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Characterization of *Myostatin* Gene in Nile Tilapia (*Oreochromis niloticus*), the Possible Association of *BsmI-exon 2* Polymorphism with Its Growth

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Abstract: *Myostatin* (*MSTN*) gene is a negative regulator of skeletal muscle. In this study, we try to evaluate *MSTN* as a candidate gene for a marker assisted selection in Nile tilapia growth trait. Molecular characterization of *MSTN* gene was done using seven designed primers. We demonstrated that, *O. niloticus MSTN* sequence and its promoter is as for all known vertebrates. Novel SNPs were identified in coding and non-coding regions compared with *MSTN* gene of *O. niloticus* x *O. aureus* hybrid, three non-synonymous SNPs were found at *MSTN* coding region; two at exon one 369 C>A, 831 T>A, and one at exon 3 2637 G>A, altering Thr 38-Pro, Glu 121-Val and Tyr 375-Cys respectively. Phylogenetic analysis revealed high similarity (99.2) with *MSTN* gene of the hybrid with *O. aurous. BsmI* induced cutting pattern at *MSTN*-exon 2 (607-bp). Among two hundred monosex male fish, two different genotypes were reported; AB genotype (607-bp, 507-bp and 100-bp) and BB genotype (507-bp and 100-bp fragments), were produced. Most large-size fish are included in AB genotype with 0.8 frequency and significantly increased body weight compared with small size fish, which are mostly included in BB genotype with 0.9 frequencies. Novel *Bsm*I-*exon 2* polymorphism of *MSTN* gene can be used as a marker assisted selection for large body weight in heterozygous Nile tilapia fish.

Keywords: Nile Tilapia, *Myostatin* Gene, Polymorphism, SNPs, RFLP

1. Introduction

Nile tilapia, a wide spread teleost fish in developing countries, is considered the second most important farmed fresh water fish. For fish, body mass is strongly related to muscle mass [1], because skeletal muscle in fish comprises the largest single tissue components representing up to 70% of total body mass. Therefore, manipulating genes that control muscle mass in fish such as *Myostatin* (*MSTN*) gene will directly lead to improved growth rates. Mutations in the *MSTN* gene were also shown to cause the double-muscling phenotype in many mammalian species [2].

MSTN homologs have been cloned and characterized in a large number of fish species [3, 4, 5, 6]. Its structure among teleost is highly conserved, compromising three exons of comparable size (300-400 nucleotides) separated by two introns [7]. Comparison of *MSTN* sequences revealed that it was extremely well conserved throughout evolution. Fish *MSTN* are over 85% identical to the mammalian homologue in the C-Terminal region. The high sequence identity suggests that this gene plays a similar role in regulating muscle growth in non-mammalian vertebrates [2, 8].

Evaluating *MSTN* as a candidate gene for marker assisted selection (MAS) for growth and the associations between its polymorphisms and growth traits has been reported in

some aquaculture species [9, 10]. However, to date, no studies on *MSTN* polymorphisms and their possible associations with growth traits have been reported in *O. niloticus*.

Association between candidate gene polymorphisms and quantitative traits have been widely applied in recent years using genetic markers. PCR-RFLPs have been demonstrated to be very useful genetic markers for candidate gene studies to reveal polymorphisms associated with quantitative traits in different species of fish [11, 12].

Fish breeding for fast-growing strains through genetic selection will in turn give considerable scope for rapid development of this aquaculture industry, so the aim of this study was to characterization of Nile tilapia *MSTN* gene, detecting SNPs and performing genetic association studies between *MSTN* polymorphisms and growth traits in Nile tilapia using PCR-RFLPs.

2. Materials and Methods

2.1. Sampling and DNA Extraction

Two hundred males Nile tilapia fish (*Oreochromis niloticus*) were obtained from private monosex fish farm at Behera governorate. The selected fish were divided according to their body weight into two groups: large size group (n=100) with weight range from 253 gm to 422 gm and small size group (n=100) with weight rang from (147 gm to 206 gm). Blood samples were collected from the caudal vein into vacationer tubes containing an anticoagulant and stored at -20°C until its use. DNA extraction was performed using TIANamp Genomic DNA Kit (cat. no. DP 304) and quantified using the Nanodrop (Uv-Vis spectrophotometer Q 5000/USA).

