Sequence and Bioinformatics Analysis on *MSTN* Gene of the Hybrid Grouper Derived from (*Epinephelus fuscoguttatus* × *Epinephelus polyphekadion*)

Liu CAO[1,2,3,4], Pan CEHN[1,3,4], Youhua ZHANG[4], Xiaomei FU[4], Hai Huang[1,2,3,4]*

1. Key Laboratory of Utilization and Conservation for Tropical Marine Bioresources of Ministry of Education, Sanya 572022, China; 2. Yazhou Bay Innovation Institute, Sanya 572022, China; 3. Hainan Key Laboratory for Conservation and Utilization of Tropical Marine Fishery Resources, Sanya 572022, China; 4. Hainan Tropical Ocean University, Sanya 572022, China

Abstract [**Objectives**] This study aimed to investigate the sequence structure and function of *Myostatin* (*MSTN*) gene in the hybrid grouper (*Epinephelus fuscoguttatus*, ♀ × *Epinephelus polyphekadion*, ♂). [**Methods**] Genetic DNA samples were extracted from the caudal fins of the hybrid grouper and its parents to amplify their *MSTN* genes. Then, *MSTN* gene sequences were analyzed using bioinformatics tools to predict their protein structures and functions. [**Results**] The hybrid grouper and its parents shared the same *MSTN* gene structure, consisting of three exons and two introns. Nucleotide sequence of the gene could be translated into 376 amino acids, including an N-terminal signal peptide, a proteolytic processing site (RXXR motif), and nine conserved cysteine residues at C-terminal, which were the typical features of transforming growth factor beta (TGF-β) superfamily proteins. Alignment of protein sequence showed that *MSTN* was highly conserved between the hybrid grouper and its parents. Especially, exon 3, an important functional domain, exhibited a sequence similarity of 100% among them. In addition, four variable amino acid residues were detected in exon 2 at positions 141, 153, 185 and 186 in the hybrid grouper, but they did not affect the secondary structure of the protein. [**Conclusion**] These results will provide molecular information for future investigation on the growth and heterosis of hybrid grouper species, and on the roles of *MSTN* gene in regulating the growth traits of the hybrid grouper.

Key words Grouper; *MSTN* gene; Growth traits; Gene structure

Grouper belongs to the family Epinephelidae (Perciformes, Percoidei), mainly distributes in the tropical and subtropical oceans. Because of its' delicious taste and high nutritional value, it is regarded as a commercially important and high-value marine fish. Grouper species are hermaphroditic, protogynous, with a long cycle of reproduction, which greatly prolong the breeding process of valuable grouper. Hybridization can effectively solve this problem. It can quickly alter the original genetic structure of parents, so that the offspring can acquire the excellent characteristics of both parents and thus exhibit heterosis[1-2]. *Epinephelus fuscoguttatus* (named as tiger grouper) is a medium-sized species distributed from the Indian Ocean to the Pacific Ocean[3] It has the advantage of being eurythermal, delicious, rich in nutrients and fast-growing, making it a high-quality grouper on the market. *E. polyphekadion* (named as camouflage grouper) is found chiefly in the Red Sea, the east coast of Africa, southern Japan, southern Queensland in Australia. The hybrid grouper derived from female tiger grouper and male camouflage grouper, exhibits significant heterosis[4-5], and has become a valued species in mariculture.

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Liu CAO(1992-), female, Ph. D., devoted to the research about fish genetics and breeding.
* Corresponding author. E-mail: huanghai74@126.com.

Myostatin (*MSTN*), also known as growth differentiation factor 8 (GDF-8), is an important gene that regulates myoblast growth. It negatively regulates myoblast growth and development by repressing the transcriptional activity of myogenic determination (Myo D) gene[6-7]. Mutations or deletions of *MSTN* gene will lead to excessive muscle development, *e. g.* double-muscling in animals[8-10]. Knockdown of MSTN gene in zebrafish results in myoblast hyperplasia[11]. MSTN gene sequence shows high homology across species, indicating that the function of MSTN protein is highly conserved during animal evolution[12]. *MSTN* gene consists of three exons and two introns, with an N-terminal signal peptide, a proteolytic cleavage site (RXXR motif) and nine conserved cysteine residues at C-terminal, which are the typical features of transforming growth factor beta (TGF-β) superfamily proteins[13]. The structure of MSTN gene has been reported in lots of fish species, such as tilapia[14], *Trachinotus ovatus*[15], *Carassius auratus*[16] and *Scomberomorus niphonius*[17], but rarely in grouper, especially that the structure of *MSTN* gene and the function of the protein have never been reported in the hybrid grouper. Therefore, this study aimed to clone *MSTN* gene from the hybrid grouper and its parents, compare and analyze the differences of nucleotide sequence and protein structure of *MSTN* between them, and predict its biological function, so as to provide molecular information for future investigation on the growth and heterosis of the hybrid grouper.

