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RESEARCH

Integrated analysis of muscle developmental features and molecular characterization of myogenic regulatory factors in two hybrid breams

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Abstract Distant hybridization combines beneficial genes from different species to create high-quality germplasm with altered phenotypes and genotypes. In our previous study, two fast-growing hybrid breams (BTBB and BBTB) were developed through a multi-step breeding strategy involving distant hybridization between blunt snout bream (*Megalobrama amblycephala*, 2n = 48, BSB, ♀) and topmouth culter (*Culter alburnus*, 2n = 48, TC, ♂), followed by two rounds of backcrossing. To elucidate the mechanisms of muscle development and rapid growth, we compared growth performance, muscle texture, antioxidant enzyme activities, and the expression of myogenic regulatory factor (MRF) genes between the hybrids and their parents. The results showed that both BBTB and BTBB had significantly greater body weight and body length than BSB ($p < 0.05$). Histological analysis showed that the two hybrid breams had significantly lower muscle fiber density ($p < 0.05$) and significantly larger muscle fiber diameter than BSB ($p < 0.05$). Muscle texture analysis indicated that BBTB had higher adhesiveness than BSB, whereas BTBB exhibited significantly greater springiness than BSB. Antioxidant analysis showed that, compared with BSB, MDA levels were significantly lower in BBTB and BTBB ($p < 0.05$), whereas CAT, SOD, and GSH levels were higher. Sequence analysis showed that the full-length cDNA sequences of *myod*, *myog*, *myf5*, and *myf6* were highly conserved between the hybrid offspring and their parents. RT-qPCR analysis revealed that the expression levels of MRFs and *mstn* were relatively high in the muscle tissues of both BTBB and BSB, whereas *mstn* expression was lower in BTBB than in BSB, suggesting that MRFs and *mstn* may act coordinately in the regulation of rapid growth in BTBB. In contrast, the expression levels of MRFs and *mstn* in BBTB were significantly lower than those in BSB, suggesting that MRF expression may increase transiently during rapid growth and then decline. Overall, the two hybrid breams exhibited distinct molecular regulatory patterns, and their superior muscle phenotypes may be associated with muscle fiber development, enhanced antioxidant capacity, and the balance between positive myogenic regulation

Zhifeng Zhou and Xiangqiong Yang contributed equally to this work.

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and *mstn*-mediated negative regulation. These findings indicate that BBTB and BTBB are promising germplasm resources for aquaculture breeding.

Keywords Distant hybridization · Growth performance · Muscle characteristics · MRFs · MSTN

Introduction

Distant hybridization is an effective breeding strategy for generating novel genetic combinations and improving economically important traits in fish, including growth performance, environmental adaptability, and stress resistance (Wang et al. 2019). Blunt snout bream (*Megalobrama amblycephala*, BSB, 2n = 48) and topmouth culter (*Culter alburnus*, TC, 2n = 48) are economically significant fish species. Through distant hybridization, we successfully developed a bisexual, fertile allodiploid hybrid (BTF_1, 2n = 48) by crossing BSB as the female parent with TC as the male parent. Subsequently, BTF_1 was utilized as the female parent in a backcross with male BSB, yielding the fertile diploid hybrid BTB (2n = 48). Further reciprocal backcrossing of BSB (♀) × BTB (♂) and BTB (♀) × BSB (♂) yielded two additional fertile diploids, designated as BBTB (2n = 48) and BTBB (2n = 48), respectively (Gong et al. 2021, 2022). These backcross hybrids provide useful materials for evaluating growth-related variation and muscle quality in hybrid bream.

Fish muscle growth depends on both hyperplasia and hypertrophy of muscle fibers, with the number of muscle fibers undergoing dynamic alterations throughout their life cycle (Wang et al. 2021). Hyperplastic expansion of muscle fibers occurs in two distinct phases: the initial stage of layered hyperplasia during the late embryonic period, which results in an increase in muscle segment volume and the differentiation of muscle fibers into red and white muscle types (Steinbacher et al. 2006). This is followed by mosaic hyperplasia, during which muscle fibers continue to proliferate, forming a mosaic pattern, with fiber numbers potentially increasing by up to fivefold (Rescan 2005; Johnston et al. 2009). Muscle fiber hypertrophy primarily takes place during the later stages of embryogenesis and continues until the fibers reach their maximal diameter (Veggetti et al. 1990). Muscle texture characteristics are critical parameters for evaluating fish muscle quality. Fish texture is typically assessed through sensory evaluation and instrumental measurements. Sensory evaluation generally encompasses attributes such as appearance, flavor, palatability, fiber perception, hardness, and elasticity (Ren et al. 2016). Instrumental measurements commonly involve stretching, shearing, extrusion, or puncturing methods to determine textural properties. The results are typically expressed using metrics such as shear force, texture profile analysis (TPA), or rupture force to characterize fish muscle texture (Zhang et al. 2017).

During aquaculture practices, factors such as fluctuating water temperatures, hypoxia, human activities, and feeding can induce stress responses in fish, potentially leading to the overproduction of reactive oxygen species (ROS). This may provoke oxidative stress and lipid peroxidation, ultimately impairing cellular function. The elevated levels of malondialdehyde (MDA), a product of lipid peroxidation, are commonly used as an indirect indicator of ROS accumulation and cellular damage (Li et al. 2008; Liu and Zhai 2012). The antioxidant defense system, comprising superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), plays a critical role in regulating ROS levels and maintaining the balance between oxidation and antioxidation (Glorieux and Calderon 2017; Panday et al. 2020; Lu et al. 2022).

At the molecular level, the myogenic regulatory factors (MRFs), including MyoD, MyoG, Myf5, and Myf6, play essential roles in muscle development, regulating muscle cell proliferation, differentiation, and fiber formation (Buckingham and Rigby 2014). These factors share three conserved domains: the basic helix-loop-helix (bHLH) domain, and adjacent N- and C-terminal regions, which contribute to their distinct functions (Davis and Weintraub 1992; Hernández-Hernández et al. 2017). MRFs are classified into early factors, such as Myf5 and MyoD, that promote muscle precursor cell specification and proliferation, and late factors, like MyoG and Myf6, which drive the terminal differentiation of muscle fibers (Asfour et al. 2018). Expression patterns vary among species, with Myf5 and MyoD typically expressed earlier than MyoG and Myf6 (Kassar-Duchossoy et al. 2004). In contrast, myostatin (MSTN), a member of the TGF-β superfamily, primarily regulates muscle growth by inhibiting fiber hyperplasia and hypertrophy (McPherron et al. 1997).

In mammals, MSTN is primarily expressed in skeletal muscle, whereas in fish, its expression extends across various tissues, with levels varying among species (McPherron and Lee 1997; Li et al. 2007). In the present study, we focused on the MRF gene family, whose members are the principal positive regulators of skeletal muscle development. Full-length cloning and sequence analysis of these genes were further conducted to determine whether the phenotypic differences among the groups were associated with coding-sequence variation or were more likely attributable to differences in gene expression regulation.

Therefore, this study systematically compared growth performance, muscle histology, texture characteristics, antioxidant status, and muscle-related gene expression among BBTB, BTBB, BSB, and BTB. By integrating phenotypic and molecular data, we aimed to clarify the biological basis underlying differences in muscle growth and quality among the two backcross hybrids and their parental lines, and to provide a theoretical basis for germplasm improvement and hybrid breeding in bream.

Materials and methods

Production of two hybrid breams

All of the fish in this study were obtained from the Yuelushan Laboratory Aquatic Variety Breeding Center, Hunan Normal University, China. A total of 40 specimen fish were selected as parents, comprising 10 females and 10 males each of BSB and BTB. BBTB was prepared by backcrossing female BSB and male BTB, and BTBB was prepared by backcrossing female BTB and male BSB. After fertilization, they hatched at 24 ~ 26 °C. Finally, all the hybrid fry were transferred to the pond for further culture.

Preparation of experimental materials

Healthy 1-year-old individuals were randomly selected from the BBTB, BTBB, BSB, and BTB, with three biological replicates collected for each group. All fish were reared in ponds of identical size (5 m × 2 m × 1.5 m) under the same husbandry and management conditions throughout the experimental period. Fish were fed twice daily with Tongwei commercial feed, consisting of sinking and floating pellets mixed at a ratio of 3:1. During the rearing period, the ponds were maintained under continuous flow-through conditions, and the water pH was maintained at 7.3 ~ 7.5. At sampling, nine fish were randomly sampled from the BBTB (weighing 136.6 ~ 152.7 g, length 17.5 ~ 18.4 cm), BTBB (weighing 135.8 ~ 148.4 g, length 18.7 ~ 19.6 cm), BTB (weighing 135.6 ~ 152.7 g, length 16.8 ~ 19.6 cm), and BSB (weighing 47.7 ~ 57.0 g, length 12.8 ~ 13.8 cm) groups. Bilateral dorsal muscle tissues were excised, immediately flash-frozen in liquid nitrogen, and subsequently stored at − 80 °C to preserve sample integrity for downstream analyses.

