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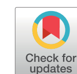
Production of diploid gynogenetic grass carp and triploid hybrids derived from the distant hybridization of female grass carp and male topmouth culter



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ABSTRACT

Distant hybridization is an effective tool to create offspring with different ploidy levels and is widely used in plants and lower organisms. In this study, the diploid gynogenetic grass carp (2nGGC, 2n = 48) and triploid hybrid (3nGT, 3n = 72) progenies of female grass carp (*Ctenopharyngodon idellus*, GC, *Leuciscinae*, 2n = 48) × male topmouth culter (*Erythroculter ilishaeformis*, TC, *Cultrinae*, 2n = 48) were successfully obtained by distant hybridization. The results regarding the chromosomal number, DNA content, 5S rDNA and fluorescence in situ hybridization (FISH) showed 2nGGC was derived from gynogenesis with 48 chromosomes and not a hybrid of GC and TC, while 3nGT was a triploid hybrid with 72 chromosomes. 3nGT exhibited significant phenotypic differences from GC and TC, but 2nGGC resembled GC. With regard to feeding, both 2nGGC and 3nGT were herbivorous. However, the total amino acid contents in the muscles of both 2nGGC and 3nGT were significantly higher than that in GC ($P < 0.01$). This is the first report of 2nGGC and 3nGT being produced by crossing GC and TC. The formation of two kinds of new offspring is very important in fish genetic breeding.

1. Introduction

Distant hybridization can combine the genomes of different species, and the offspring produced exhibit changes in phenotypes and genotypes or different ploidy levels due to hybridization (Chen et al., 2017; Liu, 2014; Song et al., 2012; Zhang et al., 2014; Huang et al., 2016; Mallet, 2007). A combination of the desirable traits from both parents often results in heterotic offspring with higher nutrient values, faster growth rates, and improved taste and disease resistance (Huang et al., 2016; Liu, 2010; Mallet, 2007). For example, in the cross between grass carp (2n = 48) and blunt snout bream (2n = 48), both the diploid (2n = 48) and triploid (3n = 72) hybrids shown faster growth rates than the female parent (He et al., 2013). The heterotic hybrids of *Haliotis discus hannai* and *H. gigantea* were more disease resistant than either parental species (Liang et al., 2017). Among the first hybrid offspring of red crucian carp (2n = 100) and blunt snout bream (2n = 48), gynogenetic red crucian carp (2n = 100), triploid hybrids (3n = 124) and allotetraploid hybrids (4n = 148) were found (Liu et al., 2007; Qin et al., 2015).

In higher eukaryotes, 5S ribosomal DNA (5S rDNA) consists of tandem repeated units, which included a conserved coding region of approximately 120 bp and a variable nontranscribed spacer (NTS) region (Martins and Galetti, 2001; Pasolini et al., 2006; Wasko et al., 2001). An indicator of organization and variability, 5S rDNA was used as a DNA marker to identify and distinguish among species of animals and plants, especially fish (Fujiwara et al., 2009; He et al., 2012; Negi et al., 2002; Suzuki et al., 1994). Therefore, we used 5S rDNA to determine the chromosome composition of the offspring of GC and TC.

Grass carp (GC, *Ctenopharyngodon idellus*, *Leuciscinae*, 2n = 48), one of the four major Chinese carps, is a global aquaculture species because of its herbivorous feeding habit, rapid growth rates and high economic value (Chilton and Muoneke, 1992; Wang et al., 2015). Topmouth culter (TC, *Erythroculter ilishaeformis*, *Cultrinae*, 2n = 48), distributed in the East Asia region, is a predatory cyprinid fish with excellent taste, rapid growth rates and strong performance in culture (Cao and Wang, 2010; Ren et al., 2014; Zhao et al., 2016). In this study, we reported for the first time the production of diploid gynogenetic grass carp (2nGGC) and a triploid hybrid (3nGT) by the distant hybridization of an herbivorous fish and a carnivorous fish, which represented the successful generation of two types of herbivorous offspring, a diploid gynogenetic grass carp and a triploid hybrid.

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2. Methods

2.1. Ethics statement

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

2.2. Animals and crosses

All the specimens in this paper were cultured in the Engineering Center for Polyploidy Fish Breeding of the National Education Ministry in Hunan Normal University. During the reproductive season (from May to June) from 2015 to 2017, 15 female GC and 15 male TC were selected as the maternal fish and paternal fish, respectively. The embryos were produced by artificial fertilization and hatched in an incubator with a water temperature of 24–26 °C. The fertilization rate (no. of embryos at the gastrula stage/no. of eggs) and the hatching rate (no. of hatched fry/no. of eggs) were calculated for 6000 embryos. Then, all the fry were transferred from the incubator to a special pond for further culture.

2.3. Measurement of DNA content and preparation of chromosome spreads

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

To further determine the ploidy level of the offspring, kidney tissue was used to prepare chromosomes from 10 GC, 10 TC, 20 2nGGC and 20 3nGT to count their chromosome numbers. The specific method could be found in Qin et al. (2015). Fifteen metaphase spreads for each sample were photographed using a microscope, and the mean chromosome numbers were obtained.

2.4. Morphological traits

We collected a random sample of 10 GC, 10 TC, 20 2nGGC and 20 3nGT at 10 months old to determine the countable and measurable morphological traits. Six countable traits of each fish (lateral scales, upper lateral scales, lower lateral scales, dorsal fins, abdominal fins, and anal fins) were recorded. The average values of six measurable traits, namely the ratios of the whole length to the body length (WL/BL), the body length to the body width (BL/BW), the body height to the head height (BH/HH), the head length to the head width (HL/HW), the head height to the head width (HH/HW), and the caudal peduncle length to the caudal peduncle height (CPL/CPH), were measured and calculated. SPSS software was used to perform ANOVA and pairwise comparisons of the data.