Table 1. *Primer sequence used for amplification of different parts of MSTN gene of Nile tilapia (Oreochromis niloticus).*

Primer name	Sequence (5'-3')	Target size
Pr1F (3'UTR)	ACATGCGAGCAAAGATATGACTG	400bp
Pr1R	TCCTTTGCACTGTGTCGGAG	
Pr2F (exon1)	GCATCTGTCTCAGATCGTGCT	370bp
Pr2R	TGCCATCATTACAATTGTCTCCG	
Pr3F (intron1/exon2)	TTTTGTGTCTCTGCGTGAGC	607bp
Pr3R	CCCTCGAATCGAAAGCGTTG	
Pr4F (intron2)	ACAGGGAGCTTGTGGTTAAGT	719bp
Pr4R	ACAAGAGAGTGTGTGTGAACAA	
Pr5F (exon3)	TGAAGATTTCAGAGGGCCCCA	357bp
Pr5R	ATCCACAACGGTCCACCAC	
Pr6F (5'UTR1)	ATGGAGAGAGAAAGGGGGTGG	607bp
Pr6R	TTGGGGACACTTAGGGGTACTA	
Pr7F (5'UTR2)	TCAGTTTCAATGTTTTCTCCCCC	551bp
Pr7R	ACATTTATCTTGCTAGGTTGCAGT	

2.2. Amplification of the MSTN Genomic Gene and Sequencing

Seven primers were used for amplification of *MSTN* gene were designed by primer 3 software (www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi) using the *MSTN* gene sequence of *Oreochromis aureus* x *Oreochromis niloticus* hybrid complete cds on the gene bank (acc. No. KC 583258.1). The primer sequences, temperature and PCR products are shown in Table 1. The PCR was performed in 25 µl reaction volume, containing 2 µl genomic DNA, 5 µl 5 x Mastermix (Thermo Scientific), 1 µl of each Primer (10 Pmol), and 16 µl dd H_2O. The final reaction mixture was placed in a thermal cycler (Veriti, Applied biosystem) and the PCR program was carried out by initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 60°C for 1 min for all primer used and 72°C for 1 min and final extension at 72°C for 10 min. PCR products stored at -20°C. The amplified DNA fragments of seven regions of *MSTN* gene were separated on 2% agarose gel (Fig. 1), stained with ethidium bromide, visualized on and photographed by Gel Documentation system (Gel Doc. Alpha-chem. imager, USA). The purified PCR products were purified using MEGAquick-spin total fragment DNA purification kit (iNtRON biotechnology) and were sent to Macrogene Company (South Korea). The results were analyzed using Chromas 1.45 (http://www.technelysium.com.au). Sequence comparisons were performed using the BLASTN program from the National Center for Biotechnology information websithttp://www.ncbi.nlm.nih.gov/.BLAST. Sequence analysis and protein alignment were performed using Geneious v. 4.8.4. software. The amino acid sequences of *MSTN* from different species used for construction of a phylogenetic tree were downloaded from GenBank.

Table 2. *Frequency of genotypes (AA, AB and BB) and alleles (A and B) in BsmI-Exon 2 of MSTN gene.*

Fish		Number/frequency of genotypes			Allele Frequency	
	Mean±S.E*	AA	AB	BB	A	B
Large size	311.57±5.85[a]	0.0	0.8	0.2	0.4	0.6
Small size	187.6±2.33[b]	0.0	0.1	0.9	0.05	0.95

*Each value represents mean±S.E of 100 fish.
Values with different letters at the same column were significantly differed. P≤0.05.

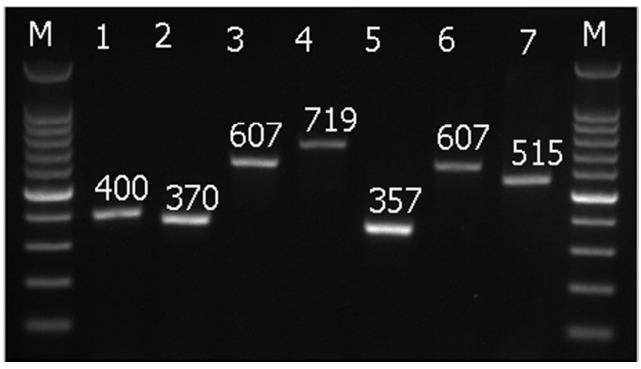

Figure 1. *Ethedium Bromide stained 1.5% agarose gel of the PCR products of different parts of the MSTN gene of O. niloticus. Lane 1, 3'UTR; 400 bp, lane 2, exon 1; 370bp, lane 3; intron 1/exon 2; 607bp, lane 4; intron 2; 719bp; lane 5; exon 3; 357bp, lane 6; 5'UTR 1; 607, lane 7; 5'UTR 2; 515bp. M. 100 bp ladder.*