Measurement of muscle fibers growth characteristics

The body weight and body length of each experimental fish were measured separately, and the muscle condition factor (K) was calculated using the formula $K = (W/L^3) \times 100$, where K represents the muscle condition factor, W the body weight (g), and L the body length (cm). Subsequently, mid-dorsal muscle tissue was excised into sections of approximately 2 cm × 0.5 cm × 0.5 cm and fixed in 1 mL Bouin's solution in an EP tube for 24 h. The samples were then dehydrated in a graded ethanol series, cleared with xylene, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) following standard protocols (Xiao et al. 2014; Wu et al. 2022). Microscopic observation was conducted, with one section per fish selected for analysis. Three regions per section were imaged at × 20 magnification, and the diameter and cross-sectional area of muscle fibers were quantified using ImageJ software. The resulting measurements were statistically analyzed using Excel software.

Determination of muscle texture properties

Pre-frozen muscle samples were sectioned into 1 cm × 1 cm × 0.5 cm rectangular blocks for analysis. TPA was conducted using a TMS-Pro texture analyzer (Food Technology Corporation, USA), equipped with a 250 N load cell and a flat-bottomed cylindrical probe (P/36) with a diameter of 8 cm. The TPA mode was configured with probe speeds of 2 mm/s prior to testing, 1 mm/s during testing, and 5 mm/s post-testing. The stroke height was set to 10 mm, with an initial force of 0.1 N, a compression ratio of 60%,

and two compression cycles with a 2seconds interval between compressions. Using this setup, the texture parameters of the muscle tissue, including hardness, adhesiveness, gumminess, elasticity, cohesiveness, and chewiness, were measured.

Determination of muscle antioxidant enzyme activity

A 0.1 g sample of muscle tissue was excised and placed into a clean EP tube, followed by the addition of 900 μL of 0.85% saline and grinding beads. The sample was homogenized using a high-throughput grinder for 1 min, cooled on ice for 1 min, and this grinding process was repeated 3–5 times. Subsequently, the homogenate was centrifuged at 4 °C for 10 min, and the supernatant was transferred to a new EP tube and kept on ice for further analyses. Total protein, MDA, CAT, SOD, and GSH levels in muscle tissue from two hybrid breams and their parental strains were measured. Assays were conducted using the protein quantification kit (TP, A045—4, BCA microplate method), MDA kit (A003—1, TBA method), CAT kit (A007—1—1, ammonium molybdate method), SOD kit (A001—3, WST-1 method), and GSH kit (A006—2—1, microplate method), all obtained from Nanjing Jiancheng Bioengineering Institute. Each assay was performed in triplicate, and the detailed procedures followed the respective kit protocols.

Full-length cloning of MRF family genes

The homologous cloning was employed to amplify partial cDNA fragments (CDS) of MRF family genes from two hybrid breams and their parents. The MRF family genes (*myod*, *myog*, *myf5*, and *myf6*) were selected for full-length cloning and sequence analysis because they are key regulators of skeletal muscle development and myogenic differentiation. The CDS sequences for the *myod* gene (KF601795.1), *myog* gene (KF577718.1), *myf5* gene (KF636496.1), and *myf6* gene (KF781549.1) of *Megalobrama amblycephala* were obtained from NCBI. Intermediate fragment primers (Table 1) were designed using Primer Premier 5.0 software to amplify these fragments. Based on the obtained intermediate fragments, terminal fragment primers were subsequently designed (Table 1). To amplify the untranslated region (UTR) cDNA fragments of MRF family genes from the two hybrid breams and their parents, 5′ and 3′ rapid amplification of cDNA ends (5′-RACE and 3′-RACE) was performed. All primers and sequencing were provided by Sangon Biotech, and specific amplification protocols were adapted from previously published methods (Huang et al. 2021). Finally, the amplified intermediate fragments, 3′-RACE fragments, and 5′-RACE fragments were assembled using BioEdit software to obtain the full-length cDNA sequences of the MRF family genes for both hybrid breams and their parents.

Sequence analysis

The coding sequences of MRF family genes in BBTB, BTBB, BSB, and BTB were predicted using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/projects/gorf/). The physicochemical properties of the corresponding proteins, including molecular weight, isoelectric point, and amino acid composition, were analyzed using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). The functional domains of the MRF family genes were predicted using the TMHMM online software (http://www.cbs.dtu.dk/services/TMHMM/). DNAMAN software was used to analyze the similarity of MRFs' amino acid sequences from the two hybrid breams and their parents with those of *Ctenopharyngodon idella*, *Cyprinus carpio*, and other species. The phylogenetic tree of MRFs' amino acid sequences from the two hybrid breams, their parents, and other species such as *Ctenopharyngodon idellus* and *Cyprinus carpio* was constructed using the neighbor-joining (NJ) method in MEGA11 software.

Real-time quantitative PCR (RT-qPCR)

In addition to the MRF family genes, *mstn* was included in the expression analysis because it functions as a major negative regulator of skeletal muscle growth. The CDS sequences of the MRF family genes in the two hybrid breams and their parents were obtained based on the full-length cDNA sequences, and the CDS sequence of the *mstn* gene (KY072939.1) from BSB was acquired from NCBI. RT-qPCR primers were designed using Primer Premier 5.0 software (Table 2), with β-actin serving as the internal reference gene. The expression levels of the MRF family genes and *mstn* in muscle tissues of the two hybrid breams and their parents were quantified by RT-qPCR

Table 1 The primer sequence of full-length cloning of MRFs

Primer name	Primer sequence (5′−3′)	
myod-F	ATACATAAAGATGGAGTTGTCG	*Myod* CDS PCR
myod-R	CAGCAGTGGATCGAAATAGT	*Myod* CDS PCR
3′ *myod*-F_1	GTCCGTGTTCTCCGCAGG	*myod* 3′-RACE
3′ *myod*-F_2	CCGTCCTGAGTGAGAGCG	*myod* 3′-RACE
5′ *myod*-R_1	CCACTGGGTGCCCTTACG	*myod* 5′-RACE
5′ *myod*-R_2	ATGAGATGGGGAAGGGAA	*myod* 5′-RACE
myog-F	AGCGTTTTTACGAAGG	*Myog* CDS PCR
myog-R	TATGATTTACCCAGCC	*Myog* CDS PCR
3′ *myog*-F_1	CACCGACCATAACAGCACTA	*myog* 3′-RACE
3′ *myog*-F_2	AGACAGTCAGGCTGGGTAA	*myog* 3′-RACE
5′ *myog*-R_1	TTGGTCAAAGCCTCCTGTCA	*myog* 5′-RACE
5′ *myog*-R_2	TTTGGAAGAAGTTATCGCCG	*myog* 5′-RACE
myf5-F	AGGGAACAGGTGGAAAA	*myf5* CDS PCR
myf5-R	AACTGCACTGGGAATCA	*myf5* CDS PCR
3′ *myf5*-F_1	CCTCTGTGGACACGGGA	*myf5* 3′-RACE
3′ *myf5*-F_2	GCAATGGGAATGAGGAA	*myf5* 3′-RACE
5′ *myf5*-R_1	CTTGAGGAGGGGCTGGC	*myf5* 5′-RACE
5′ *myf5*-R_2	GTTTTCCACCTGTTCCCT	*myf5* 5′-RACE
myf6-F	GCAGCGGAGAGGAACACG	*myf6* CDS PCR
myf6-R	AGGAGAGGCGACGAAGGC	*myf6* CDS PCR
3′ *myf6*-F_1	GCGGCGGAGTCTTCAGC	*myf6* 3′-RACE
3′ *myf6*-F_2	CAGCAGCCTTCGTCGCC	*myf6* 3′-RACE
5′ *myf6*-R_1	ATCTTTTTGAGCCGCCT	*myf6* 5′-RACE
5′ *myf6*-R_2	TGAGACACTGACCATCGC	*myf6* 5′-RACE
3′-site Adaptor	CTGATCTAGAGGTACCGGATCC	3′-RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT	5′-RACE
UPS	CTAATACGACTCACTATAGGGC	5′-RACE

Table 2 The primer sequences for RT-qPCR

Primer name	Primer sequence (5′−3′)	
Q-*myod*-F	TCGTGGAGCGAATTTCCACA	RT-qPCR
Q-*myod*-R	GAGAACACGGACTCCCTTCG	RT-qPCR
Q-*myog*-F	GTGACCATGGATCGACGGAA	RT-qPCR
Q-*myog*-R	GTTGGGATTCATGAGCGTGC	RT-qPCR
Q-*myf5*-F	GCAACAGTCCTGTATGGCCT	RT-qPCR
Q-*myf5*-R	TGCTGGACAGACACTGCAAA	RT-qPCR
Q-*myf6*-F	ACCATGTGGCTCCCAATGAG	RT-qPCR
Q-*myf6*-R	CGGCCATCTGGGAATTGGAA	RT-qPCR
Q-*mstn*-F	CGTTACGGACGGAGGAAGAC	RT-qPCR
Q-*mstn*-R	TTGACCACACCGAGAGCACC	RT-qPCR
Q-*β-actin*-F	CGGACAATTTCTCTTTCGGCTG	RT-qPCR
Q-*β-actin*-R	ACCCACACCGTGCCCATCTA	RT-qPCR

following the amplification procedure described previously (Fan et al. 2024). All primers were synthesized by Sangon Biotech. After the reaction, the data were exported and analyzed using the relative standard curve method ($2^{-\Delta\Delta CT}$ method).

