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Production of a diploid hybrid with fast growth performance derived from the distant hybridization of *Hypophthalmichthys nobilis* (female) × *Megalobrama amblycephala* (male)

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ABSTRACT

Based on the distant hybridization derived from *Hypophthalmichthys nobilis* (bighead carp, BIC, 2n = 48) (♀) × *Megalobrama amblycephala* (blunt snout bream, BSB, 2n = 48) (♂), we obtained a novel hybrid (BB) in this study. We comparatively investigated the morphological traits, chromosome numbers, DNA contents, microsatellite patterns, and DNA variations in 5S rDNA and *Hox* genes. The chromosome and flow cytometer results indicated that BB was a diploid hybrid with 48 chromosomes. We confirmed successful hybridization with microsatellite DNA markers and analyzed the *Hox* gene family in the parental and hybrid lines. The sequences of 5S rDNA in BB mapped to those in BIC and BSB, indicating that the genetic materials of BB were derived from BIC and BSB. The breeding experiment showed that BB had the characteristics of herbivore and fast growth. This diploid hybrid is of great significance for fish genetic breeding and applied research.

1. Introduction

Distant hybridization combines the genomes of distantly related species, resulting in genotypic and phenotypic variations in the offspring [1]. A combination of desirable traits from both parents often results in offspring with heterotic characteristics, such as faster growth rates and disease resistance. For example, in the cross between female grass carp (2n = 48) and male blunt snout bream (2n = 48), both the diploid (2n = 48) and triploid (3n = 72) hybrids had faster growth rates than the female parent [2]. By interspecific hybridization of Japanese white crucian carp and red crucian carp, a hybrid with strong disease resistance and fast growth rate was obtained [3]. Morphological characteristics are the external expression of genetic characteristics of species and the most direct and intuitive expression of genetic diversity. By studying the degree of similarity and dissimilarity in morphological traits among fish populations, it is helpful to understand the genetic relationship and classification system among different fish populations, and then discuss the origin and evolution process and trend of fish [4].

Hox genes and 5S ribosomal DNA (5S rDNA) have been widely employed in molecular biology research. In higher eukaryotes, 5S rDNA consists of tandem repeat units composed of a conserved coding region (~120bp) and a variable non-transcriptional spacer (NTS). Moreover, 5S rDNA has been used as a DNA marker to identify or distinguish different species in animals and plants [5]. *Hox* genes are a set of important developmental regulatory genes that are highly conserved and typically organized in a cluster. The evolutionary characteristics of *Hox* genes have accumulated abundant materials used for investigating the origin of species and their divergence. Gene duplication, mutation, and deletion are the basis for studying the evolution of *Hox* genes [6]. In vertebrates, *Hox* genes encode two exons; the highly conserved homeodomain (60 aa) is encoded by the second exon. Recent research has shown that gene duplication, sequence variation, and selective pressure have played crucial roles in the origin and evolution of *Hox* genes [7].

In this study, we compared the chromosome numbers, karyotypes, microsatellite patterns, and genetic variations in 5S rDNA and *Hox* genes of the new artificial hybrid (BB) obtained from the distant hybridization of the parental species, *Hypophthalmichthys nobilis* (bighead carp, BIC, 2n = 48) (♀) and *Megalobrama amblycephala* (blunt snout bream, BSB, 2n = 48) (♂), to obtain information on their molecular relation. The exploration of BB and its related molecular genetic relationship with the parental

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species will provide an insight into the evolution of the selected species. This study is of great importance for enhancing our understanding of fish genetic breeding and species evolution.

2. Materials and methods

2.1. Ethics statement

The guidelines established by the Administration of Affairs Concerning Animal Experimentation state that approval from the Science and Technology Bureau of China and Department of Wildlife Administration is not necessary when the fish in question are neither rare nor near extinction (first- or second-class state protection level). Therefore, approval was not required for the experiments conducted in this study. All fish were deeply anaesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) prior to dissection.

2.2. Animals and crossing procedure

This study was carried out at the State Key Laboratory for Developmental Biology of Freshwater Fishes in Hunan Normal University, Changsha, China. The BIC females (n = 10) and BSB males (n = 10) were obtained from State Laboratory for Developmental Biology of Freshwater Fishes. All BIC and BSB were tested for ploidy by flow cytometry. Three groups were simultaneously conducted during the breeding period (from April to July): in the first group, one mature female BIC and one male BSB were selected as the parents for hybridization, whereas in the other two groups, the self-mating of BIC and BSB were performed, respectively. All progeny groups were produced by artificial spawning. Spawning was induced by injecting chorionic gonadotropin (Ningbo second hormone factory, Ningbo, Zhejiang, China) in doses of 600–800 units/kg body mass, luteinizing hormone releasing hormone (LHRH) A2 in doses of 3 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China) and domperidone in doses of 1–2 μg/kg (Ningbo second hormone factory, Ningbo, Zhejiang, China). The females were given the full dosage in one injection (Human chorionic gonadotrophin (HCG) in doses of 1000–2000 units/kg body mass, LHRH A2 in doses of 5 μg/kg, Domperidone in doses of 3–5 mg/kg). The males were given the full dosage in one injection (Human chorionic gonadotrophin (HCG) in doses of 600–800 units/kg body mass, LHRH A2 in doses of 2 μg/kg). Six hours after the females were given the resolving injection, eggs and sperm were collected by stripping, and the dry method of fertilization was used (water temperature was 22–25 °C). Two minutes after fertilization, the eggs were transferred to incubation tank and incubated until the hatching of larvae at 22–25 °C. Swim-up larvae were collected in Ceramic cylinders and then transferred to rectangular cement basin for rearing for 10 months. These fish fries were reared separately. The water temperature in the cement pond was maintained at 22–25 °C. The pH of the water was between 7.5 and 8, the oxygen content in the water was above 5 mg/L, and the ammonia nitrogen content was below 0.5 mg/L.