2.3. RFLP and SNPs

Based on the sequence data of the seven regions of *MSTN* gene, a restriction map for each region was constructed using Neb cutter software. The amplified DNA fragment were digested with selected restriction enzyme which induced cut at more than one amplified fragment; *HinfI* for intron 1, exon 3 and 5'UTR 1; *Rsa 1* for intron 1 and 5'UTR 1, *MspI* for exon 1 and *BsmI* for *inton 1-exon 2*, 5'UTR 1 and 3'UTR. The reaction volume and conditions was done according to manufacturer protocol of Fastdigest-enzyme- Thermo Scientific. The reaction product was run on 2% agarose gel and visualized under Ultra-violet light (Gene Sys. V 1. 4.1.0 UV transilluminator).

Analysis for possible SNPs associated with large or small size fish were done by sequencing of exon 3 which showed different cutting pattern with restriction enzymes, in four fish (two large sizes and two small).

2.4. Data Analysis

Allele and genotype frequencies of *BsmI-Exon 2* polymorphism in *O. niloticus MSTN* gene were estimated by direct counting. Evaluation of mean frequencies between large and small size fish group by Student's t-test. Results were considered statistically significant at $P<0.05$.

3. Results

3.1. Identification of MSTN Gene

The seven PCR products represent different regions of genomic gene of Tilapia *MSTN* was recognized on 1.5% agarose as shown in Fig. 1. Sequence data of the seven regions did not overlap to span the whole gene sequence; the annotated gene of *MSTN* was deposited into GenBank and assigned the accession number (KT 987208).

It is found to be approximately 3.2 kb in length including 5' and part of 3' UTR region, three exons and two introns.

Comparison of *MSTN* sequence of Nile tilapia with *Oreochromis niloticus* x *Oreochromis aureus* hybrid (acc. KC 583258) revealed that the size of exon 1, exon 2 and exon 3 were 379 bp,

Figure 2. *Sequence of MSTN promoter of O. niloticus, showing the location of TATA boxes and CANNTG (bold and underline). Lower, amino acid alignment of the coding region compared with Oreochromis niloticus x Oreochromis aureus hybrid the highlight indicate amino acid substitution.*

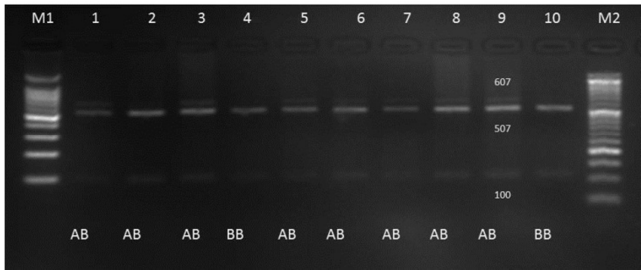

Figure 3. *Representative BsmI restriction fragment pattern of exon 2 of MSTN gene (607 bp) among 10 large size fish. Lanes 1, 2, 3, 5, 6, 7, 8, 9 are AB genotype (607-bp, 507-bp and 100-bp), lanes 4 and 10 are genotype BB (507-bp and 100-bp) and lane M 1 =Molecular marker (100-bp), Molecular marker (50-bp) 371 bp and 381 bp respectively. Sizes of intron 1 and intron 2 were 305 and 750 bp.*

The gene has typical starting codon (ATG) and termination codon (TGA) in exon 3. A putative TATA 1, TATA 2 and one CAAT boxes 225, 393, 267 bp upstream from the ATG start codon, two putative E-boxes (CANNTG motif), 137 and 355 bp upstream from the ATG starting codon.