Results

Formation of two hybrid breams

The process of backcrossing is shown in Fig. 1. After two rounds of backcrossing of female BSB × male TC lineage with female and male BSB, two hybrid breams (BBTB and BTBB) were successfully obtained.

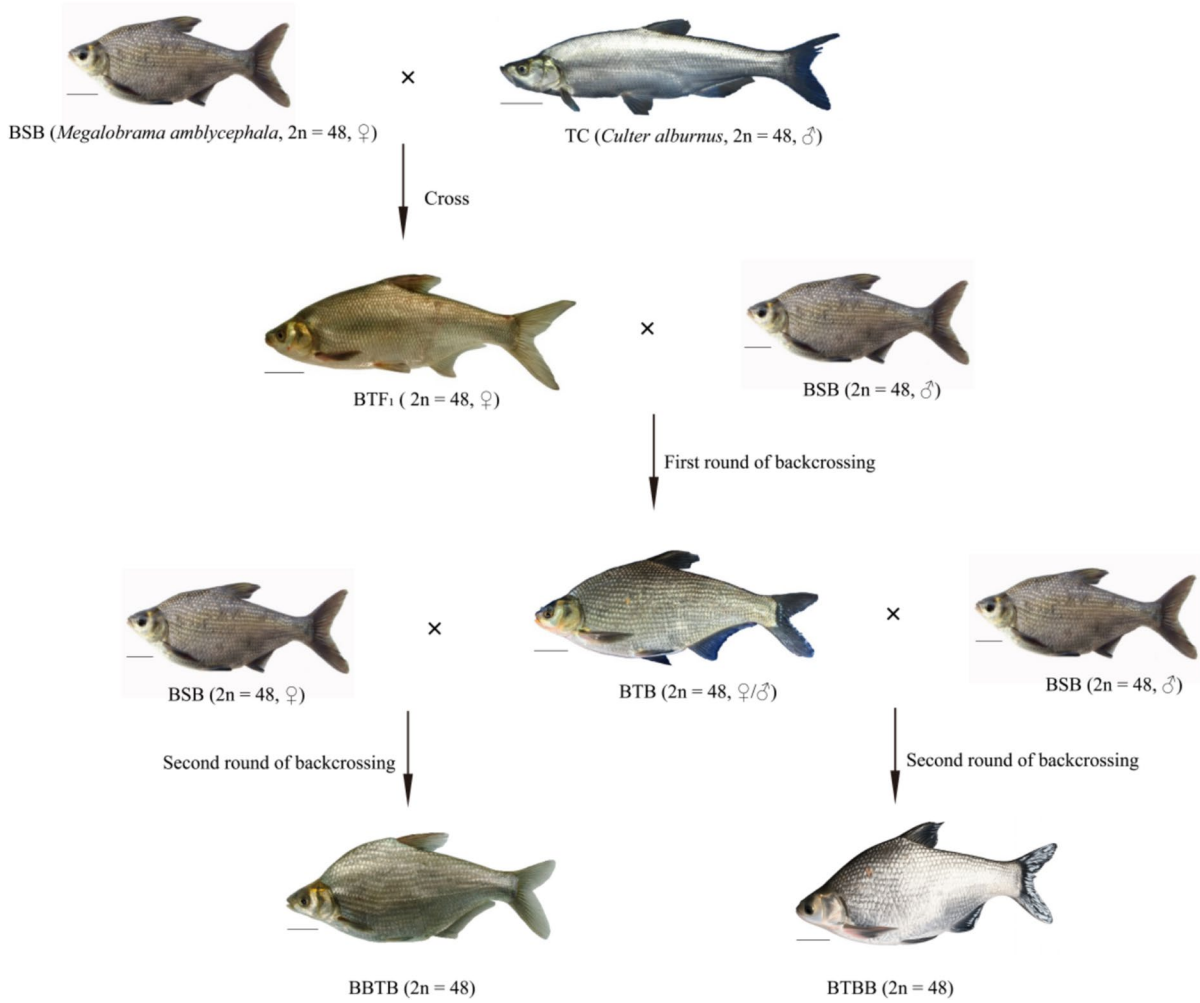


Fig. 1 The crossing and backcrossing procedure for the formation of BBTB and BTBB. BSB, blunt snout bream; TC, topmouth culter; BTF_1: BSB (♀)×TC (♂); BTB: BTF_1 (♀)×BSB (♂); BBTB: BSB (♀)×BTB (♂); BTBB: BTB (♀)×BSB (♂); bar = 5 cm

Measurement of muscle fibers growth characteristics

The body weight, body length, and muscle fatness of the two hybrid breams and their parents are summarized in Table 3. No significant differences in body weight or body length were observed among BBTB, BTBB, and BTB ($p > 0.05$), whereas all three groups showed significantly higher values than BSB ($p < 0.05$). Muscle fatness did not differ significantly among the four groups ($p > 0.05$).

The histological characteristics of muscle fibers in the two hybrid breams and their parents are shown in Fig. 2. In transverse sections, muscle fibers from both hybrid groups and their parents exhibited a polygonal morphology, with nuclei primarily located at the fiber periphery. Muscle fiber density and mean fiber

Table 3 The weight, length, and fatness of BBTB, BTBB, BSB, and BTB

Fish	Weight (g) Mean ± SEM	Body length (cm) Mean ± SEM	Fatness(g/cm^3) Mean ± SEM
BBTB	146.23 ± 4.91^b	18.03 ± 0.27^b	2.49 ± 0.03^a
BTBB	140.67 ± 3.91^b	19.03 ± 0.28^b	2.04 ± 0.03^a
BSB	50.42 ± 1.72^a	13.38 ± 0.18^a	2.11 ± 0.09^a
BTB	141.77 ± 5.48^b	18.10 ± 0.81^b	2.43 ± 0.05^a

Different letters indicate significant difference ($p < 0.05$)