2.5. Determination of amino acids and feeding habit

We fed 10 GC, 10 TC, 10 2nGGC and 10 3nGT in a special pond and cut the tail fins of the GC to distinguish them from 2nGGC. A sample of approximately 3 g of muscle from each fish was cut into small pieces and dried in a vacuum oven at a temperature of −40 °C for 36 h. Approximately 0.5 g of dry muscle samples and 10 mL of 6 M/L hydrochloric acid were added to ampoules, and the ampoules were sealed by an alcohol blast burner. These samples were hydrolyzed in a 110 °C oven for 24 h. The filtered solution was diluted 2-fold before the amino acids were analyzed with an Automatic Amino Acid Analyzer (Model Hitachi L-8900, Japan).

To confirm the feeding habit of the offspring, we fed them grass and observed whether they were herbivorous or carnivorous. Then, we dissected the heads of GC, TC, 2nGGC and 3nGT to remove their pharyngeal teeth as described in Eastman (1977). The samples of pharyngeal teeth were washed with 1% sodium hydroxide, bleached with 3% hydrogen peroxide and preserved in 100% glycerol. We observed and photographed the shape of the pharyngeal teeth with the aid of a stereomicroscope.

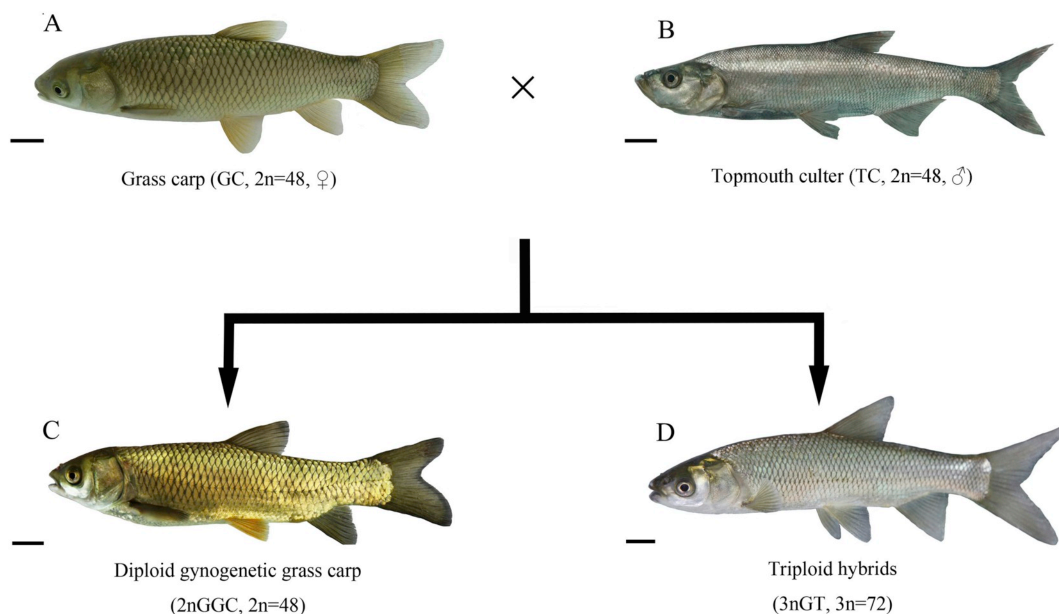

Fig. 1. The crossing procedure and appearance of GC, TC, 2nGGC and 3nGT. (A) The appearance of GC. (B) The appearance of TC. (C) The appearance of 2nGGC. (D) The appearance of 3nGT. Bar = 2 cm.

Table 1
Fertilization and hatching rates of offspring.

Year	Fertilization rates	Hatching rates
2015	92.25%	76.39%
2016	87.46%	70.38%
2017	89.43%	67.43%

Table 2
Mean DNA content of GC, TC, 2nGGC and 3nGT.

Fish type	Mean DNA content	Ratio	
		Observed	Expected
GC	60.59		
TC	71.31		
2nGGC	60.41	2nGGC/GC = 0.99[a]	1
3nGT	97.09	3nGT/(GC + 0.5TC) = 1.00[a]	1

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

2.6. Genomic DNA extraction, PCR and sequencing

We extracted total genomic DNA from the peripheral blood cells of 2nGGC, 3nGT and their parents according to the method in Sambrook et al. (1989). The 5S rDNA was amplified by polymerase chain reaction (PCR) with a pair of primers (5s-P1: 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5s-P2: 5′-CAGGTTGGTATGGCCGTAAGC-3′) designed with Primer 5.0 software and synthesized by Sagon (Shanghai, China). PCR was performed with GC, TC, 2nGGC and 3nGT templates, and the conditions were as follows: initial denaturation step at 94 °C for 5 min, followed by 30 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s), ending with 10 min of extension at 72 °C. The amplified products were analyzed in a 1.5% agarose gel using tris-acetate-EDTA (TAE) buffer, purified using a gel extraction kit (Sangon, Shanghai, China), inserted into vectors with the pClone007 Simple Vector kit (Tsingke, Beijing, China), and transferred into competent *E. coli* Top10 cells. The fragments inserted into the vector were sequenced by Sagon and analyzed by BioEdit software.

2.7. Fluorescence in situ hybridization

The 188-bp 5S rDNA fragment of TC (TC-188) was purified and labeled with Dig-11-dUTP (Roche, Germany) for fluorescence in situ hybridization (FISH). FISH was performed according to the method described by Caradonna et al. (2007), and 30 metaphase spreads of chromosomes for each individual (10 GC, 10 TC, 10 2nGGC and 10 3nGT) were counted and analyzed.

