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Short Communication

The *myostatin* gene of *Mytilus chilensis* evidences a high level of polymorphism and ubiquitous transcript expression

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ABSTRACT

Myostatin (MSTN) is a protein of the Transforming Growth Factor-β (TGF-β) superfamily and plays a crucial role in muscular development for higher vertebrates. However, its biological function in marine invertebrates remains undiscovered. This study characterizes the full-length sequence of the *Mytilus chilensis myostatin* gene (*Mc-MSTN*). Furthermore, tissue transcription patterns and putative single nucleotide polymorphisms (SNPs) were also identified. The *Mc-MSTN* cDNA sequence showed 3528 base pairs (bp), consisting of 161 bp of 5′ UTR, 2110 bp of 3′ UTR, and an open reading frame of 1257 bp encoding for 418 amino acids and with an RXXR proteolytic site and nine cysteine-conserved residues. Gene transcription analysis revealed that the *Mc-MSTN* has ubiquitous expression among several tissues, with higher expression in the gonads and mantle than in the digestive gland, gills, and hemolymph. Furthermore, high levels of polymorphisms were detected (28 SNPs in 3′-UTR and 9 SNPs in the coding region). Two SNPs were non-synonymous and involved amino acid changes between Glu/Asp and Thr/Ile. Until now, the *MSTN* gene has been mainly related to muscle growth in marine bivalves. However, the present study suggests a putative biological function not entirely associated to muscle tissue and contributes molecular evidence to the current debate about the function of the *MSTN* gene in marine invertebrates.

1. Introduction

Myostatin (MSTN), also known as growth differentiation factor 8 (GDF-8), is a protein that belongs to the Transforming Growth Factor-β (TGF-β) superfamily. Reported evidence shows that this protein has a pivotal role in muscle growth and differentiation, mainly acting as a negative regulator of the cell cycle in this tissue (McPherron et al., 1997). To inhibit muscle protein synthesis, MSTN has a major role in different signaling pathways including the Akt, MAPK, and Smad cascades (Li et al., 2008; Trendelenburg et al., 2009; Zhu et al., 2004). Generally *MSTN* cDNA encodes for proteins of around 375 amino acids in aquatic vertebrates (Roberts and Goetz, 2001), but recently *MSTN* cDNAs encoding for longer proteins have been found in marine invertebrates (Guo et al., 2012; Hu et al., 2010). Moreover, characterized sequences of *MSTN* present conserved sites such as a proteolytic processing site (RXXR) at the carboxy-terminal region and nine conserved cysteine residues after this site (McPherron et al., 1997).

The *MSTN* gene has been characterized in a wide variety of vertebrate species, including mammals, fish, and birds (Rodgers and Garikipati, 2008). However, this gene has been characterized in few invertebrate species. Regarding marine species, an important difference between the *MSTN* of fish and invertebrates has been observed in regards to the number of presented isoforms. In fish species, such as salmonids and other teleost, two different isoforms of the same gene have been identified (Roberts and Goetz, 2001), while in marine invertebrates, such as pectinids, just one isoform has been identified (Guo et al., 2012; Hu et al., 2010). Another major difference in marine species is in relation to the expression patterns that *MSTN* exhibited in different tissues. For example, in humans *MSTN* is almost exclusively expressed in muscle tissue, and so it is thought to function more specifically in the development of these tissues (Carlson et al., 1999). In contrast, for some marine invertebrates, such as the crustacean *Gecarcinus lateralis*, it is expressed in a wider array of tissues than in higher vertebrates (Covi et al., 2008), and therefore its potential function could not be precisely specified.

