± 0.59 (III+25–27)</td>
</tr>
<tr>
<td>F1</td>
<td>III+18.70 ± 0.98 (III+17–20)</td>
<td>8.60 ± 0.50 (8–9)</td>
<td>III+6.40 ± 0.68 (III+5–7)</td>
</tr>
<tr>
<td>F2–F8</td>
<td>III+18.27 ± 0.46 (III+18–19)</td>
<td>8.63 ± 0.50 (8–9)</td>
<td>III+5.45 ± 0.52 (III+5–6)</td>
</tr>
</tbody>
</table>

**FIG. 8.** The spermatozoa of RCC and F2. A) The spermatozoa of RCC. Bar = 1 μm. B) The spermatozoa of F2. Bar = 1 μm. C) The spermatozoa of RCC. Bar = 0.5 μm. D) The spermatozoa of F2. Bar = 0.5 μm.
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 F₂ 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 F₂ 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 autotetraploid hybrids in the F₃ [16, 22]. In the current study, we successfully obtain fertile F₁ (RRBB, 4n = 148) in the first generation of RCC (RR, 2n = 100) (♀) × BSB (BB, 2n = 48) (♂). We speculate that hybrid diploid embryos developed into surviving F₁ 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 (2nG₁) have two sets of RCC-derived chromosomes and the triploids (3nH) have three sets of RCC-derived chromosomes, indicating that F₁ 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 F₁ (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 F₁ are fertilized to form the autotetraploid progenies (4n = 200, RRRR) with four sets of RCC-derived chromosomes in F₂. By self-crossing, F₂ produces F₃. Until now, the autotetraploidy is stably inherited from one generation to another, and the autotetraploid population (F₃–F₈) is formed in succession, which forms an autotetraploid line.

The Bisexual Fertility of the Autotetraploid Fish

The females and males of F₁ 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 F₂ reach sexual maturity at 1 yr of age and produce a large number of mature eggs and white semen, respectively. Compared to F₁, the increased fertility of the F₂ facilitates the successful establishment of the autotetraploid fish lineage as evidence by the F₃–F₈ generations. The hybrid progeny of RCC (♀) × F₂ (♂) are triploids with 150 chromosomes, suggesting that F₂ is able to produce normal diploid sperm with 100 chromosomes. In addition, the diameter of the sperm of F₂–F₈ is about 2.40 μm, which is same as that of diploid sperm of allotetraploid hybrids (4n = 200, RCC) of the RCC (♀) × *CC (♂) [16], providing further evidence that F₂–F₈ 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 (F₃–F₈). 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 F₂–F₈.

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 F₂ and RCC. The countable traits, including the number of lower lateral scales, also differ significantly between F₂ 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 (F₂–F₈) 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.

REFERENCES