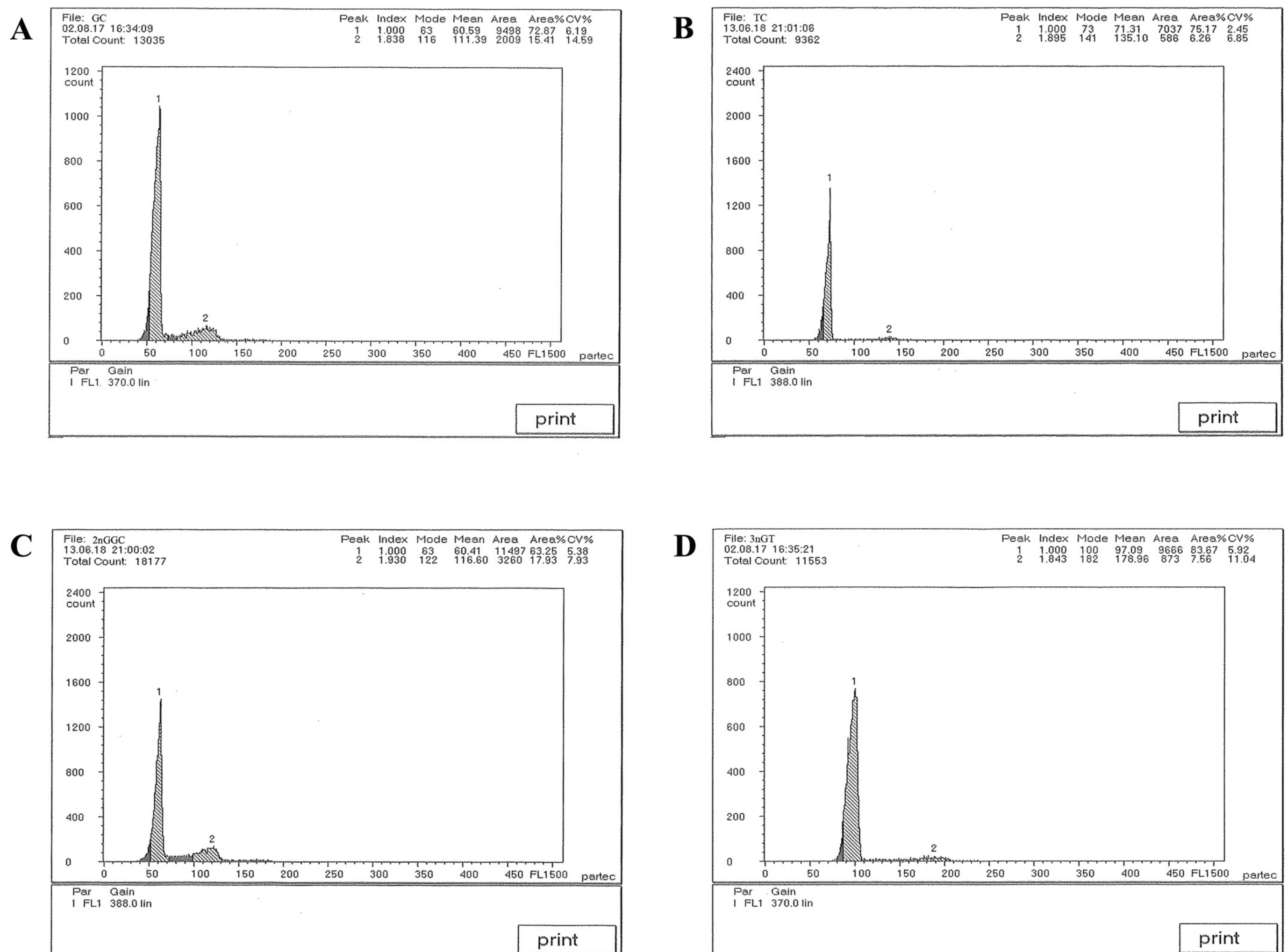

Fig. 2. Cytometric histogram of DNA fluorescence for GC, TC, 2nGGC and 3nGT. (A) The mean DNA content of GC (peak 1: 60.59). (B) The mean DNA content of TC (peak 1: 71.31). (C) The mean DNA content of 2nGGC (peak 1: 60.41). (D) The mean DNA content of 3nGT (peak 1: 97.09).

Table 3
Examination of chromosome number of GC, TC, 2nGGC and 3nGT.

Fish type	No. in metaphase	Distribution of chromosome number			
		< 48	48	< 72	72
GC	150	12	138		
TC	150	9	141		
2nGGC	300	14	286		
3nGT	300			21	279

3. Results

3.1. Formation of diploid gynogenetic grass carp and triploid hybrids

GC (2n = 48, Fig. 1A) and TC (2n = 48, Fig. 1B) are both important commercial fishes in East Asia. By crossing GC (♀) × TC (♂), we obtained two kinds of offspring, namely, 2nGGC (Fig. 1C) and 3nGT (Fig. 1D), during the reproductive seasons (from May to June) from 2015 to 2017. Six thousand embryos were examined in the process of embryonic development, and the results were presented in Table 1. Surprisingly, the hybridization resulted in a high fertilization rate (87.46% - 92.25%) and hatching rate (67.43% - 76.39%). Moreover, 2nGGC and 3nGT accounted for 0.07% and 99.93%, respectively, of the offspring at 10 months old.

3.2. Measurement of DNA content and examination of chromosome number

The distribution of the DNA content in GC, TC and their offspring was shown in Table 2 and Fig. 2. We used the sum of the DNA content of GC (Fig. 2A) and TC (Fig. 2B) as the controls. The mean DNA content of 2nGGC (Fig. 2C) was equal ($P > 0.05$) to the DNA content of GC, suggesting that 2nGGC had two sets of chromosomes from GC. The mean DNA content of 3nGT (Fig. 2D) was equal ($P > 0.05$) to the sum of that of GC and half of TC, indicating that 3nGT had one set of chromosomes from TC and two sets of chromosomes from GC.

The results of the chromosome number distribution in 2nGGC, 3nGT and their parents were shown in Table 3 and Fig. 3. Of all examined samples in GC and TC, 92% and 94%, respectively, of the chromosomal metaphases possessed 48 chromosomes (Fig. 3A and B). In the offspring of GC (♀) × TC (♂) that resembled GC in appearance, 95% of the chromosomal metaphases had 48 chromosomes, showing that they were diploids (2nGGC, 2n = 48, Fig. 3C). In the offspring of GC (♀) × TC (♂) that were intermediate between GC and TC in appearance, 93% of the chromosomal metaphases had 72 chromosomes, meaning that they were triploids (3nGT, 3n = 72, Fig. 3D). We didn't find any hybrid diploids and androgenic topmouth culter in this cross yet.