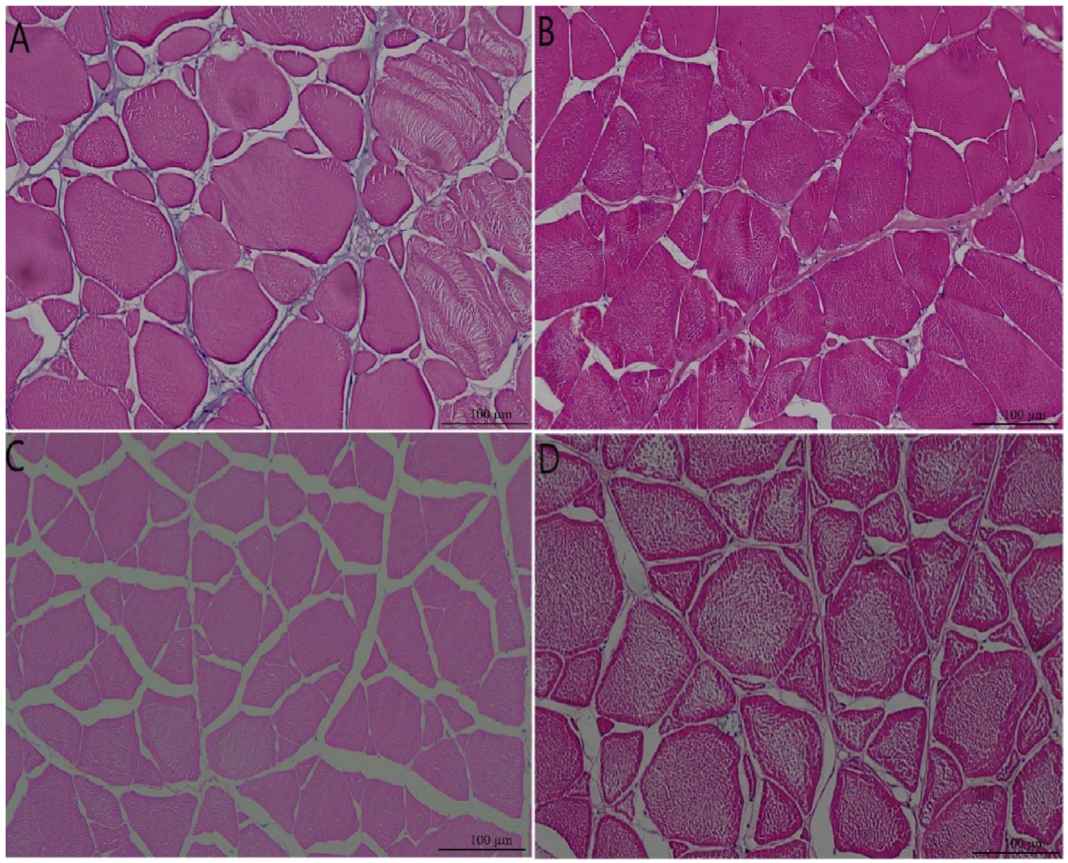


Fig. 2 Comparison of muscle fiber morphology of BBTB, BTBB, BSB and BTB. **A** BBTB muscle fibers; **B** BTBB muscle fibers; **C** BSB muscle fibers; **D** BTB muscle fibers; bar = 100 μm

diameter are summarized in Table 4. No significant differences in muscle fiber density were detected among BBTB, BTBB, and BTB, whereas all three groups showed significantly lower values than BSB ($p < 0.05$). In contrast, the mean muscle fiber diameter was significantly greater in BBTB and BTBB than in BSB ($p < 0.05$), with no significant difference relative to BTB. The distribution of muscle fiber diameters is presented in Fig. 3A. In all groups, fiber diameters were predominantly distributed within 30 ~ 60 μm, followed by 0 ~ 30 μm. Within the 0 ~ 30 μm range, the proportion of fibers was lower in BBTB and BTBB than in BSB and BTB, with the highest proportion observed in BSB. In the 60 ~ 90 μm range, BTBB exhibited the highest proportion of fibers, whereas BSB showed the lowest. Fibers with diameters exceeding 90 μm were observed in BBTB, BTBB, and BTB, but were absent in BSB. The distribution of muscle fiber area is shown in Fig. 3B. In all groups, most muscle fibers were distributed within the 0 ~ 2000 μm^2 range, with BSB showing the highest proportion of small-sized fibers in this interval.

Table 4 The muscle fiber density and mean fiber diameter of BBTB, BTBB, BSB, and BTB

	BBTB	BTBB	BSB	BTB
Fiber number/mm^2	401.80 ± 24.63^b	389.41 ± 24.90^b	865.43 ± 38.97^a	371.26 ± 35.53^b
Fiber diameter (μm)	62.11 ± 2.00^b	59.67 ± 4.86^b	40.30 ± 1.27^a	61.48 ± 4.35^b

Data are presented as MEAN ± SEM; different letters indicate significant difference ($p < 0.05$)

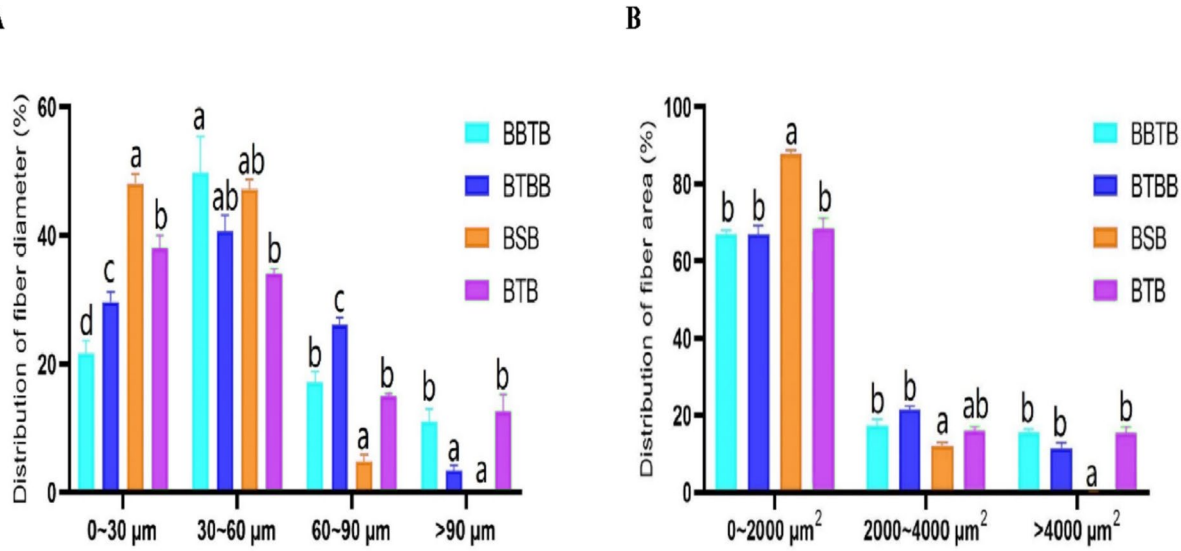


Fig. 3 Muscle fiber diameter and area distribution of BBTB, BTBB, BSB and BTB. **A** Distribution of muscle fiber diameter in BBTB, BTBB, BSB, and BTB; **B** distribution of muscle fiber area in BBTB, BTBB, BSB, and BTB. Different letters indicated significant difference ($p < 0.05$)

By contrast, BBTB, BTBB, and BTB exhibited relatively higher proportions of medium-sized fibers (2000 ~ 4000 μm^2) and large-sized fibers (> 4000 μm^2).

Comparison of muscle texture characteristics

The muscle texture characteristics of the two hybrid breams and their parents are presented in Table 5. The muscle adhesiveness of BBTB was significantly higher than that of BSB ($p < 0.05$), whereas its cohesiveness and gumminess were significantly lower than that of BSB ($p < 0.05$). For BTBB, the muscle springiness was significantly higher than those of BSB ($p < 0.05$). No significant differences in hardness were observed between BBTB, BTBB, and their parents.

Comparison of antioxidant enzyme activity in muscle

The antioxidant enzyme activity in the muscle of the two hybrid breams and their parental species is presented in Fig. 4. The muscle MDA content in BBTB and BTBB was significantly lower than that in BSB ($p < 0.05$). The activities of CAT, SOD, and GSH in BBTB and BTBB were significantly higher than those in BSB ($p < 0.05$). Furthermore, the GSH activity in the muscles of BBTB and BTBB was significantly higher than that observed in BTB ($p < 0.05$),

Table 5 The textural profile parameters of muscle of BBTB, BTBB, BSB, and BTB

Index	BBTB	BTBB	BSB	BTB
Hardness (N)	23.53 ± 2.56^{a}	27.38 ± 1.45^{a}	27.53 ± 3.47^{a}	29.54 ± 1.64^{a}
Adhesiveness (N.mm)	0.06 ± 0.01^{ab}	0.04 ± 0.01^{bc}	0.02 ± 0.01^{c}	0.08 ± 0.00^{a}
Cohesiveness	0.13 ± 0.01^{c}	0.18 ± 0.01^{b}	0.17 ± 0.02^{b}	0.23 ± 0.01^{a}
Springiness (mm)	0.99 ± 0.11^{b}	1.32 ± 0.1^{a}	1 ± 0.06^{b}	1.42 ± 0.08^{a}
Gumminess (N)	3.02 ± 0.46^{b}	4.76 ± 0.24^{a}	4.8 ± 0.91^{a}	4.16 ± 0.31^{ab}
Chewiness (mJ)	3.22 ± 0.79^{b}	6.31 ± 0.59^{a}	4.97 ± 1.02^{ab}	6.51 ± 0.32^{a}

Different letters indicate significant difference ($p < 0.05$)