MSTN is considered a highly polymorphic gene at interspecific and intraspecific levels. Previous studies have evidenced this through the identification of a high number of punctual polymorphisms in different species, mainly in mammals and in chicken, and through the association of these mutations to certain phenotypes such as muscle mass and lipid content (Grisolia et al., 2009; Johnson et al., 2009; Xianghai et al., 2007). It has been suggested that punctual mutations in the *MSTN* sequence could affect these phenotypes by inactivating the gene or by decreasing

Abbreviations: MSTN, myostatin; TGF-β, Transforming Growth Factor-β; *Mc-MSTN*, *Mytilus chilensis myostatin*; SNPs, single nucleotide polymorphisms; UTR, untranslated region; bp, base pairs; GDF-8, growth differentiation factor 8; mRNA, messenger RNA; RNA-seq, RNA sequencing; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

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the activity of the protein. In this context, a single nucleotide polymorphism (SNP) in the promoter region of *MSTN* in spotted halibut, *Verasper variegatus*, has been linked to individual growth performance (Li et al., 2012). Moreover, in the marine invertebrate *Chlamys farreri* an association analysis between growth traits and one non-synonymous SNP in the *MSTN* gene was conducted with significant results (Wang et al., 2010).

Mytilus chilensis is a marine bivalve with distribution along the Chilean coast, and is most abundant in southern Chile (Brattström and Johanssen, 1983). This species is susceptible to variations in this natural distribution as a consequence of local environmental conditions and anthropogenic activities, such as aquaculture which implies the displacement of seeds and breeders from native locations (Núñez-Acuña et al., 2012). Despite the great commercial importance of this species and its relationship with the "*Mytilus* complex," little is known about the implications of specific genes related to growth, cell development, reproduction, and immune response. The aim of this study was to characterize the *MSTN* gene as a potential candidate gene related to muscular growth, and also to identify SNPs-*MSTN*. Overall, this study provides the first report of *MSTN* mRNA in the Mytilidae family and gives relevant molecular evidence towards the understanding of its biological function in mussels.

2. Materials and methods

2.1. Myostatin gene discovery

A transcript annotated as *Myostatin* (*MSTN*) was obtained from a RNA-seq library performed for *Mytilus chilensis* (unpublished data, manuscript in preparation). Briefly, a RNA-seq library was generated through high-throughput sequencing of hemocytes of *M. chilensis* in the HiSeq 2000 platform (Illumina®, San Diego, CA, USA). Around eight hundred million reads were obtained and *De Novo* assembled, yielding over two hundred and fifty thousand contigs. Then, *MSTN* sequences from marine invertebrates were downloaded from the Genbank database in order to perform a MultiBlast analysis against all contigs by using the CLC Genomic Workbench software (version 6.0, CLC bio, Aarhus, Denmark). One contig with a significant e-value (e-value = 1.2E − 120), blasted with the *Crassostrea gigas* MSTN (Genbank accession number: EKC29862), was selected. This contig was 2753 bp in length and was used to obtain the full-length cDNA of *MSTN*.

2.2. Full MSTN cDNA characterization

To verify if the transcript was correctly annotated as *MSTN*, PCR amplifications were performed. Here, total RNA from muscle tissue of *M. chilensis* was extracted using the Trizol Reagent (Ambion®, Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. Then, from 200 ng of RNA, cDNA was synthetized using the RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific, Glen Burnie, MD, USA). In parallel, a pair of primers was designed to flank 719 bp in the C-terminal region of the ORF (Primers Mc-MSTN1-F and Mc-MSTN1-R, see sequences in Table 1) by using the Geneious 6.0.4 software (Biomatters, Auckland, New Zealand). PCR amplification was conducted using 200 ng of cDNA, 10 μM of each primer, 1.5 mM MgCl2, and 0.06U taq DNA polymerase (Thermo Scientific). PCR cycles consisted of initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 60 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. PCR products were visualized through electrophoresis in agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA) and then directly sequenced in an ABI 3730xl capillary sequencer.