3.3. Morphological traits

The physical characteristics of GC (Fig. 1A), TC (Fig. 1B), 2nGGC (Fig. 1C), and 3nGT (Fig. 1D) were illustrated in Fig. 1. Tables 4 and 5 presented the examined countable and measurable traits in the four types of fish. For the countable traits, the results showed that all the countable traits of 2nGGC, apart from the dorsal fins, were not significantly different from those of GC ($P > 0.05$) but were significantly different from those of TC ($P < 0.01$). For 3nGT, the number of lateral scales and anal fins were intermediate between those of GC and TC, but the number of abdominal fins was similar to that of GC, and the number of lower lateral scales was similar to that of TC, suggesting that 3nGT possessed a hybrid phenotype intermediate between the two phenotypes of its parents.

For the measurable traits, all the ratios measured in 2nGGC (Table 5) were not significantly different from those in GC ($P > 0.05$), indicating that the appearance of 2nGGC resembles that of GC. The WL/

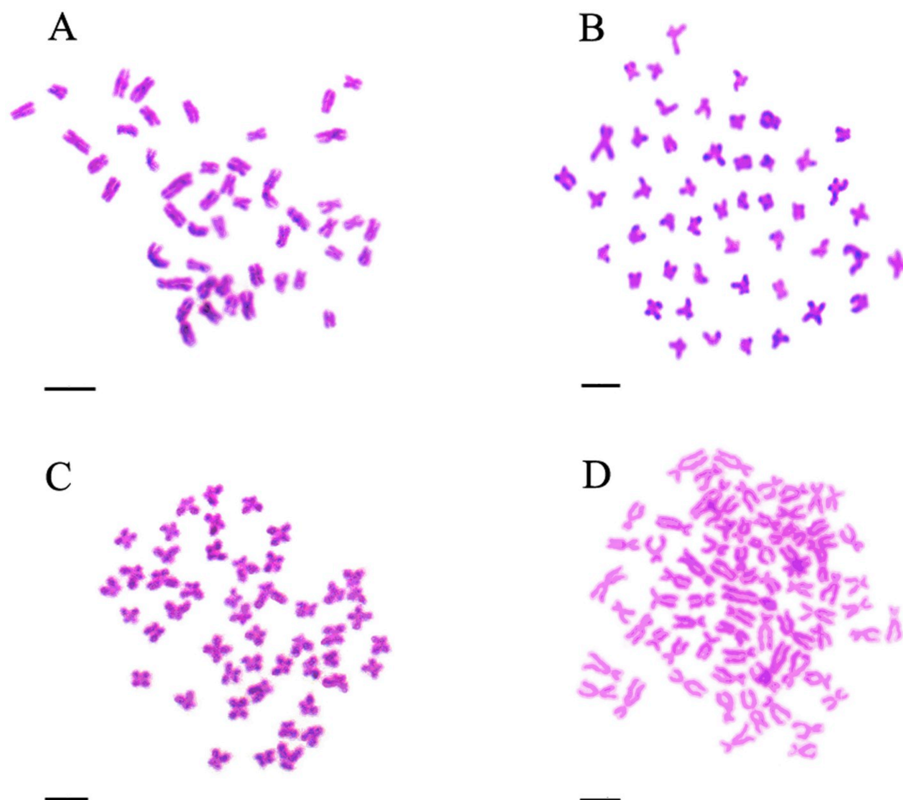

Fig. 3. The chromosomes at metaphase in GC, TC, 2nGGC and 3nGT. (A) The 48 chromosomes of GC. (B) The 48 chromosomes of TC. (C) The 48 chromosomes of 2nGGC. (D) The 72 chromosomes of 3nGT. Bar = 20 μm.

Table 4
The countable traits of GC, TC, 2nGGC and 3nGT.

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
GC	42.31 ± 0.89 (39–44)	6.43 ± 0.23 (6–7)	4.45 ± 0.34 (4–5)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 7.00 ± 0.00 (7)
TC	85.47 ± 2.58 (80–92)	17.62 ± 1.02 (16–20)	6.53 ± 0.47 (6–7)	III + 7.00 ± 0.00 (III + 7)	9.00 ± 0.00 (9)	III + 21.87 ± 1.01 (20 – 23)
2nGGC	43.71 ± 1.21 (42–45)	6.60 ± 0.23 (6–7)	4.64 ± 0.27 (4–5)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 8.00 ± 0.00 (8)
3nGT	53.89 ± 1.43 (51–56)	11.02 ± 0.96 (9–12)	6.37 ± 0.24 (6–7)	III + 7.00 ± 0.00 (III + 7)	8.00 ± 0.00 (8)	III + 10.64 ± 0.35 (10 – 11)

Table 5
The measurable traits of GC, TC, 2nGGC and 3nGT.

	WL/BL	BL/BW	BH/HH	HL/HW	HH/HW	CPL/CPH
GC	1.19 ± 0.28	6.61 ± 0.20	1.45 ± 0.01	1.45 ± 0.07	1.01 ± 0.06	1.07 ± 0.05
TC	1.16 ± 0.03	10.21 ± 0.66	1.67 ± 0.14	2.64 ± 0.14	1.50 ± 0.21	1.18 ± 0.02
2nGGC	1.17 ± 0.10	6.66 ± 0.16	1.50 ± 0.04	1.48 ± 0.06	1.01 ± 0.07	0.98 ± 0.04
3nGT	1.21 ± 0. 13	9.12 ± 0.57	1.21 ± 0.04	2.11 ± 0.15	1.30 ± 0.11	1.10 ± 0.09

BL ratio was higher in 3nGT than in either GC or TC ($P < 0.05$). Moreover, the ratio of BH/HH in 3nGT was lower than that in either parent ($P < 0.05$), indicating a variable phenotype. The other measurable traits of 3nGT were numerically intermediate between those of GC and TC.