Materials and Methods

Materials

Thirty hybrid groupers, 30 tiger groupers and 30 camouflage

groupers were bought from Hainan Chenhai Aquatic Products Co., Ltd. Genomic DNA samples were extracted from their caudal fins using the Genomic DNA Purification Kit bought from Sangon Biotech (Shanghai) Co., Ltd, preserved at −20 ℃.

Methods

***MSTN* gene amplification** Primers were designed using Primer 5.0 software based on the published sequence of *MSTN* gene of *Epinephelus coioides* (accession number: KR269814.1), shown in Table 1. The *MSTN* gene of *E. coioides* is over 2 000 bp long, so two pairs of primers were used to amplify the two fragments of the gene. The PCR reaction system was 10 μl, containing 5 μl of Master Mix, 0.5 μl of each primer, 1 μl of DNA template and 3 μl of sterile water. The reaction procedure was started with a pre-denaturation step at 94 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 35 s, annealing at 50 ℃ for 35 s and extension at 72 ℃ for 35 s, and a final extension at 72 ℃ for 5 min. Then, the PCR products were subjected to electrophoresis on a 1% agarose gel, and the target gene fragments were recovered using the DNA Gel Extraction Kit from Sangon Biotech (Shanghai) Co., Ltd., then ligated to pMD18-T vector and sequenced.

Table 1 Primers for *MSTN* gene amplification

Primers	5′-3′	Size of target fragments//bp
MSTN1-F	TTTTAAACCAAACTGCACAC	About 1 200
MSTN1-R	CAGCAGTAAATGCTACCAATAG	
MSTN2-F	TACTATTGGTAGCATTTACTGC	About 1 100
MSTN2-R	CTCTCACCAGGATCTCCGTCC	

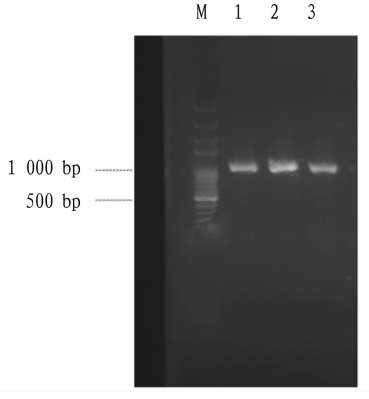
Sequence and bioinformatics analysis on *MSTN* gene The sequences of above PCR products were aligned and spliced using Bioedit software to yield the complete *MSTN* gene sequence. ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) software was used to identify the open reading frame of *MSTN* gene, SignalP 6.0 software (https://services.healthtech.dtu.dk/service.php? SignalP) to predict the signal peptide, Simple Modular Architecture Reach Tool (SMART) (http://www.smart.embl-heidelberg.de/) to identify the domain architectures of MSTN protein, NetSurfP-2.0 (https://services.healthtech.dtu.dk/service.php? NetSurfP-2.0) to predict the secondary structure of the protein, SWISS-MODEL (https://swissmodel.expasy.org/interactive) to predict the tertiary structure of the protein.

Evolutionary analysis The phylogenetic tree was generated based MSTN amino acid sequences of camouflage grouper, tiger grouper, the hybrid grouper, *Epinephelus coioides*, *Epinephelus lanceolatus* (JN681176.1), *Etheostoma spectabile* (XP_032385540.1), *Perca flavescens* (XP_028447210.1), *Sebastes schlegelii* (ABD-96100.1), and *Danio rerio* (NP_998140.1) by maximum likelihood method (bootstrap repeat is 1 000) in MEGA 7.0 software.