2.3. Measurement of DNA contents and preparation of chromosome spreads

To measure the mean DNA contents of erythrocytes in BIC, BSB, and BB, we collected 20 BIC, 20 BSB, and 100 BB at 10 months of age and drew 0.2–0.5 mL blood from the caudal veins of each individual into syringes containing 250–350 units of sodium heparin. All samples were processed following previously described methods [8] and measured by flow cytometry (Partec). The $\chi 2$ test with Yate's correction was used to test for deviations from the expected ratio of the DNA content ratios of the offspring to the sum of BIC and BSB. To further determine the ploidy level of the offspring, kidney tissue were used to prepare chromosomes from 20 BIC, 20 BSB, and 20 BB individuals to count their chromosome numbers following described methods as follows: the experimental fish were injected with PHA (Phytohaemagglutinin) for 1–3 times, with a dose of 2–8 μg/g body weight per time, and the interval time was 12–24 h. Colchicine was injected 2–6 h before sampling, with a dose of 2–4 μg/g body weight. The kidney tissue was removed and cut into pieces with scissors in a petri dish containing 0.8% saline. Subsequently, moved the tissue fragments into the tube and blew in the tube. Centrifuged with 1000 r/min for 5 min and discard supernatant. The collected cells were immersed in 0.075 mol/L KCl hypotonic solution for 0.6–1 h and gently blew during hypotonic process. Then centrifuged and collected precipitation. Fixed, centrifuged and refasten in methanol and acetic acid (3:1) mixture, repeated 2–3 times. Dropped on the frozen slide to dry by flame. Next, Stained by Giemsa dye for microscopic examination. Then, photographed with Pixerapro 600 ES (the USA) digital microscopic camera system. One hundred metaphase spreads from each sample were photographed using a microscope, and the mean chromosome numbers were obtained. The ratios of the diploid hybrid DNA content to the total BIC and BSB DNA content were calculated, and a $\chi 2$ test with Yates's correction was used to test for deviations from the expected ratio.

2.4. Hybrid identification using Hox genes

To determine if BB individuals genetically originated from BIC or BSB, we inferred the genomic composition from *Hox* gene haplotypes using STRUCTURE [9]. We ran STRUCTURE for K-values ranging from 1 to 6 with 20 replicates for each K. Each replicate had a total of 100,000 iterations with the first 50,000 discarded as burn-in. The optimal K-value was 2 based on the STRUCTURE HARVESTER analysis [10]. We combined 20 runs at K = 2 using CLUMPP [11] and constructed a graphic using DISTRUCT [12].

2.5. Test method for measuring body weight

In the first ten days of March 2019, when the fries grew to 2–3 g, 20 individuals randomly selected, and the left fin and right fin were cut as markers. These fries were cultured in the same ceramic cylinder. Three parallel test groups (No. 1, No. 2, and No. 3) were set up with a total of 120 fish (60 BIC and 60 BSB). The fish were raised under the same conditions, and all fish in each ceramic tank were weighed after 30 days of culture. The absolute growth rate of body mass (Average Daily Gain, ADG) was calculated according to the following formula. Absolute growth rate (g/d) =(W2–W1)/(t2-t1)(1). Where W1 and W2 were the body mass measurements (g) at times t1 and t2, respectively. The body mass of fish was measured by an electronic balance (accurate to 0.1 g). The average value and standard deviation of growth data from each parallel group was calculated by Excel software. The significance test of differences was analyzed by SPSS software [13]. When the P value was less than 0.05, the difference was significant, and when the P value was less than 0.01, the difference was extremely significant.

2.6. Morphological traits

At 1 year of age, 20 BIC, 20 BSB, and 20 BB individuals were randomly selected for morphological examination following previously described methods [14]. SPSS v17.0 (IBM Corp., NY, USA) was used to analyze the covariance of the measurable and countable morphological traits among fish samples. The level of statistical significance was set at $p < 0.05$. We fed 10 BIC, 10 BSB and 10 BB in an open pond to observe their feeding habits. By feeding with aquatic plants (Eichhornia crassipesSolms) and feed in the pond, we observed the fishes' food intake directly and found that both the grass and feed were ingested.

2.7. Extraction, PCR amplification, cloning, and sequencing

The total genomic DNA was extracted from the peripheral blood cells of 10 BIC, 10 BSB, and 10 BB following previously described methods [15]. A set of primers (5S forward primer: 5′-GCTATGCCCGATC TCGTCTGA-3'; 5S reverse primer: 5′-CAGGTTGGTATGGCCGTAAGC-3′)

and 12 combinations of degenerate PCR primers (*HoxA4a*, *HoxA9a*, *HoxB2a*, *HoxB3a*, *HoxB1b*, *HoxB5b*, *HoxB6b*, *HoxC4a*, *HoxC6b*, *HoxD4a*, *HoxD9a*, and *HoxD10a*) were used to amplify the DNA. The PCR primers were shown in Table S1. The PCR assays and thermally programmed steps were conducted following previously described methods [16]. The PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China). The plasmids were transformed into *Escherichia coli DH5a*, purified, and sequenced with vector-specific primers using the primer-walking method on an ABI 3730XL automatic sequencer (ABI PRISM 3730; Applied Biosystems, CA, USA).

2.8. Microsatellite DNA cloning and sequencing

Total genomic DNA was isolated from whole blood collected from the caudal vein of 10 BIC, 10 BSB, and 10 BB individuals using a standard phenol-chloroform procedure. The DNA concentration and quality were assessed using agarose gel electrophoresis. The primer pair was obtained from the whole genome of experimental fish [17]. The microsatellite loci were amplified, and sequencing was performed following previously described methods [18].