3.4. Determination of amino acids, appearance of pharyngeal teeth and feeding habits

The amounts of 17 amino acids presented in GC, TC, 2nGGC and 3nGT were shown in Table 6, including 7 essential amino acids, 2 semi-essential amino acids and 8 nonessential amino acids. The moisture contents of the four types of fish ranged between 80.00% and 81.46% in fresh muscle. The total amino acid contents (TAAs) of 100 g of dry muscle were 53.74 g in GC, 63.31 g in TC, 58.76 g in 2nGGC and 61.90 g in 3nGT. Meanwhile, the total essential amino acids (EAAs), which ranged between 19.83 and 23.57 g/100 g dry muscle, accounted for 39.45–40.10% of TAAs. Moreover, the total delicious amino acids (DAAs), with values ranging from 21.20–25.08 g/100 g dry muscle, accounted for nearly 40% of TAAs. The amount of TAAs was higher in TC than in GC; there were also significant differences in the amounts of the total EAAs and DAAs between GC and TC ($P < 0.01$). Glutamate was the most common amino acid in all the fish muscle samples, with values ranging 8.68 g/100 g to 10.48 g/100 g dry muscle, and cysteine was the least common amino acid, with values ranging from 0.47 g/100 g to 0.55 g/100 g dry muscle.

The physical characteristics of the pharyngeal teeth in the four types of fish were presented in Fig. 4. In GC, we observed that there were two rows of teeth on the pharyngeal bone, including 4 major and 2 lateral pharyngeal teeth, and many striated grooves on the surface of the pharyngeal teeth (Fig. 4A and B). However, in TC (Fig. 4C and D), there were rows of needle-sharp teeth on the pharyngeal bone, with 4 main pharyngeal teeth, 1 middle pharyngeal tooth and 3 lateral pharyngeal teeth. In the offspring, there was no significant difference in the appearance of pharyngeal teeth between 2nGGC (Fig. 4E and F) and GC, but 3nGT (Fig. 4G and H) possessed 4 main pharyngeal teeth and 3 lateral pharyngeal teeth with striated grooves.

Regarding their feeding habits, GC were herbivorous, and TC were carnivorous. However, both 2nGGC and 3nGT were herbivorous.

3.5. 5S rDNA analyses

PCR products, generated by a primer for the 5S gene, were separated into different band patterns by gel electrophoresis (Fig. 5). GC exhibited 3 DNA bands (approximately 180 bp, 360 bp and 540 bp), TC exhibited 2 DNA bands (approximately 180 bp and 360 bp), 2nGGC exhibited 3 DNA bands (approximately 180 bp, 360 bp and 540 bp) and 3nGT exhibited 3 DNA bands (approximately 180 bp, 360 bp and 540 bp). Twenty clones for each band were sequenced. The sequencing results revealed that these sequences, which were longer than 200 bp, were a simple unit composed of a 120-bp coding sequence (CDS) of the 5S gene and a NTS region (approximately 60 bp). Comparing the first bands (approximately 180 bp, Fig. 6) of the four types of fish, we found that the sequence in GC was a 180-bp fragment with a 68-bp NTS, in TC it was a 188-bp fragment with a 76-bp NTS, in 2nGGC it was a 180-bp fragment with a 68-bp NTS, and in 3nGT it was a 180-bp fragment with a 68-bp NTS and a 188-bp fragment with a 76-bp NTS. Moreover, we found two significant SNPs in this sequencing result, which were important for distinguishing parents from offspring.

3.6. Fluorescence in situ hybridization

The FISH results were shown in Fig. 7. For the probe of TC-188, two

Table 6
The mean amino acid contents of GC, TC, 2nGGC and 3nGT muscle (g/100 g).

		GC	TC	2nGGC	3nGT
Wet weight		3.58	3.19	2.40	3.68
Dry weight		0.69	0.59	0.48	0.71
Moisture content		80.83	81.46	80.00	80.55
Essential amino acids	Thr	2.28	2.66	2.84	2.69
	Met	1.88	2.18	1.76	2.11
	Ile	2.38	2.90	2.45	2.80
	Leu	4.29	5.20	4.69	5.08
	Lys	4.06	4.66	6.16	4.62
	Val	2.71	3.27	2.98	3.14
	Phe	2.22	2.69	2.51	2.54
Semi-essential amino acids	His	1.67	2.28	1.87	2.03
	Arg	3.16	3.34	2.30	3.47
Nonessential amino acids	Asp*	6.42	8.27	6.80	7.77
	Glu*	8.68	9.97	10.48	10.14
	Gly*	2.75	3.05	2.64	2.96
	Ala*	3.34	3.80	3.64	3.82
	Cys	0.55	0.54	0.47	0.53
	Tyr	1.98	2.42	1.98	2.22
	Pro	2.29	2.26	2.40	2.47
	Ser	3.06	3.67	2.79	3.52
ΣTAAs		53.74	63.15	58.76	61.90
ΣEAAs		19.83	23.57	23.38	22.97
ΣDAAs		21.20	25.08	23.56	24.68
ΣEAAs/ΣTAAs		36.89	37.32	39.79	37.12
ΣDAAs/ΣTAAs		39.45	39.72	40.10	39.88

Notes: 1. *mean delicious amino acid. 2. ΣDAAs indicates total delicious amino acids. 3. ΣEAAs indicates total essential amino acids. 4. ΣTAAs indicates total amino acids. 5. Tryptophan is destroyed in acid hydrolysis and is not detected.