Full-length cDNA of *MSTN* was obtained by the rapid amplification of cDNA ends (RACE) technique using the SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA). Primers for amplifying the 5′UTR and 3′UTR ends were designed according manufacturer's instructions (Table 1). Amplicons were purified and cloned using the TOPO TA cloning kit (Invitrogen, Life Technologies) and then transformed into *E. coli* JM109 in LB/amp/IPTG/Xgal. Positive clones were selected by galactosidase reaction visualization, and then its plasmids were purified using the E.Z.N.A® Plasmid DNA Mini kit II (Omega Bio-tek, Doraville, GA, USA). Purified plasmids were sequenced in both reverse and forward directions using M13 universal primers. Sequence analyses were carried out in the Geneious software, and consisted of quality visualizations, assembly, and BLASTn against the Genbank non-redundant database.

Table 1
Primers used in the present study. The "Specification" column shows the reaction in which each primer was used.

Primer name	Sequence (5′ to 3′)	Specification
Mc-MSTN1-F	TCAACTTCCTCCAACGGAAC	end-point PCR
Mc-MSTN1-R	TCGGCAAGGTTGTGAATTGA	
Mc-MSTNq-F	AACAAGTGGATGCGATACCC	real-time PCR
Mc-MSTNq-R	TCGGCAAGGTTGTGAATTGA	
α-tubulin-F	GAGCCGTCTGCATGTTGAGC	endogenous control (qPCR)
α-tubulin-R	TGGACGAAAGCACGTTTGGC	
Mc-MSTN_R5-i	CTCGCCTCAACTGCTCGCTAG	RACE 5′ inner
Mc-MSTN_R5-o	ACTCGAGCAGGATTGCCGTCC	RACE 5′ outer
Mc-MSTN_R3-i	GGTGCACAACTAGTGCTG	RACE 3′ inner
Mc-MSTN_R3-o	TAGTATAAATGAGAAATAGACC	RACE 3′ outer

2.3. MSTN gene transcription analysis

Total RNA extractions were performed from different tissue samples of mussels ($N = 10$); the adductor muscle, mantle, gills, gonad, digestive gland, and hemolymph. Extractions were performed using the Trizol Reagent® (Invitrogen, USA) with the same conditions as in the previous section. Regarding hemolymph, minor changes to the protocol were made. Specifically, centrifugation at 1200 ×*g* for 30 min at 4 °C after hemolymph collection and homogenization, after the Trizol was added, in the Mixer Mill Retsch MM200 (Retsch Inc., Düsseldorf, Germany) at 20 Hz for 5 min were performed.

Primers for qPCR analysis were designed by Geneious software (Table 1). End-point PCR reactions were conducted to standardize the optimal conditions for the amplification by using the same reagents described above but with a new PCR program consisting of initial denaturation at 95 °C for 2 min, 35 cycles denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Then, a dynamic range analysis was conducted to obtain qPCR efficiencies and optimal conditions for qPCR runs. For this, five serial dilutions of cDNA were prepared starting with 80 ng and with a serial factor of 1:5 in order to generate an amplification curve with both pairs of primers designed for qPCR analysis. The qPCR runs were assayed in an ABI StepOnePlus™ Mastercycler (Applied Biosystems®, Life Technologies) with the Maxima Kit® SYBR Green/ROX qPCR Master Mix (Thermo Scientific®) following manufacturer's instructions. The run method comprised of a holding stage at 95 °C for 10 min to activate the enzyme, then 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. After the amplification stage, a melting curve was performed from 57 °C to 95 °C, and data was collected every 0.3 °C to determine the existence of a sole amplification product and to verify the inexistence of contaminations and primer dimers. Efficiencies were calculated in the StepOnePlus™ Software (version 2.2, Applied Biosystems®). To quantify mRNA relative levels, the $\Delta\Delta C_T$ method was used adding the α-tubulin gene as an endogenous control (primers in Table 1). Finally, statistical analyses were performed in Microsoft Excel and GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

2.4. SNP identification

Three pools of total RNA ($n = 10$ mussels for each pool) were used to identify SNPs associated to the *MSTN* gene. SNP identification was

conducted in the CLC Genomic Workbench software using a minimal allelic frequency of 0.25 and minimal depth of 20 reads in the polymorphic site. Then, SNPs were visualized in alignments previously constructed in the Geneious software in order to determine their positions at 5′UTR, ORF, and 3′UTR.