2.9. Sequence comparison and analysis

The sequence homology and variation among the fragments amplified from BIC, BSB, and BB were analyzed using BioEdit [19,20]. To increase the probability of detecting duplicated paralogs and circumventing errors resulting from the PCR assay of 5S rDNA and 12 *Hox* genes, we sequenced 20 clones of each gene from BIC, BSB, and BB. The obtained sequences were screened for 5S rDNA and *Hox* gene fragments using BLAST [21] to determine their origin. The *Hox* gene sequences were aligned using a Maximum Likelihood (ML) phylogenetic tree was constructed using MEGA 7.0 [22]. We assigned PCR fragments based on the identity of the subtree in which they were located. A phylogenetic analysis was performed using the ML method, and the best fit nucleotide substation model with the lowest BIC score was determined using MEGA v7.0 [22]. Conserved regions were determined using Gblocks. ML analyses were performed using the GTR + G model, and the robustness of the tree topology was assessed with 1000 bootstrap replicates [23].

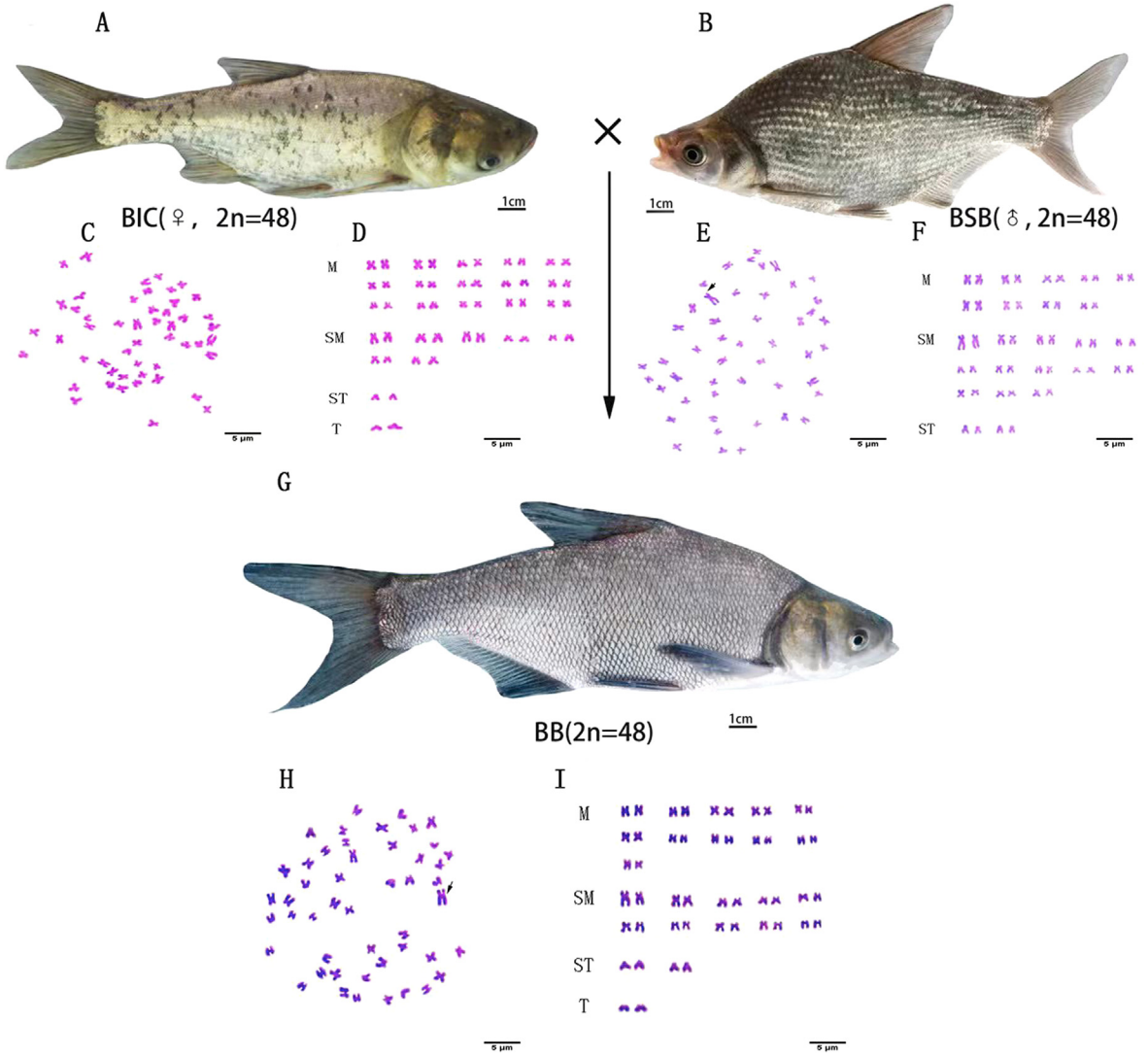

Fig. 1. Formation procedure, chromosomal traits, and appearance of BIC, BSB, and BB (A) The appearance of BIC (B) The appearance of BSB (C) The chromosome number of BIC (D) The chromosome karyotype of BIC (E) The chromosome number of BSB (F) The chromosome karyotype of BSB (G) The appearance of BB (H) The chromosome number of BB (I) The chromosome karyotype of BB Black arrows indicate larger pairs of submetacentric chromosomes.

3. Results

3.1. Formation of the new diploid hybrid

In the present study, BB (Fig. 1G) was artificially produced by crossing female BIC (2n = 48) (Fig. 1A) with male BSB (2n = 48) (Fig. 1B). BB exhibited a high fertilization rate (>95.8%) and hatching rate (>89.6%) (Table S2). In this experiment, the fertilization rate and hatching rate of BIC and BSB were 90.36%, 91.25% and 85.37%, 86.29%, respectively. Quantifiable trait ratio results showed that the body of BB was flatter than that of BIC. The head of BB was similar to that of BIC, and the body of BB was similar to that of BSB (Fig. 1).