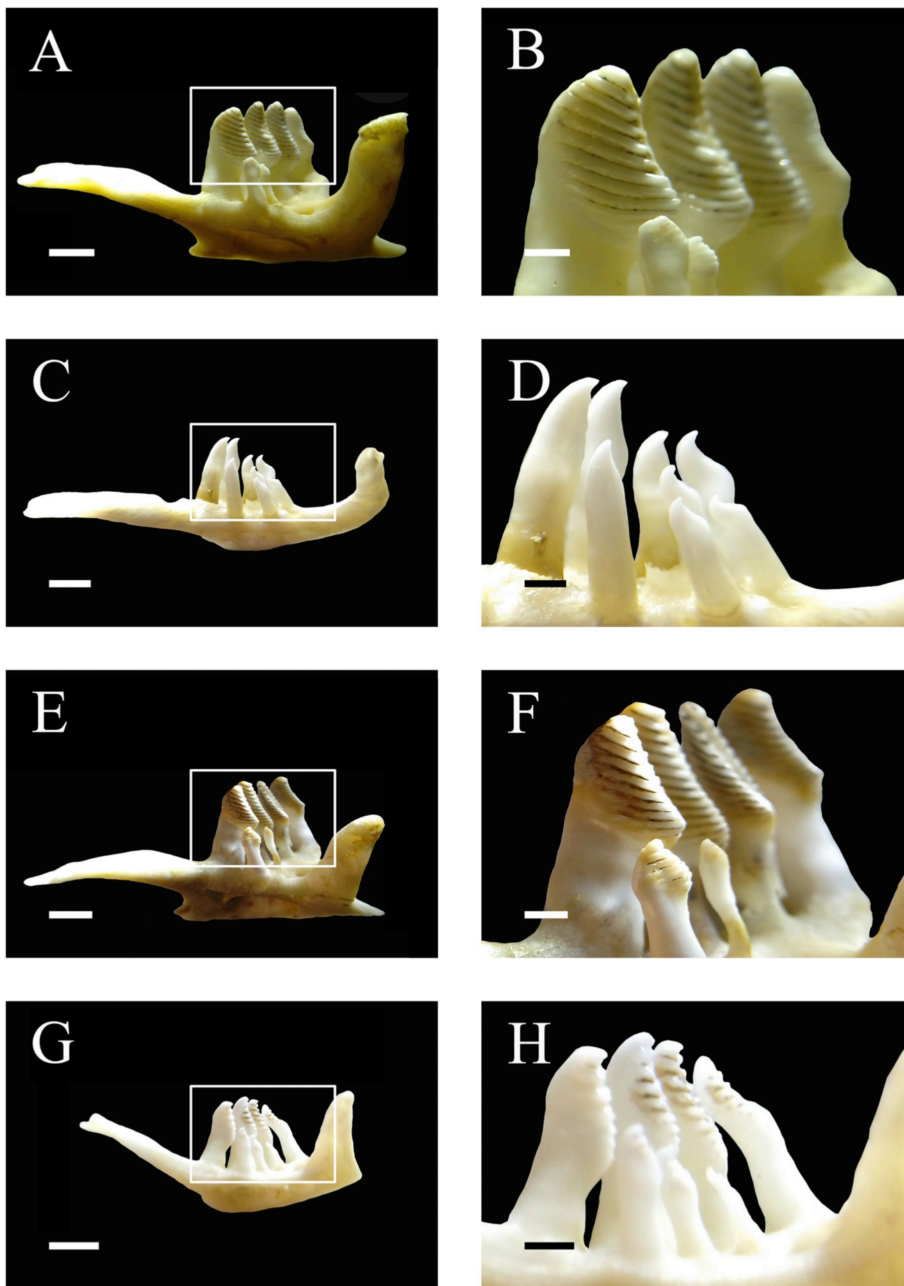

Fig. 4. The appearance of the pharyngeal teeth in GC, TC, 2nGGC and 3nGT. (A) The pharyngeal teeth of GC with 4 major and 2 lateral pharyngeal teeth. Bar = 2.1 cm. (B) The same as (A). Bar = 0.7 cm. (C) The pharyngeal teeth of TC with 4 main pharyngeal teeth, 1 middle pharyngeal tooth and 3 lateral pharyngeal teeth. Bar = 2.1 cm. (D) The same as (C). Bar = 0.7 cm. (E) The pharyngeal teeth of 2nGGC with 4 major and 2 lateral pharyngeal teeth. Bar = 2.1 cm. (F) The same as (E). Bar = 0.7 cm. (G) The pharyngeal teeth of 3nGT with 4 main pharyngeal teeth and 3 lateral pharyngeal teeth. Bar = 2.1 cm. (H) The same as (G). Bar = 0.7 cm.

strong signals and two weak signals were detected in TC (Fig. 7B), one strong signal and one weak signal were detected in 3nGT (Fig. 7D), and no signals were detected in GC (Fig. 7A) or 2nGGC (Fig. 7C). This result revealed the genetic origin of 2nGGC and 3nGT at the chromosomal level.

4. Discussion

Distant hybridization, which can combine different genomes and different traits of different species, plays an important role in improving the genetic resources of species (Liu, 2014; Wang et al., 2018; Abbott et al., 2013; Payseur and Rieseberg, 2016). Unfortunately, compared with plants, there are very few reports of distant hybridization in animals, especially in freshwater fish (Liu et al., 2016). In this study, distant hybridization of female GC (2n = 48, Fig. 1A) and male TC (2n = 48, Fig. 1B), which belong to different subfamilies of fish (*Leuciscinae* and *Cultrinae*), was accomplished by artificial fertilization. We detected the ploidy level of the offspring of this hybridization by flow cytometry and chromosome preparation and confirmed two types of offspring, namely, 2nGGC (2n = 48, Fig. 1C) and 3nGT (3n = 72, Fig. 1D).