Four SNPs at the 5'UTR were detected (150 G>C, 391 del-A, 394 Ins-G, 397 Ins-A), one SNP at intron 2 (1584 del-T) and 2 SNPs at 3'UTR (2737 A>G, 2728 C>T) and four successive insertion A, C, A, C at 2899, 2900, 2901, 2902. Three non-synonymous SNPs were found at *MSTN* coding region which constitute 376 amino acids; two at exon one 369 C>A, 831 T>A, and one at exon 3 2637 G>A, altering Thr 38-Pro, Glu 121-Val and Tyr 375-Cys respectively (Fig. 2). No SNPs has been detected at intron 1 or exon 2.

3.2. RFLP and SNP Analysis

The cutting pattern of *HinfI* in intron 1 and 5'UTR 1; *Rsa 1* in intron 1 and 5'UTR 1; *MspI* for exon 1; *BsmI* for 5'UTR 1 and 3'UTR are homozygous cutting pattern in both large and small size fish.

BsmI induced cutting pattern at intron 1-exon 2 PCR product. Restriction analysis of PCR-RFLP- *BsmI* of intron 1-exon 2 PCR product (607-bp) showed two different genotypes: AB genotype (607-bp, 507-bp and 100-bp) and BB genotype (507-bp and 100-bp fragments), genotype AA is absent (Figure 3). The AB is the most common genotype among large size fish with 0.8 frequency and significantly increased body weight compared with BB which is the most common genotype among small size fish with 0.9 frequencies (Table 2). *BsmI* of *intron 1-exon 2* PCR product was located at exon 2.

AluI induced cutting pattern at exon 3 (357 bp), two different genotypes are produced, one genotype Hh (357-bp, 300-bp and 57-bp) at large size fish, hh genotype (300-bp and 57-bp) at all other fish, the genotype HH is absent. The cutting pattern of *AluI* at exon 3 is not informative (data is not shown). Further sequence analysis for exon 3 for two large size and two small size fish revealed little number of SNPs repeated in both small and large size fish which were not informative when correlated to the growth trait (Data is not shown).

3.3. Phylogenetic Analysis

Alignment of the deduced protein sequence of *MSTN* gene of Nile tilapia with that of some fish species and other vertebrates is shown in figure 4. Phylogenetic analysis revealed a close relation among fish *MSTN* genes. Two distinct clades among different fish species was observed, the similarity in the first clade ranged from 73-99% and in the other clade is 62-65% indicate that there is considerable variation among fish species in *MSTN* gene amino acid sequence. Also, the similarity is 60-65% between *MSTN* gene of Nile tilapia and that of mammals and birds.

4. Discussion

On account of the increasing worldwide aquaculture importance of Nile tilapia, attempt for increasing production through selective breeding were done.

In the present study, *MSTN* gene of Nile tilapia was isolated and characterized which consisted of three exons and two introns as for all known vertebrate *MSTN* and encodes for 376 amino acid long peptide [3, 4, 5, 6]. Nile tilapia *MSTN* has a size of 379 bp, 371 bp and 381 bp respectively for the exons and 305 and 750 bp for the introns. The same size of exons and intron to *O. niloticus* x *O. aureus* hybrid were previously reported and were submitted to gene bank acc. KC 583258. Though different sizes of exons and introns were reported in other fish species [13, 14].

We also demonstrated that MSTN promoter sequence of Nile tilapia has the same features common to the other fish promoters, as several TATA and E. boxes in close proximity to the transcription start sites [14, 15].