Results and Analysis

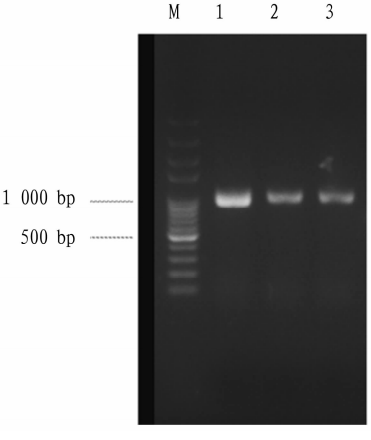
Sequence analysis of *MSTN* gene

The *MSTN* sequences of the hybrid grouper and its parents were PCR amplified and gel isolated. Fig. 1 shows the *MSTN* bands obtained using *MSTN*1 primer pair and Fig. 2 shows those obtained using *MSTN*2 primer pair. The electrophoretic bands were clearly distinguished. The *MSTN* gene fragments amplified from the hybrid grouper and its parents were aligned and spliced via multiple sequence comparison to obtain the complete sequence (2 418 bp). The exons and introns were identified using ORF Finder software, and the exons were translated into amino acid sequences. The *MSTN* nucleotide sequences of the hybrid grouper and its parents all consisted of three exons and two introns, could be translated into 376 amino acids. The exon 1 was 379 bp, exon 2 was 371 bp and exon 3 was 378 bp. By comparing the *MSTN* gene encoding regions between the hybrid grouper and its parents, it was found that they shared extremely high sequence similarity (>98%), and only four variable sites were found in exon 2 (at amino acid residues 141, 153, 185 and 186). The three amino acids different between camouflage grouper and tiger grouper are amino acid residues 141, 185 and 186 (which were leucine, serine and lysine in camouflage grouper, and phenylalanine, asparagine and arginine in tiger grouper). Amino acid residues 141, 185 and 186 in the hybrid grouper were heterozygous sites and presented the genotype of both parents. In addition, the amino acid at residue 153 in the hybrid grouper was different from both its parents (Fig. 3 and Fig. 4).



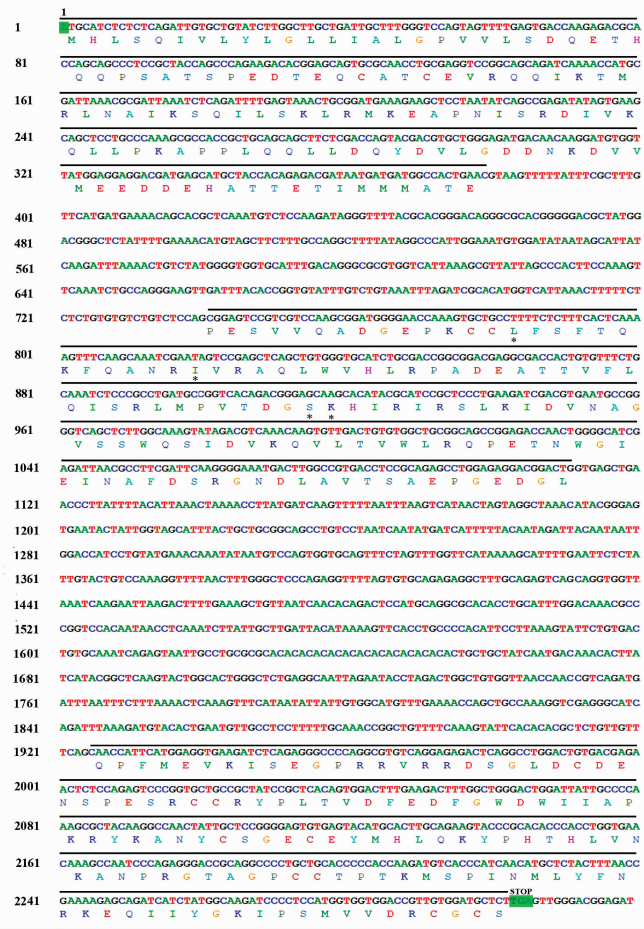
Lane M, 100 bp DNA Marker; lane 1, tiger grouper; lane 2, camouflage grouper; lane 3, the hybrid grouper.

Fig. 1 Electropherogram of PCR products amplified using *MSTN*1 primers



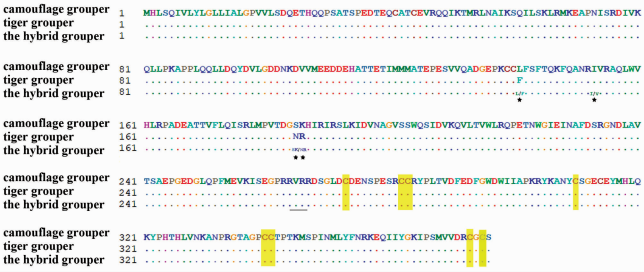
Lane M, 100 bp DNA Marker; lane 1, tiger grouper; lane 2, camouflage grouper; lane 3, the hybrid grouper.