3.2. Measurement of DNA contents and examination of chromosome numbers

Using the total DNA contents of BIC and BSB as references, the mean DNA content of BB was equal to half of the sum of the BIC and BSB DNA contents ($P < 0.05$) (Fig. 2; Table 1), suggesting that BB might inherit one set of chromosomes from each parental line.

For the karyotype analysis, 100 metaphase spreads from both parental species and the hybrid were photographed (Fig. 1; Table 2). Of the photographs, 95.0% showed 48 chromosomes in BIC (Table 2); thus, we inferred that BIC was diploid with a karyotype of 2n = 2x = 48 (Fig. 1C) and 30 m + 14sm + 2st + 2t (Fig. 1D). Of the BSB photographs, 95.0% showed that BSB was diploid and had 48 chromosomes with 18 m + 26 sm + 4st (Fig. 1E and F). A larger pair of submetacentric chromosomes was observed in BSB (black arrow), which could be an ideal chromosomal marker for this species (Fig. 1E). Additionally, 90.0% of the chromosomal metaphase supported that BB was diploid and had a karyotype of 22 m + 20 sm + 4 st + 2 t (Fig. 1 H, I). We also found a large pair of submetacentric chromosomes in BB, which supports these candidate chromosome markers for identification of the BB hybrid.

Table 1
Mean DNA content of BIC, BSB, BB.

Fish type	Mean DNA content	Ratio	
		Observed	Expected
BIC	49.64		
BSB	65.25		
BB	58.03	(BIC + BSB)/2*BB = 0.99[a]	1

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

Table 2
The chromosome numbers of BIC, BSB, and BB

Fish type	Distribution of chromosome number			
	No. in metaphase	<48	48	>48
BIC	100	3	95	2
BSB	100	4	95	1
BB	100	5	92	3

3.3. Morphological traits

As shown in Table 3, there were obvious differences in the morphological traits (countable and quantifiable traits) between BB and the parental species (BIC and BSB). All morphological traits of BB were intermediate between the parental phenotypes. The number of dorsal fin of

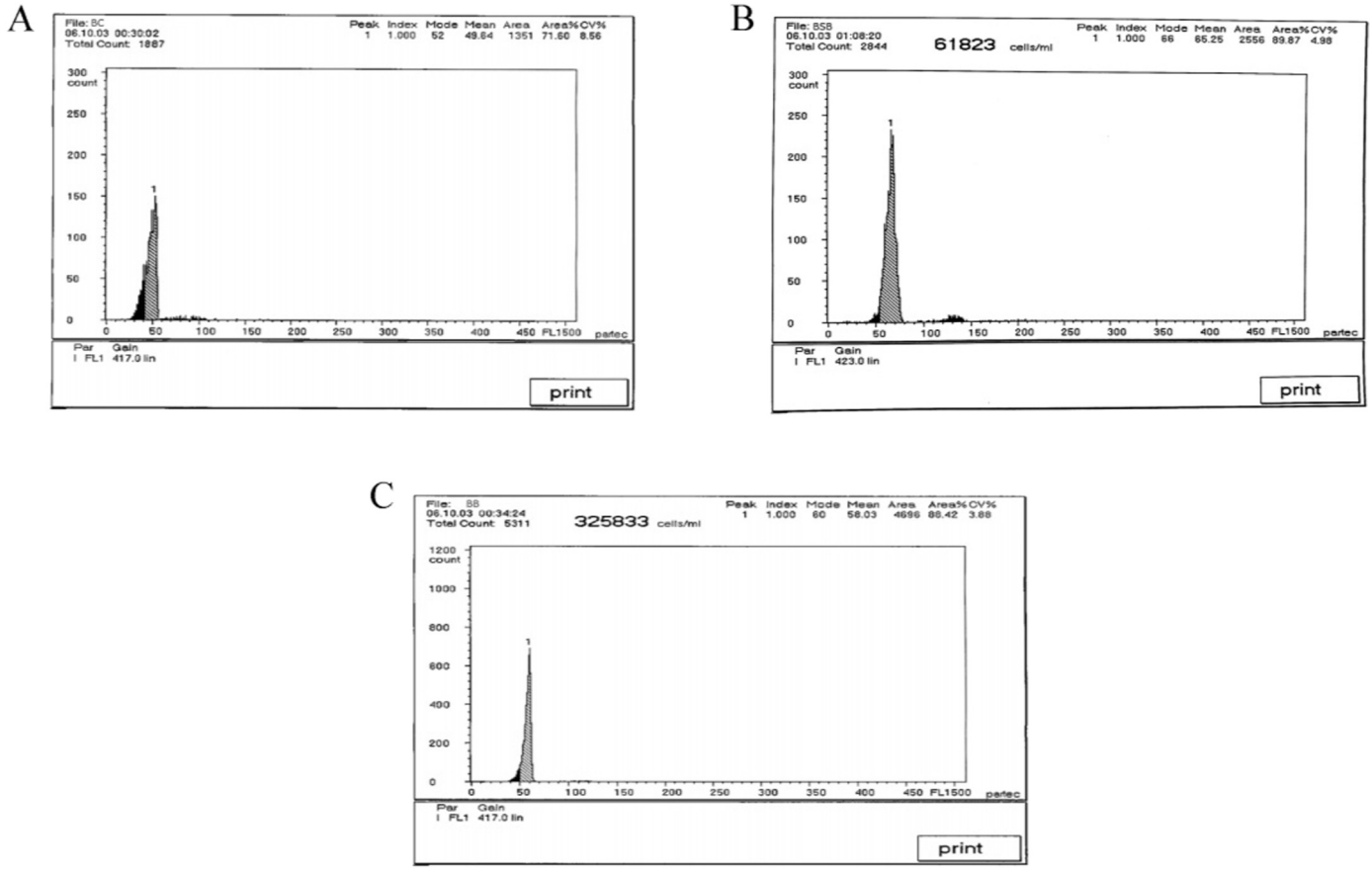

Fig. 2. Cytometric histograms of DNA fluorescence for BIC, BSB, and BB (A) The mean DNA content of BIC (peak, 1:49.64)(B) The mean DNA content of BSB (peak, 1: 65.25) (C) The mean DNA content of BB (peak, 1:58.03).