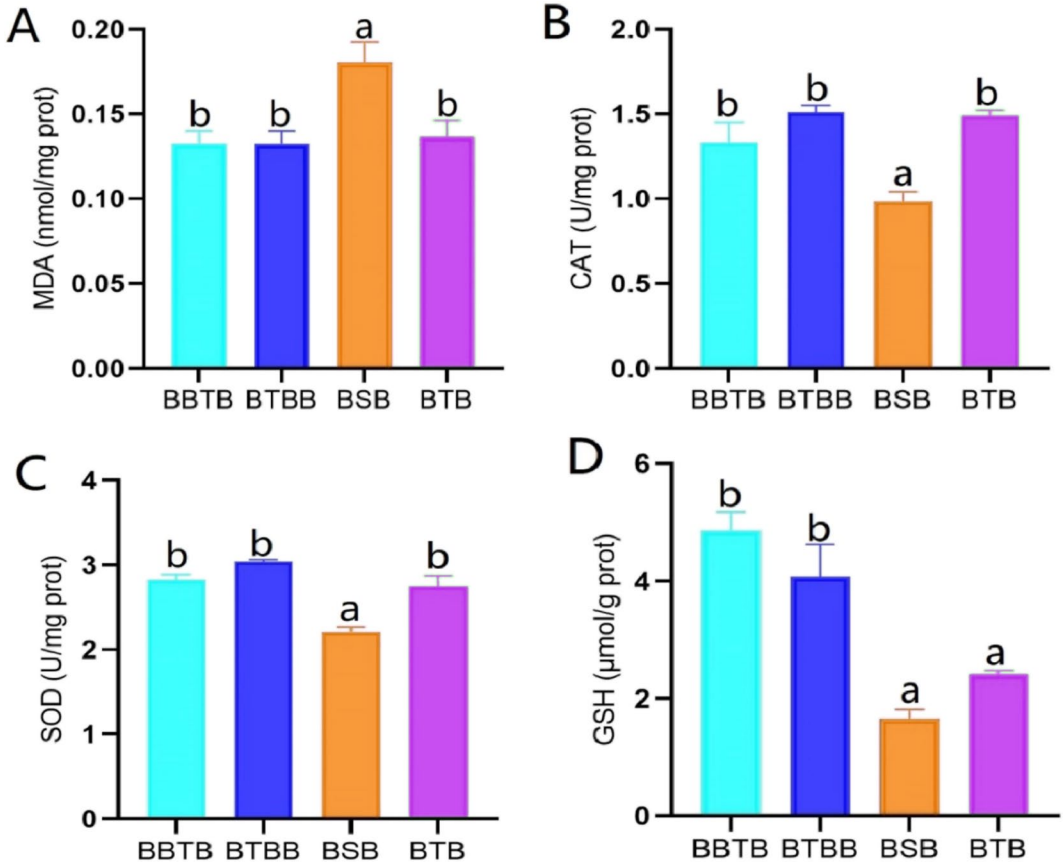


Fig. 4 Comparison of muscle antioxidant enzyme activity of BBTB, BTBB, BSB, and BTB. **A** Malondialdehyde (MDA) content; **B** catalase (CAT) activity; **C** superoxide dismutase (SOD) activity; **D** glutathione (GSH) content; different letters indicated significant difference ($p < 0.05$)

while no significant differences were detected in the activities of the other enzymes across these groups.

Full-length sequence of MRF family genes

The full-length cDNA of *myod* gene and the predicted amino acid sequence of the two hybrid breams and their parents are shown in Fig. 5. The full length of *myod* of BBTB (Fig. 5A), BTBB (Fig. 5B), BSB (Fig. 5C), and BTB (Fig. 5D) was 1631 bp, with an open reading frame of 828 bp, encoding 275 amino acids. The 5′ non-coding region was 187 bp, and the 3′ non-coding region was 616 bp. The molecular weight of the proteins predicted by BBTB, BTBB, and BSB *myod* was 30,810.10, the isoelectric point was 5.46, and the most abundant amino acid was Ser (12.4%). The corresponding protein molecular weight of BTB *myod* was 30,824.13, the isoelectric point was 5.47, and the most abundant amino acid was Ser (12.4%). The full-length cDNA of *myog* gene and the predicted amino acid sequence of the two hybrid breams and their parents are shown in Fig. 6. The full length of *myog* of BBTB (Fig. 6A), BTBB (Fig. 6B), BSB (Fig. 6C), and BTB (Fig. 6D) was all 1486 bp, with an open reading frame of 765 bp, encoding 254 amino acids. The 5′ non-coding region was 158 bp and the 3′ non-coding region was 563 bp. The molecular weight of the corresponding protein was 28,192.38, the isoelectric point was 6.09, and the most abundant amino acid was Ser (13.4%). The full-length cDNA of *myf5* gene and the predicted amino acid sequence of the two hybrid breams and their parents are shown in Fig. 7. The full length of *myf5* of BBTB (Fig. 7A), BTBB (Fig. 7B), and BSB (Fig. 7C) was 1317 bp, and the open reading frame was 723 bp, encoding


A
B
C
D

◂**Fig. 5** Full length and predicted amino acid sequence of *myod* genes in BBTB, BTBB, BSB and BTB. Black box, basic domain; shadow, HLH domain; underline, LCD domain; lowercase letters, non-coding area; capital letters, code area

240 amino acids. The 5′ non-coding region was 49 bp, and the 3′ non-coding region was 545 bp. The full length of *myf5* in BTB (Fig. 7D) was 1321 bp, and the open reading frame was also 723 bp, encoding 240 amino acids. The 5′ non-coding region was 53 bp and the 3′ non-coding region was 545 bp. The predicted protein molecular weight of BTBB and BSB *myf5* genes was 26,324.38, the isoelectric point was 6.22, and the most abundant amino acid was Ser (15.0%). The predicted protein molecular weight of BBTB and BTB *myf5* was 26,296.32, the isoelectric point was 5.97, and the most abundant amino acid was still Ser (15.0%). The full-length cDNA of *myf6* gene and the predicted amino acid sequence of the two hybrid breams and their parents are shown in Fig. 8. The full length of *myf6* of BBTB (Fig. 8A), BTBB (Fig. 8B), BSB (Fig. 8C), and BTB (Fig. 8D) was 1282 bp, with an open reading frame of 720 bp, encoding 239 amino acids. The 5′ non-coding region was 24 bp and the 3′ non-coding region was 538 bp. The molecular weight of the corresponding protein was 26,973.99, the isoelectric point was 5.78, and the most abundant amino acid was Ser (9.2%).

The MyoD, MyoG, Myf5, and Myf6 proteins of the four fish species all had common structural features, and each protein contained a basic domain, a HLH domain, and a low complexity domain (LCD). Specifically, the basic domain of MyoD was located at amino acids 1–89, the HLH domain was located at amino acids 90–141, and the LCD was located at amino acids 204–215. The basic domain of MyoG was the 1–107 amino acid, the HLH domain was the 108—159 amino acid, and the LCD was the 178—204 amino acid. The Basic domain of Myf5 was located at amino acids 1–72, the HLH domain was located at amino acids 73–124, and the LCD was located at amino acids 131–150 and 221–232, respectively. The Basic domain of Myf6 was located at amino acids 2–97, the HLH domain was at amino acids 98–149, and the LCD was at amino acids 203–224.

Amino acid sequence similarity analysis of MRFs

The similarity of the MyoD amino acid sequences between BBTB, BTBB, and other species is shown in Fig. S1. The MyoD amino acid sequences of BBTB and BTBB were 100% identical to those of BSB and 99.6% similar to BTB. Figure S2 presents the alignment results for MyoG amino acid sequences, showing 100% similarity between the MyoG sequences of BBTB and BTBB with their parental species. In the alignment of Myf5 amino acid sequences (Fig. S3), the similarity between BBTB and BTBB was 99.5%. The Myf5 sequence of BBTB was 99.5% similar to BSB and 100% identical to BTB, while the Myf5 sequence of BTBB was 100% identical to BSB and 99.5% similar to BTB. For Myf6 amino acid sequence alignment (Fig. S4), the sequences of BBTB and BTBB showed 100% similarity.

The MyoD amino acid sequences of BBTB and BTBB exhibited over 95% similarity with those of *Culter alburnus*, *Ctenopharyngodon idellus*, and *Cyprinus carpio*. Their similarity with *Capra hircus*, *Sus scrofa*, *Mus musculus*, *Bos taurus*, and *Homo sapiens* was significantly lower, approximately 60%. For the MyoG amino acid sequences, BBTB and BTBB showed more than 95% similarity to *Ctenopharyngodon idellus*, *Cyprinus carpio*, and *Schizothorax prenanti*, while their similarity to *Sus scrofa*, *Mus musculus*, *Bos taurus*, and *Homo sapiens* was only about 50%. The Myf5 and Myf6 amino acid sequences of BBTB and BTBB were highly similar to those of *Ctenopharyngodon idellus*, *Cyprinus carpio*, *Danio rerio*, and *Schizothorax prenanti*, but displayed lower similarity to *Sus scrofa*, *Mus musculus*, *Bos taurus*, and *Homo sapiens*.