Fig. 2 Electropherogram of PCR products amplified using *MSTN*2 primers



The exons are overlined; the positions where the transcription starts and ends are indicated by green color; polymorphic amino acid residues are indicated by asterisks.

Fig. 3 MSTN nucleotide and amino acid sequences



The RXXR motif is underlined; polymorphic amino acid residues are indicated by asterisks; cysteine residues are indicated by yellow color.

Fig. 4 Comparison and analysis of MSTN amino acid sequences between the hybrid grouper and its parents

Structural analysis of MSTN protein

Signal peptide of MSTN protein was predicted using SignalP-6.0, which showed that MSTN protein had a signal peptide from amino acid residues 1 to 22, and a cleavage site between amino acid residues 22 and 23. This indicated that MSTN protein had the characteristics of a secretory protein (Fig. 5). The functional domains of MSTN protein were analyzed using SMART, and the results showed that the protein had a TGFb_ propeptide with an E-value of 1.30e-32 at amino acid residues 38 to 269, and a TGFβ domain with an E-value of 1.18e-47 at amino acid residues 282 to 376.

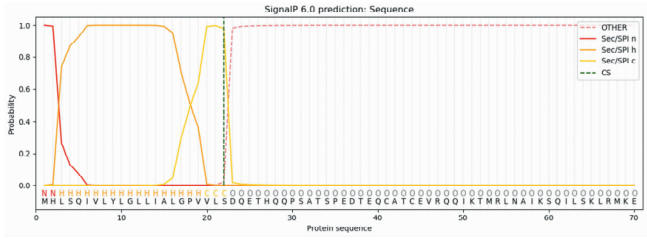
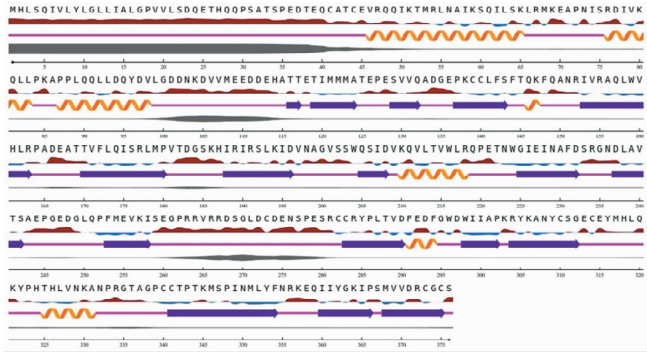


Fig. 5 Predicted signal peptide of MSTN protein



Relative solvent accessibility: red indicates the amino acid residues exposed to the outside, and blue indicates the amino acid residues embedded inside, with a threshold value of 25%; α-helices, extended chains; random coils; irregular; the thickness of the lines is the possibility of disordered residues.

Fig. 6 Secondary structure f MSTN protein of the hybrid grouper

Secondary and tertiary structures of MSTN protein

By predicting the secondary structure of MSTN protein in the hybrid grouper, it was found that the polymorphic sites did not change the secondary structure of the protein, *i. e.*, MSTN protein of the hybrid grouper and its parents shared the same secondary structure. The α-helices formed mainly at amino acid residues 45-65, 76-83, 87-99, 145-147, 210-218, 291-294 and 326-331. The extended chains formed mainly at amino acid residues 116-117, 119-124, 129-132, 137-143, 153-163, 170-180, 188-196, 205-208, 225-232, 237-242, 253-258, 283-290, 298-302, 304-312, 341-354, 360-366 and 368-375 (Fig. 6). The tertiary structure of MSTN protein was similar to a V-shape (Fig. 7).

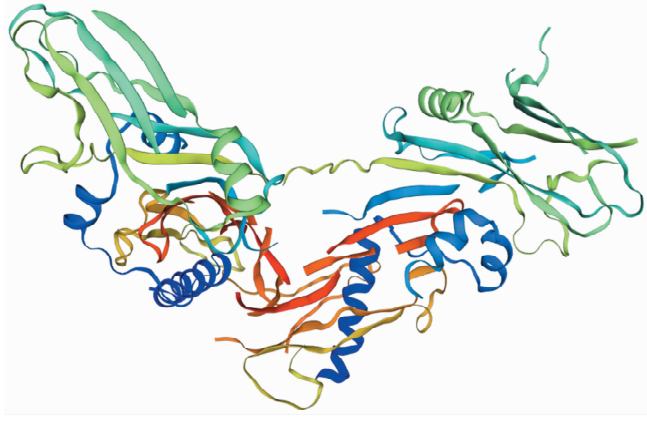


Fig. 7 Tertiary structure of MSTN protein of the hybrid grouper

Evolutionary analysis based on MSTN sequence

As shown by the phylogenetic tree, all the grouper species clustered together. *P. flavescens* and *E. spectabile* were closely related and clustered together. *Barchydanio rerio* was distantly related to other species (Fig. 8).