3. Results and discussion

3.1. Molecular characterization of Mc-MSTN

This study was carried out in the frame of a wide transcriptomic sequencingperformed in the Chilean mussel *Mytilus chilensis* (this data will be publicly available soon in a new manuscript that describes the RNA-seq analyses). From this RNA-seq experiment, it was possible to characterize novel genomic knowledge of this species, which has great interest from an ecological and commercial point of view. This transcriptomic information was able to characterize not only *Mc-MSTN*, which is addressed in the present study, but to perform studies that provide more understanding mainly in the immune response and physiology of this species at transcriptomic level. Because of this reason, novel studies regarding this sequencing effort will be published soon.

The complete *Mc-MSTN* cDNA sequence was characterized by 3528 bp containing an open reading frame of 1257 bp, which coded for 418 amino acids (Genbank accession number: KF040495). Within its sequence, a conserved RXXR cleavage site and nine cysteine residues were identified (Fig. 1). Untranslated regions of 5′ UTR and 3′ UTR were also identified with 161 bp and 2110 bp, respectively, and the latter contained the polyadenilate signal AATAAAA and a poly-A site. Homology analysis among related species showed a 58.5% similarity with *Argopecten purpuratus* (Morales-Collío et al., submitted for publication), 57.6% with *Argopecten irradians* (Guo et al., 2012) and *Chlamys farreri* (Hu et al., 2010), 51.5% with *Peneaus monodon* (De Santis et al., 2011), and 50.9% with *Litopenaeus vannamei* (JQ045427). These similarities increased when alignments used the conserved C-terminal region (from the RXXR site to the stop codon); sharing 62.6% with *A. irradians*, 62.1% with *C. farreri*, 61.5% with *A. purpuratus*, 56.9% with *L. vannamei*, and 56.3% with *P. monodon*. These values are lower than similarities evidenced among pectinids or crustaceans, but this was expected, as this is the first available *MSTN* sequence for the entire Mytilidae family.

3.2. Expression profile of Mc-MSTN

The gene transcription profile of the *Mc-MSTN* was performed through quantitative PCR analysis. The *MSTN* gene was detected among all analyzed tissues, and was significantly up-regulated in the gonad ($p < 0.05$), followed by expression in the mantle and finally in adductor muscle tissue. Progressively lower levels of transcript expression were found in the gills, digestive gland, and hemolymph, respectively (Fig. 2). The expected results, according to previous studies in mollusks such as the *Argopecten* and *Chlamys* genus (Guo et al., 2012; Hu et al., 2010), were that *MSTN* would be mainly associated to muscular tissue. However, our transcript expression data suggests that the *Mc-MSTN* is not exclusively associated to muscle tissue growth, thus providing novel information for a differential biological function of this gene in mollusk bivalves. So far, there are few studies that report ubiquitous transcript expression in marine invertebrates. For instance, *Nematostella vectensis MSTN* gene expression has been associated with a wide array of tissues and is not restricted to or directly related with muscular tissues, suggesting that *MSTN* could have alternative functions such as the formation and/or regulation of cell development (Saina and Technau, 2009). Other studies in crustaceans have shown a similar ubiquitous expression of transcript in all tissues (De Santis et al., 2011). For example, the American lobster *Homarus americanus* evidences higher relative levels of *MSTN* during natural molting and eyestalk ablation in a wide array of tissues (MacLea et al., 2010). The function of *MSTN* in invertebrates is still open for debate, and findings from authors Lo and Frasch (1999) show a homolog to *myostatin* called *myoglanin*, which was related to synaptic process and neuromuscular junctions. Until now, the *Mc-MSTN* is a unique *MSTN*-like found in the entire Mytilidae family and which provides novel molecular insights of biological functions not exclusively related to muscle growth.