Table 3
The countable and measurable traits of BIC, BSB, and BB.

Phenotypes	Types of fish		
	BIC	BSB	BB
BL/WL	0.75 ± 0.01	0.82 ± 0.02	0.78 ± 0.01[a]
BH/BL	0.30 ± 0.01[a]	0.43 ± 0.03[b]	0.31 ± 0.01[a]
HL/BL	0.33 ± 0.04[a]	0.22 ± 0.01[b]	0.24 ± 0.02[b]
HH/HL	0.72 ± 0.05[a]	0.86 ± 0.02[b]	0.67 ± 0.01[a]
HH/BH	0.78 ± 0.01[a]	0.44 ± 0.03[b]	0.55 ± 0.03[c]
CPH/CPL	0.48 ± 0.10[a]	1.05 ± 0.01[b]	0.73 ± 0.01[c]
No. of lateral scales	103–105	49–52	67–69
No. of upper lateral scales	28–29	8–10	15–18
No. of lower lateral scales	15–16	9–11	11–14
No. of dorsal fins	III+7-8	III+8-9	III+7-9
No. of abdominal fins	III+7-9	III+8-10	III+8-9
No. of anal fins	III+13-14	III+25-27	III+17-18

The different letters represent significant differences ($P < 0.05$).

BB was more similar to that of BIC. The number of dorsal fin differed significantly between BB and BSB ($P < 0.05$). For the number of lower lateral scales, BB exhibited numbers outside the range of both parental trait values.

Feeding with aquatic plants and feed separately and long term follow-up observation, it was found that BIC all ingested two kinds of food. In addition, we put together a feed preparation (feed and aquatic plants) and found that BSB and BB ingested aquatic plants. Therefore, we concluded that BB were herbivorous fish.

3.4. Hybrid identification and microsatellite DNA

To determine if BB was hybridized from BIC and BSB, we examined the genomic composition of BIC, BSB, and BB using STRUCTURE. The two parental species (BIC and BSB) corresponded to the second cluster and were exclusively assigned to one of the two clusters. All BB individuals showed mixed ancestries, suggesting that the BB individuals genetically originated from BIC and BSB (Fig. 3).

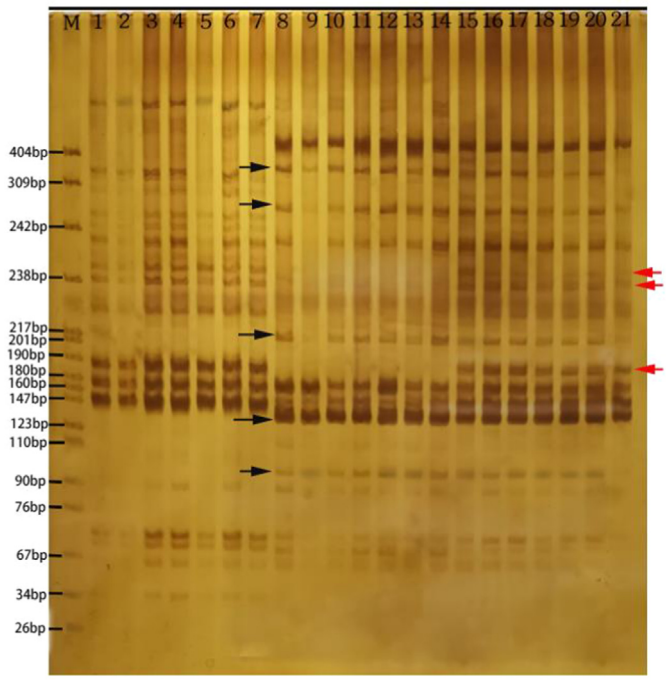

Fig. 4. Microsatellite patterns of BIC (1–7), BSB (8–14), and BB (15–21) Electropherogram of microsatellite DNA patterns produced by the primer pair MFW-M in BIC, BSB, and BB. Lanes 1–7 represent BIC; Lanes 8–14 represent BSB; Lanes 11–15 represent BB. Black arrows indicate DNA bands derived from BSB. Red arrows indicate DNA bands derived from BIC. M represents the pBR322 DNA/Mspl Marker.

To detect microsatellite DNA patterns among BIC, BSB, and BB, we amplified fragments using the TTF-EST primers. BB exhibited some DNA fragments similar to those of BIC (Fig. 4, red arrows) and some similar to