In the process of distant hybridization, the first hybrid progenies tend to integrate the advantageous parental traits and show heteroses (Groszmann et al., 2015; Li et al., 2016). We measured the amino acids and moisture contents in the four types of fish (Table 6). The results showed that compared with TC, GC had significantly lower levels of TAAs, EAAs, and DAAs. Compared with GC, 2nGGC and 3nGT had 9.35% and 15.18% more TAAs, 17.94% and 15.88% more EAAs, and 11.13% and 16.42% more DAAs, respectively. Meanwhile, the total amino acid contents of 3nGT were higher than that in 2nGGC. The results demonstrated that distant hybridization could not only combine different genomes but could also be an effective way of improving the contents of amino acids in the muscle. Similar results presented in the hybridization of *Carassius auratus cuvieri* (♀) and *Carassius auratus* red var. (♂) (Liu et al., 2017). For these reasons, first, the hybrid nature of the 3nGT had the potential to present higher total amino acids than

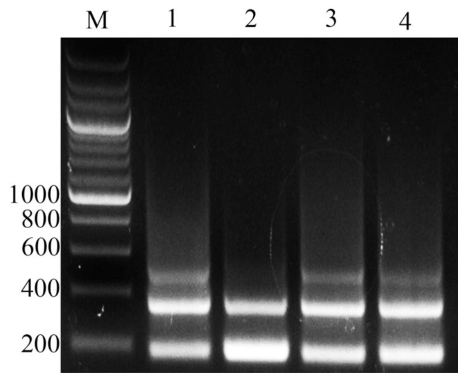

Fig. 5. Agarose gel electrophoresis detection of 5S rDNA in GC, TC, 2nGGC and 3nGT. M, DNA ladder markers (200-bp increments). In lane 1, there are three DNA fragments (approximately 180, 360, and 540 bp) from GC. In lane 2, there are two DNA fragments (approximately 180 and 360 bp) from TC. In lane 3, there are three DNA fragments (approximately 180, 360, and 540 bp) from 2nGGC. In lane 4, there are three DNA fragments (approximately 180, 360, and 540 bp) from 3nGT.

2nGGC. In addition, the subgenome of TC in 3nGT made it exhibit a higher heterosis than 2nGGC in total amino acid contents. Last, we suggested that the offspring selectively expressed the parental genes and exhibited heterosis in the contents of amino acids as well as some other features (Ren et al., 2017). Until now, it was unclear which genes caused an increase in muscle amino acid contents.

It was unclear what feeding habits would be exhibited by the offspring of the crossing of a herbivorous fish and a carnivorous fish. In the hybridization of female GC (herbivorous) and male bighead carp (omnivorous), both types of offspring were herbivorous (Wiley et al., 1986). However, the hybrids of female GC (herbivorous) and male blunt snout bream (herbivorous) were omnivorous (He et al., 2013). Surprisingly, both two types of offspring resembled GC in their feeding behavior when fed grass. Then, we evaluated and compared the pharyngeal teeth 2nGGC, 3nGT and their parents (Fig. 4). The structure of the striped groove in the pharyngeal teeth of GC is related to grinding fibrous grass tissue, while the needle-shaped pharyngeal teeth of TC are associated with a predatory feeding behavior, which reflect the fact that the structure of pharyngeal teeth is related to feeding ecology. The structure of the pharyngeal teeth of the offspring was similar to that of GC, which suggested that these offspring had a wider food source as herbivores. Furthermore, we obtained excellent experimental material to study the inheritance and evolution of fish feeding ecology.

Through distant hybridization, the genetic information in the GC eggs and TC sperm was integrated into the zygote. In previous reports, 5S rDNA, consisting of a variable NTS region and a conserved 5S rRNA region, has been a useful genetic marker for species identification (Wasko et al., 2001), and it has been widely used in the parenthood analysis of distant hybrids with regard to genetics and variation (Hu et al., 2018; Pendas et al., 1995). In this study, the DNA marker of 5S rDNA was used to identify the parentage of 2nGGC and 3nGT. According to the gel electrophoretogram (Fig. 5), GC had 3 bands, TC had only 2 bands, and both 2nGGC and 3nGT had 3 bands. Although we were not sure whether there were TC genome components in the offspring, we could determine that both 2nGGC and 3nGT inherited 5S rDNA from GC, represented by the bands at approximately 540 bp. However, the sequencing results revealed that the coding region was highly homologous in the parents, and the NTS region was enough to distinguish GC from TC. Interestingly, we found two recombined SNPs in the sequencing results (Fig. 6) at positions 132 and 157, respectively. The 'G' → 'A' transition at position 132 was an important point to distinguish 2nGGC from GC because the 'A' was derived from TC, but no other SNP variations were observed. For the sequence at 3nGT-180, theoretically it should be from GC, but the 'G' → 'A' transition at position 132 and the 'T' → 'C' transition at position 157 revealed a genetic recombination of GC and TC at a molecular level.

The chromosome localization of the 5S rDNA (Fig. 7) showed that the probe of TC-188 had two strong signals and two weak signals in TC (Fig. 7B) but no fluorescence signal in GC (Fig. 7A), indicating that the probe exhibited specificity for distinguishing the GC genome from the TC genome. No fluorescence signal was found in 2nGGC (Fig. 7C), meaning they were a diploid gynogenetic GC with two sets of GC chromosomes, not a hybrid of GC and TC. There was one strong and one weak signal in 3nGT (Fig. 7D), suggesting that 3nGT had only one set of chromosomes from TC. The distant hybridization of female GC and male TC revealed that hybridization could cause not only SNPs and gene recombination at the molecular level but also chromosome recombination at the cellular level.

Gynogenesis, a process without syngamy, is a unique reproductive method (Stanley and Sneed, 1974). In a previous study, only a few fish reproduced by natural gynogenesis; however, gynogenetic individuals are much rarer during distant hybridization (Kobayasi et al., 1973; Li et al., 2000; Xiao et al., 2011). In this study, we confirmed the genetic composition of 2nGGC by 5S rDNA analysis and FISH, which revealed that 2nGGC was a gynogenetic GC, not a hybrid of GC and TC. This is the first report of a gynogenetic GC derived from the interspecific hybridization of female GC and male TC, with the same chromosome number, although the formation mechanism was unclear. We believed that the formation of 2nGGC might occur through a process similar to artificial gynogenesis, including sperm inactivation and chromosome doubling. It was difficult to satisfy these two conditions at the same time, which might explain why 2nGGC only accounted for a small part of the offspring. In contrast, 3nGT had two sets of GC chromosomes and