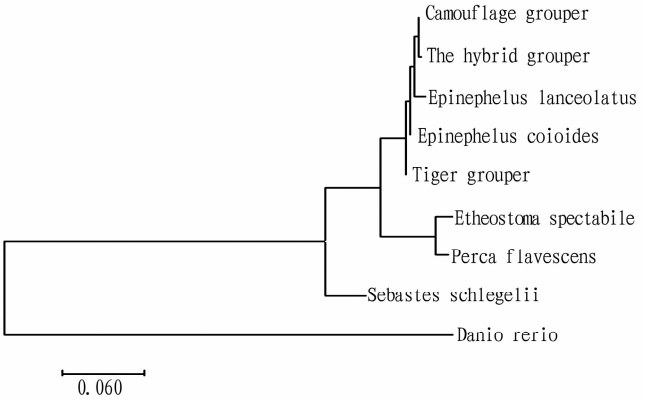


Fig. 8 The phylogenetic tree generated based on MSTN amino acid sequences by maximum likelihood method

Conclusion and Discussion

MSTN gene in fish have three exons and two introns, the same as in mammals. It also has the typical features of TGFβ family members: an N-terminal signal peptide, a proteolytic processing site (RXXR motif) and nine conserved cysteine residues at C-terminal[18-19]. Multiple sequence alignment indicated that *MSTN* genes in the hybrid grouper and its parents all consists of three exons and two introns, as in other fish species. The three exons are 379, 371 and 378 bp in length, respectively, and can be translated into 376 amino acids. The MSTN proteins share the same structure, consisting of a signal peptide, a proteolytic processing site (RXXR motif), TGF-β pre-peptide domain and TGF-β functional domain. The above results indicated that the protein encoded by *MSTN* gene is a functional protein.

Multiple sequence comparison showed that *MSTN* gene has a high similarity between camouflage grouper and tiger grouper, with only three amino acid residues different between them (residues 141, 185 and 186 are leucine, serine and lysine in camouflage grouper, phenylalanine, asparagine and arginine in tiger grouper). All of the three variable residues are located within exon 2. MSTN amino acid sequence of the hybrid grouper acquired polymorphism from both parents at residues 141, 185 and 186, and a mutation at residue 153. However, these residues do not affect the predicted secondary structure of MSTN protein. The functional domain of MSTN protein is composed of 109 amino acids, which are mainly located within exon 3, and the nine cysteines located in exon 3 are import to the proper functioning of the domain. It has been reported that an 11-bp deletion at exon 3 in double-muscled cattle eventually causes the loss of the active region of MSTN molecule, which in turn leads to a massive increase in muscle[20]. In Piedmont cattle, amino acid mutations in exon 3 result in the complete or near complete loss of MSTN function and increased skeletal muscle[21]. The results of this study showed that MSTN gene is highly conserved between the hybrid grouper and its parents. The amino acid sequence in exon 3 of MSTN is highly conserved, which is important for maintaining gene function. Furthermore, evolutionary analysis revealed that all the grouper species cluster closely together to form a branch of the phylogenetic tree. *E. spectabile* and *P. flavescen* belong to different genera in the family Percidae in (Perciformes), while grouper belongs to the family Serranidae (Perciformes), and *S. schlegelii* belongs to Scorpaeniformes. Therefore, in the phylogenetic tree obtained based on MSTN amino acid sequence, all the grouper species cluster together with *E. spectabile* and *P. flavescen* first, and then with *S. schlegelii*, which is consistent with their taxonomic status.

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The above results indicated that the eukaryotic expression system established in this study could efficiently express rhFX, and the corresponding affinity chromatography purification method could extract the target protein with a purity of 93%. The rhFX eukaryotic mammalian cell expression system constructed in this study provides a certain reference basis for the large-scale preparation of rhFX, and also lays a foundation for studying various physiological functions and action mechanisms of FX and the development of drugs for treating FX deficiency.

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