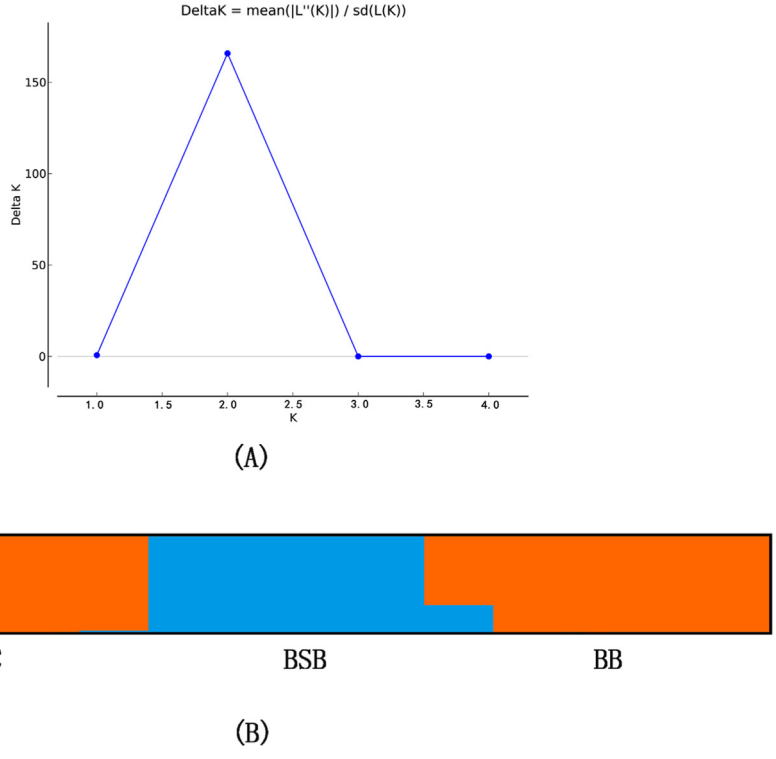

Fig. 3. Genetic relation of BIC, BSB and BB STRUCTURE displayed for the optimal number of clusters (K = 2)(A), BSB cluster was blue, BIC cluster was yellow (B).

those of BSB (Fig. 4, red arrows), suggesting that BB inherited genomic DNA from both BIC and BSB. BIC, BSB, and BB were aligned by BioEdit to analyze sequence homology and variation.

3.5. Results of the weight comparison

We used a balance to measure weights of BIC and BB from each ceramic cylinder. The comparison of the growth rates of BIC and BB was shown in Table 4. Table 4 showed that the absolute growth rate of the mass of No. 1 BB was 0.47 ± 0.03 g/d, while that of BIC was 0.32 ± 0.03 g/d, showing that the growth of BM was 46.87% higher than that of BSB. The value for No. 2 BB was 0.47 ± 0.03 g/d, while that of BIC was 0.33 ± 0.03 g/d, showing that the growth of BM was 46.67% higher than that of BSB. The value for No. 3 BB was 0.47 ± 0.03 g/d, while that of BIC was 0.32 ± 0.03 g/d, showing that the growth of BM was 46.87% higher than that of BSB. The results showed that there was a significant difference in body the growth rate between BIC and BB.

3.6. Genetic variation analysis of 5S rDNA sequences

Both parents and the hybrid offspring showed two bands in 5S rDNA gene electrophoresis. We further sequenced 20 clones for each band. The sequencing results revealed that these sequences were a simple unit composed of a 120-bp coding sequence (CDS) for the 5S rDNA gene and an NTS region (~60 bp).

The sequence similarity analysis showed that the similarity of the 178-bp sequences from BIC and BB was 96.6%, and the similarity of the 186-bp sequences from BSB and BB was 90.4%. The 178 -bp bands of BB were both from BIC, and the 367-bp sequences was a chimeric sequence (the NTS1 was from BIC and the NTS2 was from BSB). (Figs. 5 and 6).

3.7. Phylogenetic analysis

The organization of the *Hox* clusters in BIC, BSB, and BB was shown in Tables S3, S4 and S5. Thirteen *Hox* gene family members from BIC, BSB, and BB were analyzed using the HKY + G + I model in MEGA v7.0 to estimate the genetic divergence between these species. The phylogenetic tree of the genes was constructed using the ML method based on the genetic distance. The confidence level was tested using 1000 bootstrap replicates (Fig. 7). The phylogenetic tree showed that the genetic material of BB originated from BIC and BSB. Through different *Hox* genes subunits, it was proved that BB was a hybrid of BIC and BSB.

4. Discussion

Long-term biological research on the evolutionary aspects of fish hybridization has shown that hybridization was an effective way to increase genetic diversity and leads to both diploid and polyploid hybrids. It had an important role in germplasm creation and genetic improvement of animals [1]. Our laboratory had carried out a long and systematic research work on distant hybridization and successfully produced several excellent lines such as the allotetraploid crucian carp line (F_3–F_{30}) [17], the autotetraploid fish line (F_2–F_{13}) [24] and the allodiploid hybrids derived from the female *Megalobrama amblycephala* × male *Erythroculter ilishaeformis* [25]. These distant hybridization offspring showed obvious genetic and variation at DNA level. The good features of both parents were integrated in appearance.

In this study, we obtained BB hybrids by crossing BIC and BSB. These hybrids differed from their parental species. Of the traits analyzed in this study, BB was phenotypically intermediate to BIC and BSB and significantly differed from the parental species. The appearance characteristics of results showed that all measurable and countable traits of BB were between BIC and BSB, which indicated that BB had obvious heterozygote, and the measurable traits were in line with intermediate type. In the quantifiable traits, BB was significantly different from its parents ($P < 0.05$), indicating that the appearance of BB has a certain degree of

Table 4
Comparison and analysis of growth rate in BIC and BB.

Group number	Fish name	Initial mass/g	Terminal mass/g	Absolute growth rate of body mass ($g.d^{-1}$)
No. 1	BIC	2.52 ± 0.08	11.80 ± 0.98	0.32 ± 0.03
	BB	2.44 ± 0.10	13.52 ± 0.76	0.47 ± 0.03
No. 2	BIC	2.50 ± 0.12	11.94 ± 0.91	0.33 ± 0.03
	BB	2.48 ± 0.08	16.02 ± 0.86	0.47 ± 0.03
No.3	BIC	2.54 ± 0.11	11.95 ± 0.92	0.32 ± 0.03
	BB	2.42 ± 0.15	16.08 ± 0.84	0.47 ± 0.03