Phylogenetic tree of MRFs

In the phylogenetic tree of the MyoD gene (Fig. S5A), BTBB and BSB clustered closely into a single branch, which then grouped together with BBTB. In the phylogenetic tree of the MyoG gene (Fig. S5B), BTBB and BBTB formed a closely related branch, which clustered together with BSB. For the Myf5 gene

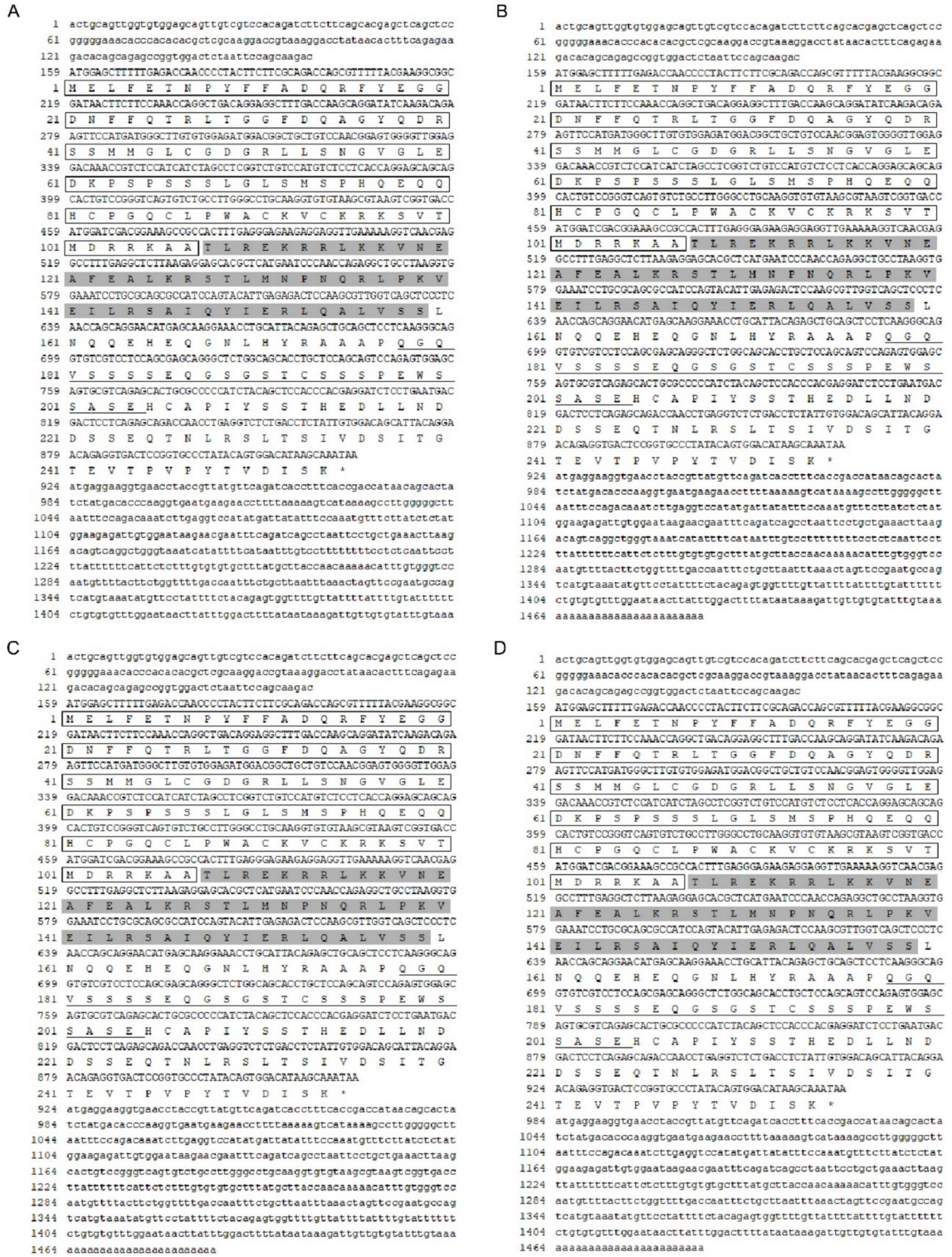


Fig. 6 Full length and predicted amino acid sequence of *myog* genes in BBTB, BTBB, BSB and BTB. Black box, basic domain; shadow, HLH domain; underline, LCD domain; lowercase letters, non-coding area; capital letters, code area

phylogenetic tree (Fig. S6A), BTBB and BSB clustered into one branch, while BBTB and BTB grouped into another branch; ultimately, all four species clustered into a larger branch. In the phylogenetic tree of the Myf6 gene (Fig. S6B), the parental BSB and BTB clustered closely into one branch, while BBTB and BTBB formed a separate branch. These two branches subsequently grouped into a single larger cluster containing all four fish species.

Expression analysis of MRFs and *mstn* genes in the muscle of two hybrid breams and their parents

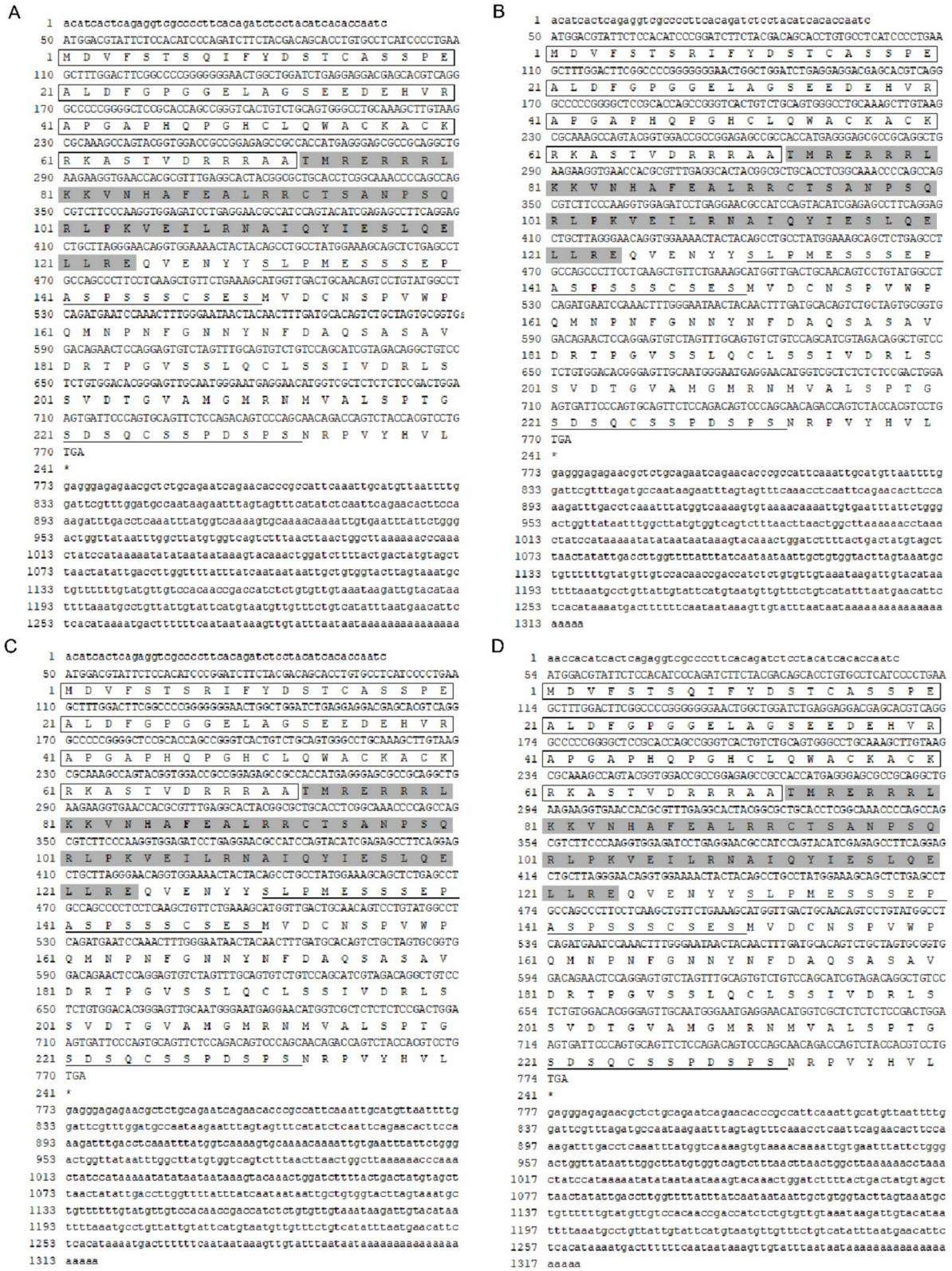
The expression levels of growth-related genes in the muscles of the two hybrid breams and their parents are presented in Fig. 9. The analysis revealed that the expression levels of MRFs (*myod*, *myog*, *myf5*, *myf6*) and *mstn* in BBTB muscle were significantly lower than those in BSB ($p < 0.05$). The expression level of *myf5* in BBTB was significantly lower than that in BTB ($p < 0.05$), whereas the expression level of *mstn* was significantly higher than that in BTB ($p < 0.05$). No significant differences were observed in the expression levels of *myod*, *myog*, and *myf6* between BBTB and BTB. In BTBB muscle, the expression levels of *myod*, *myf6*, and *mstn* were significantly lower than those in BSB ($p < 0.05$), while the expression of *myog* was significantly higher compared to BSB ($p < 0.05$). No significant difference was observed in the expression level of *myf5* between BTBB and BSB. The expression levels of the MRF family genes in BTBB were significantly higher than those in BTB ($p < 0.05$), whereas the expression level of *mstn* did not differ significantly between BTBB and BTB.