Since this transcript it is new for mytilidae species, and also it is not enough homologous with other higher taxa myostatins, it might not be possible to use currently developed antibodies for this protein to detect *Mc-MSTN* at protein level. Nonetheless, it is known that validation of gene expression data through proteomic assays is undoubtedly necessary, particularly due to the results obtained in this current study. In the case of *myostatin* gene, a monoclonal Anti-MSTN antibody was successfully constructed in posthatch broiler and tested to evaluate the function of myostatin in this species. The results of that previously described study showed that this antibody was able to bind MSTN and to test the effect of this protein in muscle mass (Kim et al., 2006). Furthermore, a polyclonal antibody for this protein was developed in the same species, which had more affinity with the MSTN propeptide than with the mature protein (Kim et al., 2007). Ongoing research in our group is currently aimed at developing recombinant Mc-MSTN protein to better establish *in vitro* models to validate the gene expression data evidenced in this study.

3.3. Identification of polymorphisms in the Mc-MSTN

SNP identification was conducted on the contig annotated as *MSTN*-like from a *De novo* assembly for *Mytilus chilensis* transcriptome. A total of thirty-seven SNPs were found, with nine of these localized on the coding sequence and twenty-eight in the 3′ UTR region. No SNPs were found in the 5′ UTR. Seven SNPs located in the coding sequence were synonymous, while two SNPs were involved in amino acid changes (non-synonymous SNPs). One A/C substitution at the 257th position implied a change from Glu to Asp, and a C/T substitution at the 349th position implied a change from Thr to Ile (Table 2).

This is the second time that SNPs were identified in a *MSTN* gene from a marine invertebrate. A previous work in marine bivalves was with *Chlamys farreri*, and two non-synonymous SNPs were associated with growth traits, with one of these being correlated to differential growth phenotypes (Wang et al., 2010). Those two polymorphisms were not the same SNPs found in the present study. Regarding other marine species, in the spotted halibut *Veraspes variegatus* five SNPs were found in the promoter region of *MSTN*, and these could be associated to growth traits of females in this species (Li et al., 2012). On the other hand, in the cyprinid *Cyprinus carpio* six SNPs were identified within the *MSTN* gene, and two were positively correlated to body weight and condition factor traits (Sun et al., 2012). Despite that the current study did not correlate the identified SNPs markers with traits of interest, further studies will be aimed to correlate other biological functions such as reproduction and shell growth. The high level of polymorphism discovered could be related to mechanisms of post-transcriptional regulation. MicroRNAs associated to the *MSTN* gene have been recently reported (Allen and Loh, 2011). The MSTN 3′-UTR contains a putative recognition sequence for specific microRNAs that are conserved across a wide range of vertebrate species and which activate *MSTN* promoters. Future studies will explore the potential contribution of posttranscriptional mechanisms and the role of microRNAs on MSTN expression of marine invertebrates.

4. Conclusions

The present study has reported for the first time an *MSTN* gene from the Mytilidae family, allowing establishing new comparisons in the field of genetics in relations to other MSTN sequences from different species. Identification was based on molecular characteristics and conserved regions such as the RXXR proteolytic cleavage site and nine cysteine