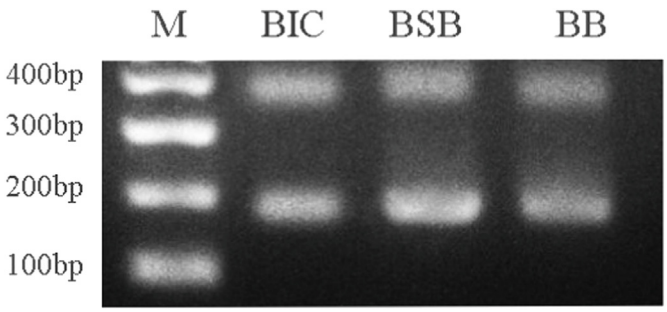

Fig. 5. PCR amplification map of 5S rDNA from BIC, BSB, and BB DNA bands amplified from BIC, BSB, and BB. M: DNA ladder (100 bp increments); Lane 1 (BIC): two DNA bands (~200 and ~400 bp) from BIC; Lane 2 (BSB): two DNA bands (~200 and ~400 bp) from BSB; Lane 3 (BB): two DNA bands (~200 and ~400 bp) from BB.

variation. However, the BH/BL and HH/HL of BB were more similar to that of BIC, showing the characteristics of large head. The BL/WL, HL/BL and HH/BH of BB were more inclined to BIC, showing the characteristics of lateral flattening. It could be seen that BB has inherited the unique shape characteristics of its parents. In the countable traits, the number of dorsal fin and abdominal fin of BB was more similar to that of BIC. The results showed that BB inherited the appearance characteristics of its parents, showing a certain hybridization.

In terms of phenotype, the hybrid offspring integrated the advantages of both parents, so that the offspring shown heterosis in appearance, growth rate, survival rate and disease resistance [26]. In this study, *Aristichthys nobilis* and *Megalobrama amblycephala*, were selected as parents with the same chromosome number (48) and different dominant traits. *Aristichthys nobilis* belongs to the *Aristichthys* and *Megalobrama amblycephala* belongs to the *Megalobrama*. The two crosses were intergeneric crosses. The genetic characteristics and heterosis of the hybrid offspring were analyzed by fertilization rate, hatching rate, ploidy, *Hox* genes, microsatellite DNA patterns and 5SrDNA. The results showed that the hybrids of bighead carp and blunt snout bream had high fertilization rate and hatching rate with very neat specifications, which were close to the corresponding data of their parents during self-crossing, which were significantly different from the low fertilization rate and hatching rate exhibited by most distant crosses. In terms of feeding, the hybrid fish had herbivore characteristics and obvious breeding advantages. The results of DNA content determination and chromosome preparation showed that the hybrid offspring were still diploid fish, and there was no polyploidization, which was different from that of other distant hybridization combinations.

In terms of chromosomes and karyotypes, both BIC and BSB had 48 chromosomes. BB also had the same chromosomes with a karyotype of 22m + 20sm + 4st + 2t. We determined that the chromosomal composition of BB consisted of one set from BIC and one set from BSB. Based on

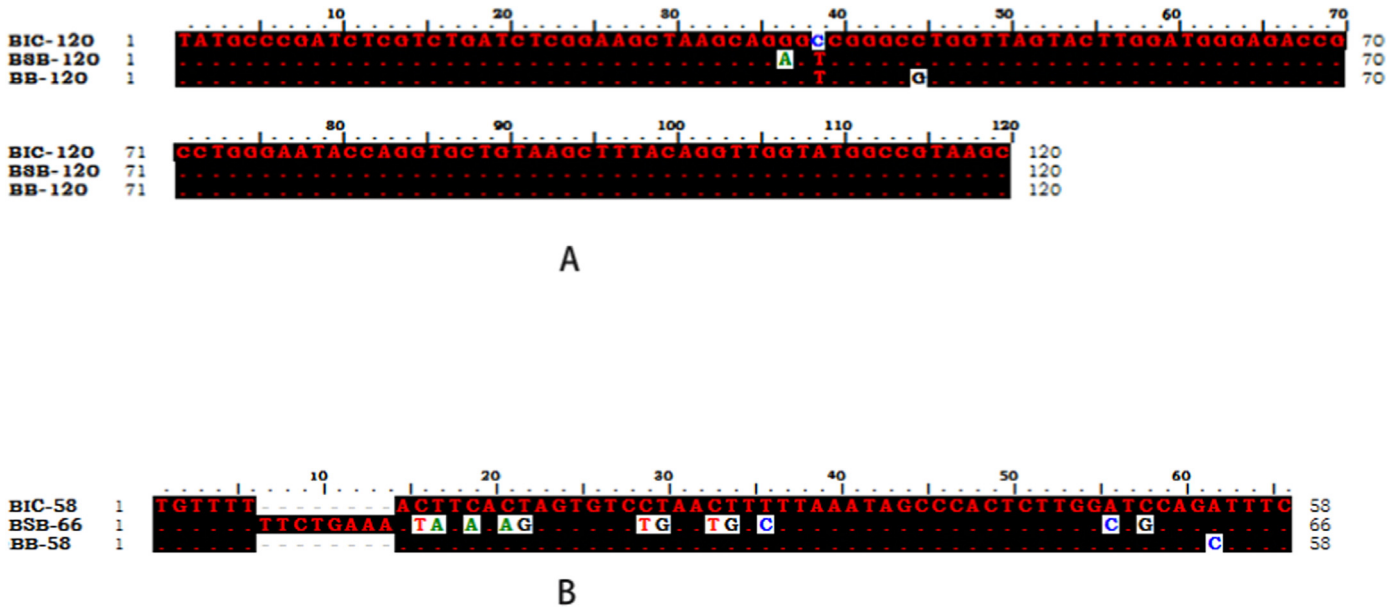

Fig. 6. Comparison of the NTS sequences from BIC, BSB, and BB Dots indicate identical nucleotides.