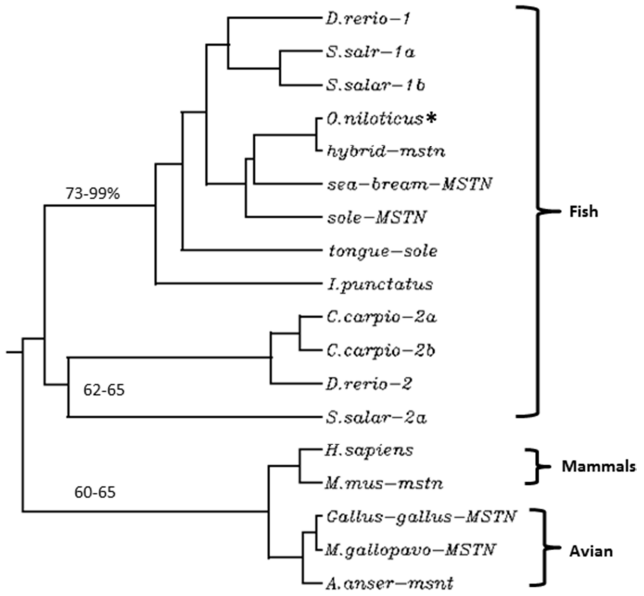

Figure 4. *Phylogenetic analysis of O. niloticus MSTN amino acid sequences (KT 987208) (marked with *) compared with the sequences of some fish species, mammals and birds are as follows: O. niloticus x O. aureus hybrid (KC 583258), Sea bream (S. aurata MSTN-AAK 53545), Sole (S. senegalensis-ACL12507), Tongue sole (C. semilaevis-ABU25352), S. salar MSTN1 a (CAC51427), S. salar MSTN1 b (CAC19541), D. rerio MSTN1 (AAB86693), I. punctatus MSTN (AAK84666), S. salar MSTN 2 a (ABN72587), D. rerio MSTN2 (NP_001019991), C. carpio MSTN2 a (ACY01747), C. carpio MSTN2 b (ACY01748), H. sapiens MSTN (AAC96327), M. musculus MSTN (AAO46885), A. anser MSTN (AAL35276), G. gallus MSTN (AAB86688), M. gallopavo MSTN (O42221).*

Compared with MSTN gene of O. niloticus x O. aureus hybrid, we demonstrated novel four SNPs at 5'UTR, one SNP at intron 2, two SNPs at 3'UTR and three non-synonymous SNPs at the coding regions. Polymorphism of the MSTN gene from other fish species has been studied; several mutations have been also associated with growth trait in fish. Sun et al. [16] detected two SNPs in exon 3 in common carp MSTN, which were significantly associated with body weight (BW). Cheng and Sun [17] identified two non-synonymous SNPs and two synonymous SNPs in MSTN exon 2 in hybrid of Culter alburnus and Ancherythroculter nigrocauda fish and association between non-synonymous SNP in exon 2 with total length, body length, body height, head length, and body weight was present.

Many authors identified synonymous and non-synonymous SNPs in exon 3 in many species of fish and associate them with body weight [16, 18]. We identified one synonymous SNPs at exon 3 (2637 G>A, altering Thr 38-Pro) furthermore, sequence analysis of exon 3 in a number of small and large size fish revealed little number of SNPs repeated in both small and large size fish which were not informative when correlated to the growth trait, this results may be due to few number of sequence sample.

Baylan et al. [19] did not find any polymorphism on the *MSTN* gene of *Oreochromis aureus* and *Oreochromis*

niloticus in exons 1, 2 or 3 using the PCR-RFLP technique. This agree with our data, only three SNPs has been detected in the coding region of *MSTN* of *O. niloticus* compared with *O. niloticus* x *O. aureus* hybrid, two in exon 1 and one at the end of exon 3, no SNPs has been detected in exon 2. This also reflect homozygous cutting pattern of five restriction enzymes used in this study in seven different regions.

Furthermore, we demonstrated polymorphic cutting pattern of *BsmI* at exon 2. with two distinct genotypes; AB (heterozygous) which is the most common among large size fish with 0.8 frequency and significantly increased body weight compared with BB which is the most common among small size fish with 0.9 frequencies. This novel *BsmI-exon 2* polymorphism of *MSTN* gene can be used as a marker assisted selection for body weight in Nile tilapia. Sánchez-Ramos et al. [12] associated between heterogeneity of genotypes of *MSTN*-1 using RFLPs and growth traits in Marine Commercial Species of fish.

With regard to the protein sequence of the isolated gene, *MSTN* shares high similarity with most known teleost fish. The phylogeny of the *MSTN* gene has been widely assessed by previous authors and is largely consistent with the current study [5, 14].

5. Conclusion

The characteristics of *O. niloticus MSTN* sequence and its promoter is as for all known vertebrates. Novel SNPs were identified in coding and non-coding regions. Novel *BsmI-exon 2* polymorphism of *MSTN* gene can be used as a marker assisted selection for large body weight in heterozygous Nile tilapia fish.

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