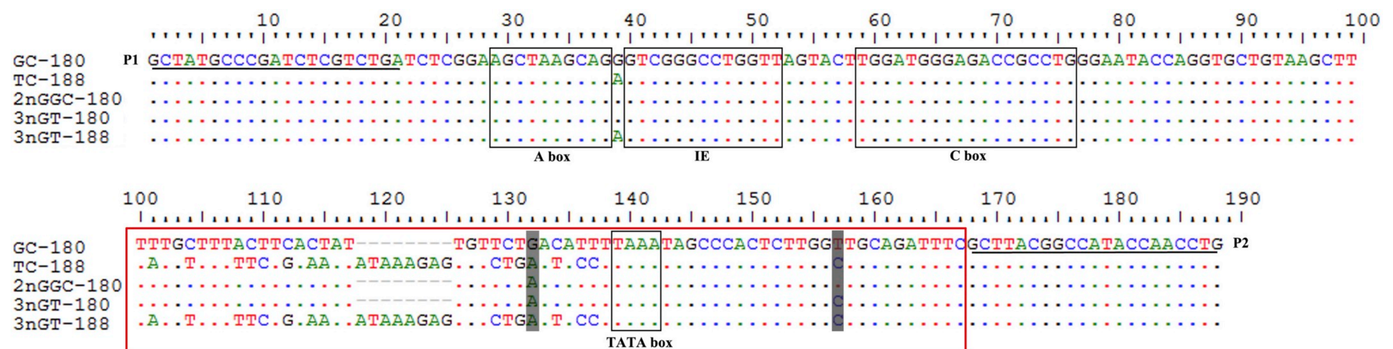

Fig. 6. Nucleotide sequence alignment of the repeated unit in GC, TC, 2nGGC and 3nGT. The NTS region is marked by a red box, and the other regions are the *5S rRNA* genes. The black boxes show the four regulatory elements (A box, IE, C box and TATA box). The shaded areas are recombinational SNP sites in the NTS. The dots indicate sequence identity, and the hyphens represent insertions/deletions. The primers (P1/P2) of 5S rDNA are underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

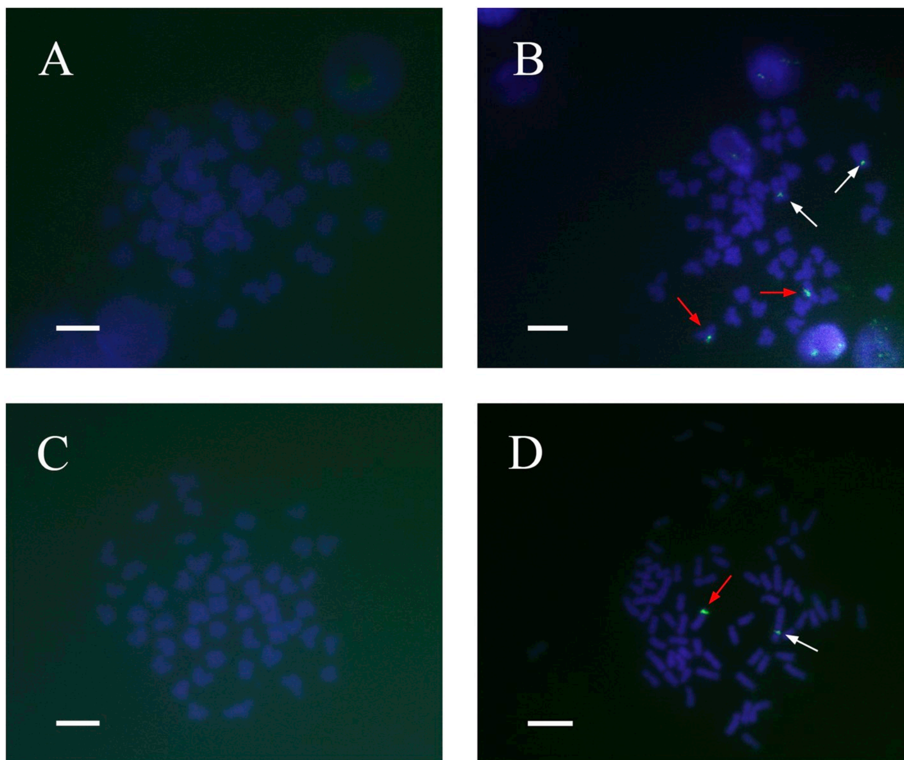

Fig. 7. Examination of hybridizing signals by fluorescence in situ hybridization (probe TC-188) in GC, TC, 2nGGC, and 3nGT. (A) There is no hybridizing signal in GC. (B) There are two strong hybridizing signals (red arrow) and two weak hybridizing signals (white arrow) in TC. (C) There is no hybridizing signal in 2nGGC. (D) There is one strong hybridizing signal (red arrow) and one weak hybridizing signal (white arrow) in 3nGT. 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.)

one set of TC chromosomes and might have been formed by the sperm entering the egg normally, followed by inhibition of the second polar body extrusion during the metaphase of meiosis II, which would cause the GC sister chromatids to fail to separate. In our previous study, we produced the artificial gynogenetic red crucian carp by crossing the female red crucian carp with the male blunt snout bream, and the males and females were found in the artificial gynogenetic red crucian carp (Liu et al., 2010). The artificial gynogenetic red crucian carp lineage was established by the self-crossing of the males and females, forming a new improved fish lineage. In this study, it was very possible to get the males and females in the 2nGCC, which also potentially to form a new improved grass carp lineage with great significance in grass carp breeding.

In summary, we obtained diploid gynogenetic GC and triploid hybrids in the distant hybridization of female GC and male TC, which belong to different subfamilies. The ploidy and genetic composition of the offspring were confirmed by analyzing the DNA content, chromosome number and 5S rDNA and by FISH. Although the male parent is carnivorous, we confirmed that both 2nGGC and 3nGT were herbivorous. However, compared to the female parent, the offspring had higher contents of amino acids in their muscles, indicating that they had a wider food source, lower cost and higher nutritional value for aquaculture. Furthermore, 2nGGC had broad market application prospects in GC aquaculture. Moreover, these progenies also provided good material to study changes in feeding habits, gene recombination and genome fusion.

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