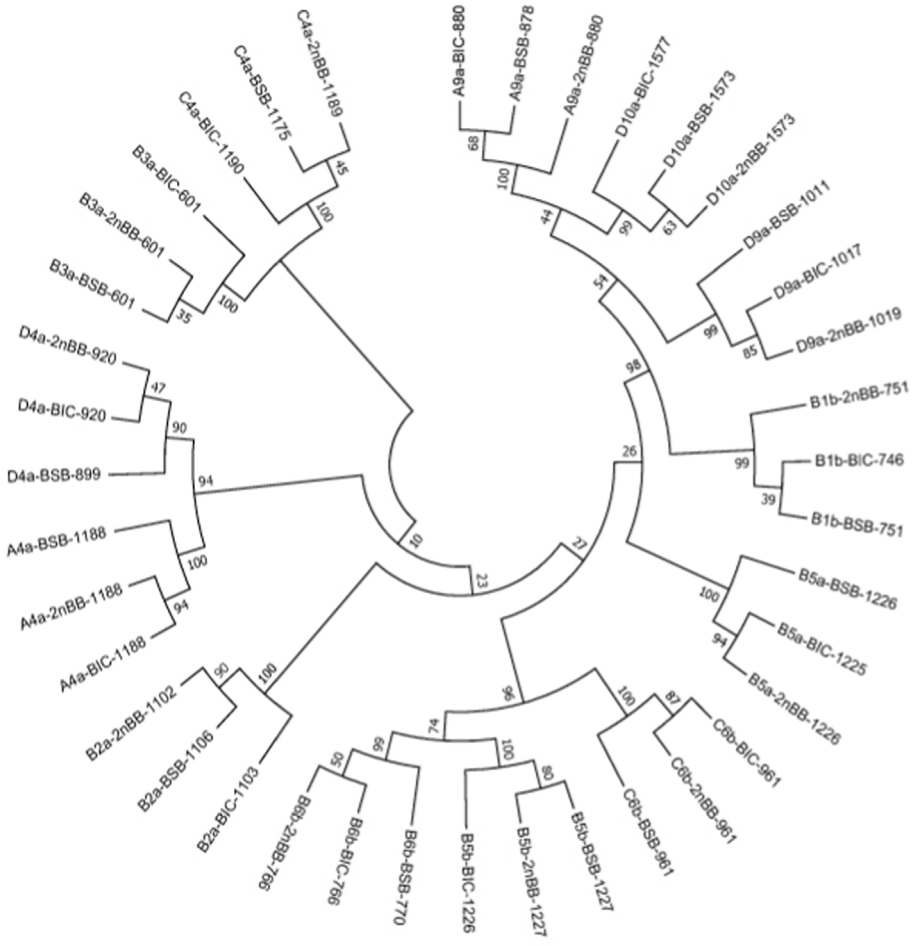

Fig. 7. Nucleotide-based ML tree representing the phylogenetic relationships of the putative *Hox* genes obtained from BIC, BSB, and BB The numbers at each node represent the percentage bootstrap value of 1000 replicates. The numbers along the horizontal lines represent the self-expanding value.

these findings, we inferred that BB was a diploid hybrid.

In this hybrid combination, the hybrid progenies showed certain hybrid characteristics in appearance, and the countable and proportional traits were between the parents. The hybrid progenies showed obvious

hybrid characteristics in appearance, but did they also have changes in genetic material? Therefore, its molecular genetic characteristics were studied.

Microsatellite markers exhibited high polymorphism and rich informational content. Microsatellite markers were widely used in gene linkage and genetic map construction, genetic diversity research, pedigree and development research, and individual and pure line tests [27]. In this study, TTF-EST primers were used to amplify a DNA fragment from BIC, and this DNA fragment appeared in BB. The DNA fragment from BSB was also inherited. Notably, there were several BIC and BSB DNA fragments inserted into the BB genome, resulting in substantial variation of their performance.

The 5S rDNA amplification and sequencing results of BIC, BSB, and BB showed that there was two 5S rDNA structural unit in BB. According to the sequence comparisons of the common CDS, only a few base changes were found. By comparing the NTS regions of BIC, BSB, and BB, we found that BB was highly similar to BIC and BSB, supporting the notion of genetic similarity between BIC and BSB. The average similarity between BB and BIC was obviously higher than between BB and BSB, indicating that the genetic materials of BB were mainly inherited from BIC. There were two types of 5S rDNA fragments in BB, among which 180bp type was from BIC and 368bp type was a chimeric sequence (the first sequence was from BB and the second sequence was from BIC), showing hybrid characteristics. The 5S rDNA of BB and their parents were sequenced and analyzed. The results showed that BB inherited the 5S rDNA structure unit of parents, showing high heterozygote.

The results of the *Hox* gene phylogenetic tree showed that most of the *Hox* genotypes in BB inherited the genotype of BIC, and BB was more similar to BIC. At the same time, there was a certain degree of variation in the base sites in the recombinant genotype, such as base deletion and mutation. It might be due to the distant relatives of the parents, resulting in the fusion of incompatible genome combinations with each other, resulting in mutation sites.

In summary, the establishment of the BB hybrid was novel in respect to the genetics and breeding of different subfamily hybrids. BB had obvious growth advantages and thus has potential economic benefits for the aquaculture industry.

The acquisition of the hybrid of *Aristichthys nobilis* and *Megalobrama amblycephala* not only provided a new material to study the genetic variation of hybrid offspring, but more importantly in terms of production applications. This hybrid fish, which combined the advantageous characteristics of *Aristichthys nobilis* and *Megalobrama amblycephala*, could be used to cross with *Aristichthys nobilis* to obtain improved *Aristichthys nobilis* and with *Megalobrama amblycephala* to obtain improved *Megalobrama amblycephala*, which had great application prospects in fish breeding and production.

Author contributions

YD.W, YX. L, SJ. L designed the study, carried out the analyses and prepared and draft the manuscript. SJ.L, YD.W, C. G, RR. Z and AM. L performed the technical discussions. HF. T, S. W, KK. L, JJ. Y and YX.L participated in data simulation and discussions. QB. Q, C. Z and M. T were involved in the statistical analysis. All the authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.repbre.2022.05.002.

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