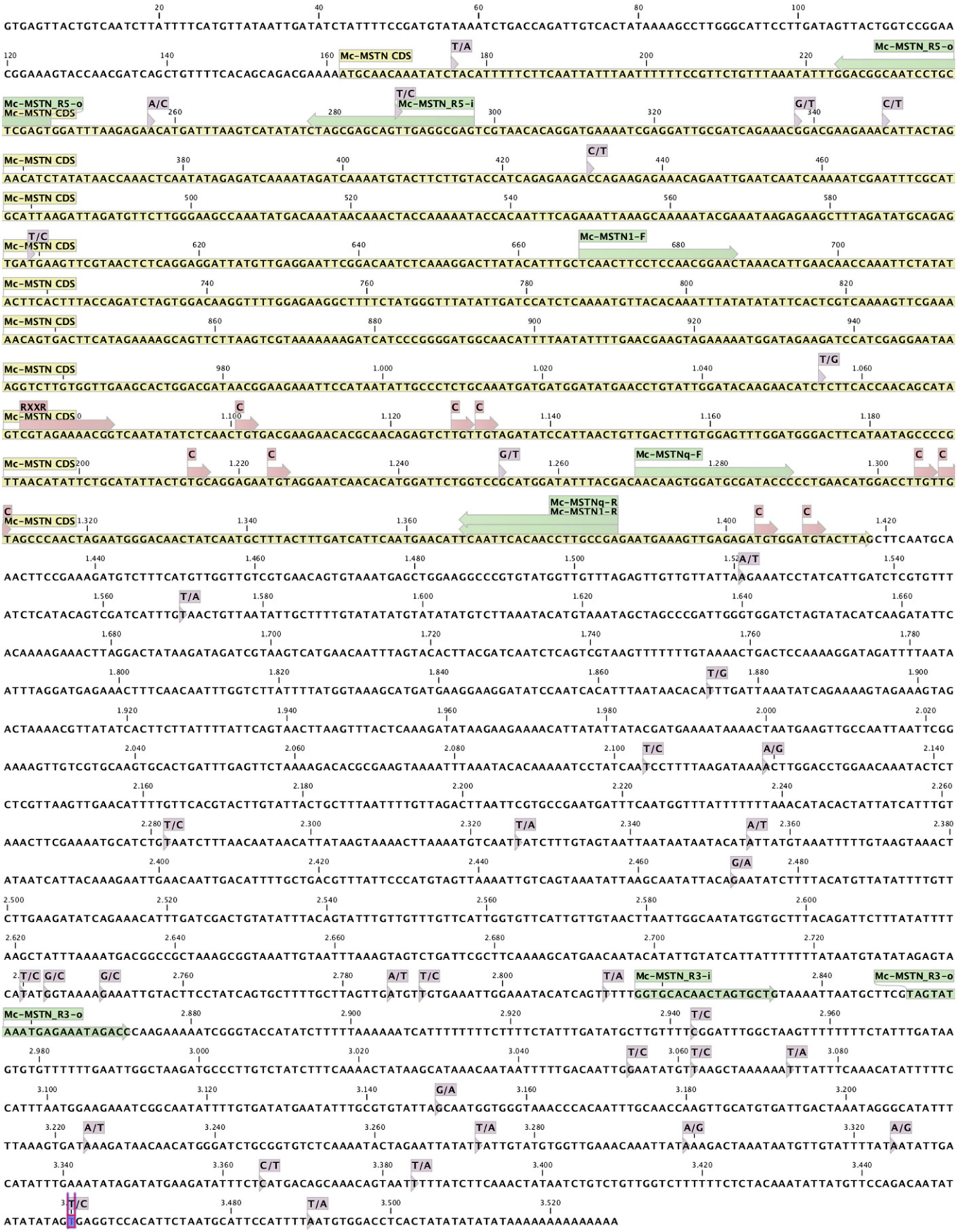

Fig. 1. Complete sequence of *Mc-MSTN*. The open reading frame is marked in gray from 168th nucleotide to 1418th. The RRKR proteolytic cleavage state and the nine-cysteine residues are marked in gray boxes. SNPs markers are shown with the respective polymorphism change involved and the sequences of primers used are shown with green arrows.