Discussion

Flesh quality and growth performance are among the most important economic traits in aquaculture breeding, as they directly influence production efficiency, product value, and the potential for commercial application. In the present study, the average body weight and body length of BBTB, BTBB, and BTB at 1 year of age were all significantly greater than those of BSB ($p < 0.05$), whereas no significant differences were detected among BBTB, BTBB, and BTB. These results indicate that, under identical rearing conditions, both backcross hybrids retained the growth advantage of BTB and consistently outperformed BSB, suggesting that their enhanced growth performance may be associated with heterosis. Although no significant differences in condition factor were observed among the four groups, BBTB exhibited the highest value, implying a potential relative advantage in edible flesh yield.

Fish muscle growth is closely associated with the dynamics of muscle fiber hyperplasia and hypertrophy. Previous studies have reported a positive association between muscle fiber diameter and growth rate in teleosts (Xu et al. 2014). In the present study, muscle fiber density in BBTB, BTBB, and BTB was significantly lower than that in BSB ($p < 0.05$), whereas mean muscle fiber diameter was significantly larger than that of BSB ($p < 0.05$). These findings suggest that the growth advantage of the two hybrid breams was closely related to muscle fiber hypertrophy, consistent with the findings of Xu et al. (2014). In addition, numerous small-diameter fibers (< 30 μm) were observed alongside large, mature fibers (> 90 μm) in muscle cross sections, indicating that a certain degree of mosaic hyperplasia was still present at 1 year of age. Taken together, these observations suggest that muscle growth in the two hybrid breams resulted from the combined effects of muscle fiber hypertrophy and continued recruitment of new fibers, with hypertrophy likely serving as the principal structural basis for increased body size and enhanced growth performance. This interpretation agrees with previous reports describing the coexistence of fiber hypertrophy and hyperplasia during later stages of fish muscle growth (Wang et al. 2021). Accordingly, the muscle differences observed between BSB and the other three groups may primarily reflect divergence in overall growth performance.

Changes in muscle fiber structure may affect not only growth but also muscle texture. Muscle texture is an important determinant of flesh quality and processing suitability and is influenced by multiple factors, including muscle histomorphology, nutrient composition, and physiological condition. TPA showed that adhesiveness was significantly higher in BBTB than in BSB ($p < 0.05$), whereas springiness was significantly higher in BTBB than in BSB ($p < 0.05$). Because adhesiveness contributes to sensory satisfaction during consumption, BBTB may possess superior palatability; similarly, greater


A
B
C
D

◂**Fig. 7** Full length and predicted amino acid sequence of *myf5* genes in BBTB, BTBB, BSB and BTB. Black box, basic domain; shadow, HLH domain; underline, LCD domain; lowercase letters, non-coding area; capital letters, code area

springiness is generally associated with a crisper texture, suggesting that BTBB may have an advantage in this regard (Cao et al. 2022). Previous studies have demonstrated that adhesiveness and springiness are closely associated with muscle lipid and protein contents, with greater nutrient deposition generally corresponding to improved texture traits (Tan et al. 2006). Earlier work further showed that muscle lipid and protein contents in BBTB and BTBB were higher than those in BSB (Li et al. 2022). Notably, although the two hybrid breams exhibited superior growth performance, no deterioration in muscle texture was observed; instead, they outperformed their parents in certain texture-related traits. This finding indicates that growth improvement in these hybrids did not compromise flesh quality. Therefore, the superior texture characteristics of the two hybrid breams were likely attributable to the combined effects of muscle fiber structural remodeling and improved muscle nutritional composition.

In addition to histological characteristics and texture properties, redox status represents another important physiological basis of flesh quality. Under normal conditions, the production and scavenging of reactive oxygen species (ROS) remain in dynamic equilibrium. However, excessive oxidative stress can induce lipid peroxidation, protein damage, and membrane disruption, ultimately compromising muscle integrity and flesh quality (Mateus et al. 2017; Afonso 2020). In the present study, MDA content in BSB muscle was significantly higher than that in BBTB, BTBB, and BTB ($p < 0.05$), indicating that BSB experienced the most severe lipid peroxidation and oxidative damage. Meanwhile, SOD and CAT activities in the muscle tissues of BBTB, BTBB, and BTB were all significantly higher than those in BSB ($p < 0.05$), and GSH content in BBTB and BTBB was significantly higher than that in BSB and BTB ($p < 0.05$). These results indicate that the two hybrid breams possessed a more favorable redox balance and stronger antioxidant defense capacity. Enhanced antioxidant capacity may reduce oxidative injury, preserve muscle fiber structural integrity, and provide a more favorable intracellular environment for the development of desirable flesh texture. Thus, improved antioxidant status may represent an important physiological basis underlying the superior muscle phenotype of the two hybrid breams.

The myogenic regulatory factor (MRF) family genes, including *myod*, *myog*, *myf5*, and *myf6*, play crucial roles in fish growth and development and function as key upstream regulators of skeletal muscle determination, differentiation, and maturation (Buckingham and Rigby 2014). In the present study, the coding sequences and protein domains of *myod*, *myog*, *myf5*, and *myf6* were highly conserved among BBTB, BTBB, BTB, and BSB. Amino acid sequence similarity of the MRF genes between the two hybrid breams and their parents exceeded 99%, with some genes showing 100% identity. In the NJ phylogenetic tree, the MRF genes of these four groups clustered tightly together and were positioned adjacent to those of other cyprinids, indicating a high degree of genetic stability and evolutionary conservation of MRF genes within *Cyprinidae*. These findings suggest that the muscle phenotypic differences observed among the four groups are unlikely to arise from substantial functional variation in the MRF coding regions themselves, but are more likely attributable to differences in gene expression regulation.

Based on the MRF sequences obtained, RT-qPCR was further performed to investigate the molecular basis underlying the rapid growth of the two hybrid breams by examining the expression of MRFs and *mstn* in muscle tissues of the hybrids and their parents. The results showed that the two hybrid breams exhibited distinct molecular response patterns during muscle growth regulation, indicating that MRFs and *mstn* play pivotal roles in this process. In BTBB muscle, MRF expression was comparable to that in BSB, whereas *mstn* expression was significantly lower than that in BSB ($p < 0.05$). At the same time, both muscle fiber diameter and fiber area in BTBB were significantly greater than those in BSB ($p < 0.05$). This concordance between phenotype and gene expression suggested that the muscle advantage of BTBB may be closely associated with the coordinated effects of enhanced promyogenic signaling and reduced inhibitory signaling. This interpretation is consistent with previous studies showing that muscle fiber volume increases markedly in *mstn*-knockout mice, supporting the inhibitory role of *mstn* in muscle fiber hypertrophy (Matsakas et al. 2010). Notably, although both



Fig. 8 Full length and predicted amino acid sequence of *myf6* genes in BBTB, BTBB, BSB, and BTB. Black box, basic domain; shadow, HLH domain; underline, LCD domain; lowercase letters, non-coding area; capital letters, code area

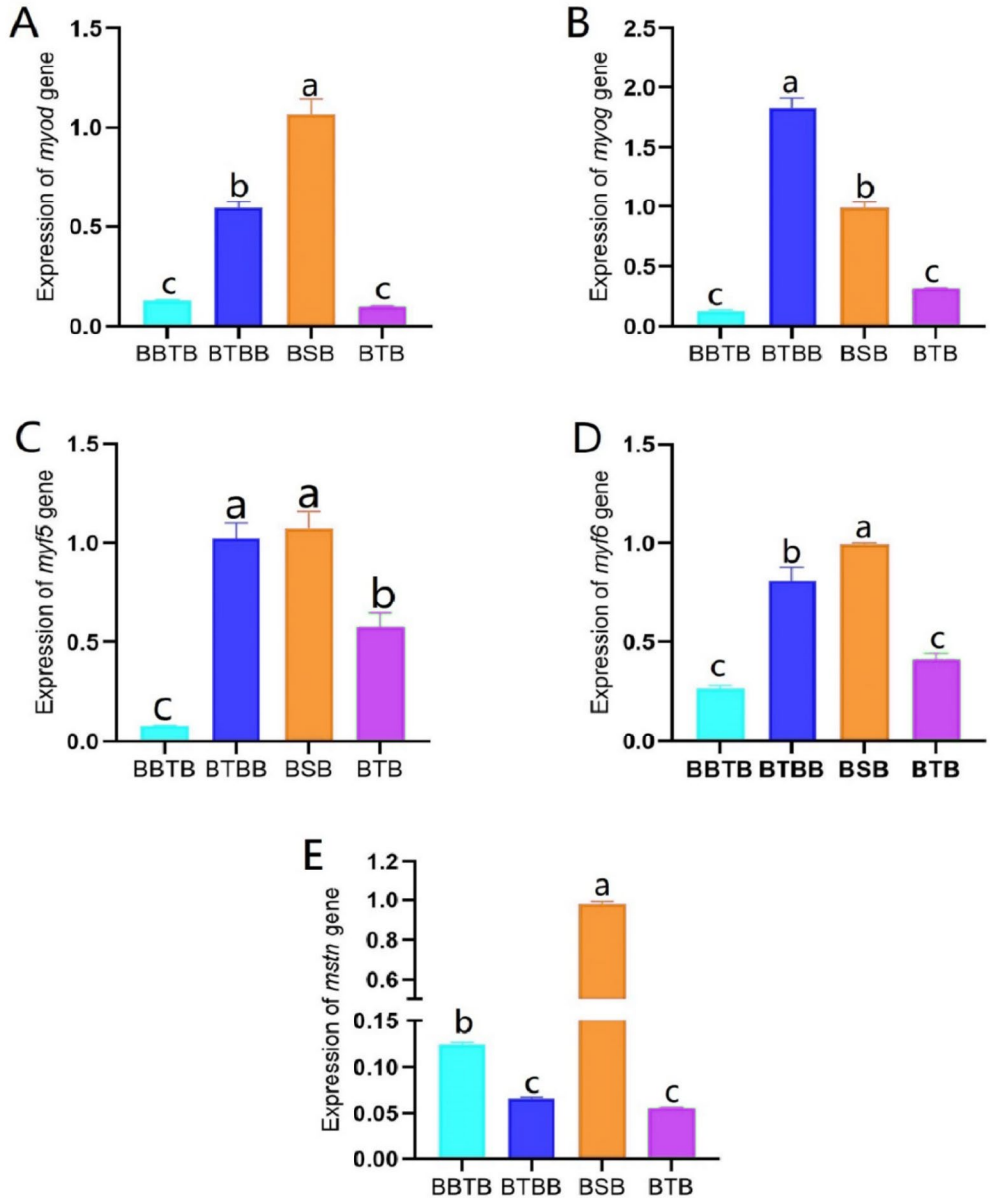


Fig. 9 Expression of growth-related genes in muscles of BBTB, BTBB, BSB, and BTB. **A** Expression of *myod* gene in muscle; **B** expression of *myog* gene in muscle; **C** expression of *myf5* gene in muscle; **D** expression of *myf6* gene in muscle; **E** expression of *mstn* gene in muscle; different letters indicated significant difference ($p < 0.05$)

MRFs and *mstn* were highly expressed in BSB muscle, its growth rate remained relatively low. We therefore speculate that elevated *mstn* expression may, at least in part, suppress the promyogenic effects of MRFs on muscle growth. In contrast to BTBB, BBTB displayed a different phenotype–gene expression relationship. Although BBTB also exhibited larger muscle fibers and faster growth, the expression levels of both MRFs and *mstn* were significantly lower than those in BSB ($p < 0.05$). This finding indicates that MRF expression does not necessarily show a simple linear relationship with the final growth phenotype. Fish muscle growth is a dynamic developmental process, and gene expression measured at a single time point is more likely to reflect

the transient physiological state at sampling than the cumulative outcome of the entire muscle growth trajectory. Previous studies have shown that, during the growth of *Monopterus albus*, MRF expression does not remain continuously elevated; rather, it increases initially and then gradually declines with increasing body weight (Tok et al. 2011). It is therefore reasonable to infer that BBTB may have undergone a more active phase of myogenic regulation and muscle fiber enlargement at an earlier developmental stage and had already entered a relatively stable phase by 1 year of age, thereby exhibiting lower MRF expression at the time of sampling.

It is also noteworthy that MRF expression in BBTB muscle was significantly lower than that in BTBB ($p < 0.05$), whereas *mstn* expression in BTBB was significantly lower than that in BBTB. Despite these distinct transcriptional patterns, no significant differences in growth traits were observed between the two hybrid breams. These findings indicate that the final muscle growth phenotype cannot be explained solely by the expression level of any single myogenic regulatory gene. Rather, it is more likely determined by the integrated balance among positive myogenic regulation, *mstn*-mediated negative regulation, and cellular redox status. Nevertheless, fish muscle growth is not regulated exclusively by MRFs and *mstn*. Other genes, such as fibroblast growth factor 6 (FGF6) and myosin heavy chain (MyHC), also play important roles. FGF6 is critically involved in myoblast proliferation, differentiation, muscle repair, regeneration, and hypertrophy (Armand et al. 2003; Xu et al. 2019), whereas MyHC is highly expressed in yellowtail muscle and participates in the regulation of muscle growth and locomotor function (Bao et al. 2021).

Overall, fish muscle growth is a complex biological process governed by the coordinated action of multiple genes. Future studies should incorporate continuous sampling across different developmental stages and include additional muscle growth-related factors, such as FGF6, MyHC, and MyLC, to more systematically elucidate the molecular mechanisms underlying muscle growth and flesh quality formation in the two hybrid breams. Taken together, the present study demonstrates that the two hybrid breams exhibit faster growth, larger muscle fibers, and stronger antioxidant capacity while also showing trends toward superior flesh quality traits relative to the parents. Therefore, the superior muscle phenotype of the two hybrid breams is likely the result of the combined effects of muscle fiber development, antioxidant defense, MRF-mediated positive myogenic regulation, and *mstn*-mediated negative regulation.

Conclusion

In this study, we systematically compared growth performance, muscle histomorphology, texture properties, antioxidant capacity, and the expression patterns of myogenic regulatory genes in two hybrid breams and their parents. The results showed that both hybrid breams exhibited growth performance comparable to that of BTB and significantly superior to that of BSB, indicating that they retained the growth advantage of BTB and may possess promising value for aquaculture production. Histological analysis further demonstrated that the superior growth of the hybrids was closely associated with increased muscle fiber diameter and reduced fiber density, suggesting that muscle fiber hypertrophy, together with continued recruitment of new fibers, contributed to their enhanced muscle development. In addition, the hybrids displayed improved flesh quality–related traits, including favorable texture characteristics and stronger antioxidant capacity, as reflected by lower MDA content and higher activities or contents of SOD, CAT, and GSH. These findings indicate that the improved growth of the hybrid breams was not achieved at the expense of flesh quality, but was accompanied by an overall enhancement of muscle phenotype.

Furthermore, the coding sequences and protein domains of the MRF family genes were highly conserved among the two hybrid breams and their parents, suggesting that the observed phenotypic differences were more likely attributable to variation in gene expression regulation than to structural differences in the genes themselves. At the level of gene expression, BTBB exhibited MRF expression patterns broadly comparable to those of BSB, but showed significantly lower *mstn* expression, implying that reduced negative regulation by *mstn* may contribute to its enhanced muscle growth. In contrast, although BBTB also showed superior growth and larger muscle fibers, both MRF and *mstn* expression levels were lower than those in BSB,

indicating that muscle growth phenotype cannot be explained by the expression level of a single gene. Rather, the superior muscle traits of the hybrid breams are more likely the result of the coordinated action of muscle fiber development, antioxidant regulation, MRF-mediated promyogenic signaling, and *mstn*-mediated inhibitory signaling. Overall, this study provides new evidence for understanding the physiological and molecular basis of heterosis-related muscle growth in hybrid breams and offers an important theoretical foundation for future studies on genetic improvement and flesh quality regulation in aquaculture species.

Author contribution Z. Z. provided direction and ideas, designed the study, performed data collection and wrote the original manuscript; X. Y. designed the study, performed formal analysis, data collection and revised the manuscript; X. O. and X. Y. validated the experiments; X. C. carried out the investigation; Y. H. and K. C. developed the software; S. F. and S. L. designed the study and performed visualization; M. T. provided direction and ideas, acquired funding, administered the project and revised the manuscript.

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Data availability The data supporting this study's conclusions are provided in the article and its supplementary materials.

Declarations

Ethics approval The animal protocol utilized in this study was approved by the Ethics Committee of Hunan Normal University (Approval Code 610). All procedures strictly complied with the Administration of Affairs Concerning Animal Experimentation Guidelines (China) and institutional animal welfare policies.

Competing interests The authors declare no competing interests.

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