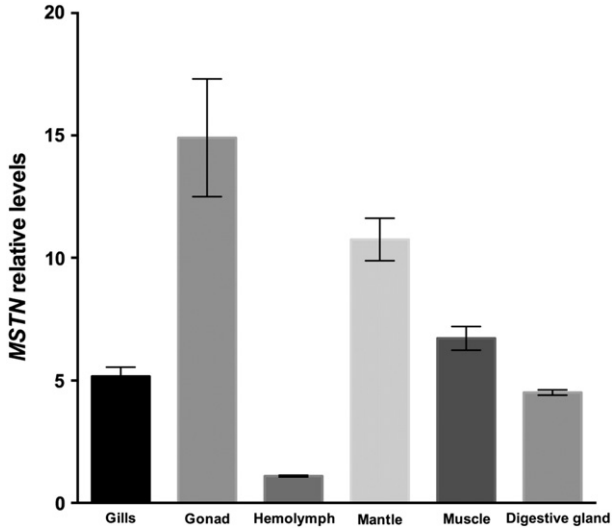

Fig. 2. mRNA relative levels of *Mc-MSTN* in different tissues. Y-axis showed ΔΔCt values normalized to the control endogenous (*α-tubilin*).

residues. The evidence from this work suggests that it is not directly related with muscular growth in adult individuals. On the other hand, polymorphisms found in the *Mc-MSTN* sequence could be related to mechanisms of post-transcriptional regulation due the high abundance of these molecular markers in the 3′ UTR region, which in many genes has shown relevant regulatory regions such as microRNA binding sites. Since in Chile there is great concern about the aquaculture activities of *Mytilus chilensis*, this work contributes with this novel gene and molecular markers useful to association studies.

Therefore, applications of the knowledge gained from this study could be aimed at genetic association studies between the SNP markers identified in the 3′-UTR region of this novel gene and specific and desired traits. Also, further ongoing work is needed to validate of the biological function of this gene through the development of Mc-MSTN recombinant protein that could be used to establish its function through *in vitro* bioassays.

Table 2
Summary of SNPs identified in *Mc-MSTN*. The "SNP position" column corresponds to the position of the nucleotide that had the polymorphism start counting from the first nucleotide of the 5′–UTR. S: synonymous SNP; NS: non-synonymous SNP.

SNP position	Polymorphism	Location	S/NS	Amino acid change
176	T/A	CDS	S	–
257	A/C	CDS	NS	Glu-Asp
288	T/C	CDS	S	–
336	G/T	CDS	S	–
349	C/T	CDS	NS	Thr-Ile
431	C/T	CDS	S	–
599	T/C	CDS	S	–
1055	T/G	CDS	S	–
1253	G/T	CDS	S	–
1521	A/T	3′-UTR	–	–
1570	A/T	3′-UTR	–	–
1874	T/G	3′-UTR	–	–
2104	T/C	3′-UTR	–	–
2119	A/G	3′-UTR	–	–
2282	T/C	3′-UTR	–	–
2326	T/A	3′-UTR	–	–
2355	A/T	3′-UTR	–	–
2472	G/A	3′-UTR	–	–
2740	T/C	3′-UTR	–	–
2743	G/C	3′-UTR	–	–
2750	G/C	3′-UTR	–	–
2786	A/T	3′-UTR	–	–
2790	T/C	3′-UTR	–	–
2813	T/A	3′-UTR	–	–
2943	T/C	3′-UTR	–	–
3054	T/C	3′-UTR	–	–
3062	T/C	3′-UTR	–	–
3074	T/A	3′-UTR	–	–
3149	G/A	3′-UTR	–	–
3224	A/T	3′-UTR	–	–
3273	T/A	3′-UTR	–	–
3299	A/G	3′-UTR	–	–
3325	A/G	3′-UTR	–	–
3365	C/T	3′-UTR	–	–
3384	T/A	3′-UTR	–	–
3460	T/C	3′-UTR	–	–
3490	T/A	3′-UTR	–	–

Conflict of interest statement

The authors declare no conflict of interest in the current study.

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