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Investigation of the polymorphism of the myostatin gene by using PCR-RFLP in two tilapia species (*Oreochromis aureus* and *Oreochromis niloticus*)

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Abstract

The aim of the study was to investigate the restriction fragment length polymorphism (RFLP) of the exon 1–3 fragments from the myostatin gene in two tilapia species, *Oreochromis aureus* and *Oreochromis niloticus*. Tissue samples were collected from a total of 50 fish, and the total genomic DNA was extracted and used for PCR amplification. Particular regions of exon 1–3 from the myostatin gene (390, 435 and 342 bp, respectively) were amplified with specific primers for each exon region by using PCR. The PCR-amplified products of each exon region were digested with appropriate restriction endonuclease enzymes and analysed using agarose gel electrophoresis.

Key words: Tilapia, myostatin, polymorphism, PCR-RFLP.

Introduction

Myostatin (MSTN) is an important negative regulator gene of the skeletal muscle mass and has a potential application in both agriculture and aquaculture. The myostatin gene, also known as GDF-8 (growth and differentiation factor-8), inhibits muscle growth[1]. It is a member of the TGF-b (transforming growth factor-beta) superfamily, which is comprised of proteins that have important roles in cell growth and signal transduction. Because of its regulator role in muscle development and growth, MSTN has been studied for productivity, growth and performance in livestock, including cattle [2], pigs [3], sheep [4], chickens [5, 6], rabbits [7], and some commercial fish species, such as rainbow trout [8], Mozambique tilapia [9], white perch [10], Atlantic salmon [11], shi drum [12], gilthead sea bream [13], European sea bass [14], white bass [9], and catfish spp.[15–17]. Several polymorphisms have been identified in the gene, indicating that the MSTN gene is highly variable [8, 10]. Since the discovery of MSTN'sprimary role in skeletal muscle growth and development, a considerable number of MSTN polymorphisms have been investigated. Several researchers showed polymorphisms of this gene in two breeds of cattle, the Piedmontese and the Belgian blue, both of which cause double muscling phenotype naturally occurring mutations (11 bp deletion) of the myostatin gene[1, 2, 18–20]. These polymorphisms,including an 11-base pair deletion in the third exon of the gene [2, 18], are directly related to the double muscling phenotype [21–24]. This gene consists of three exons and two introns in fish. Exon 1, responsible for the N-terminal signal sequence for secretion, includes the highest inter-specific variability, while exons 2 and 3 are highly conserved across species and are translated into the pro-peptide and C-terminal bioactive dimer [16, 25]. Sun *et al.*[26] investigated partial genomic fragments of the MSTN gene in common carp (*Cyprinus carpio* L.) for single nucleotide polymorphisms (SNPs). They also identified two SNPs in intron 2 and two synonymous SNPs in exon 3. Wang *et al.* [27] found two mutations by screening 103 cultivated mollusc *Chlamys farreri* specimens for polymorphisms in the MSTN gene using both DNA sequencing methods and PCR-single strand conformation polymorphisms (PCR-SSCP).

The aim of this study was to investigate the variability of three exon fragments located on the myostatin gene of two tilapia species by using polymerase chain reactions (PCR) for the amplification of the region of interest by using appropriate restriction enzymes, followed by the restriction fragment length polymorphism (RFLP) method.

Material and Methods

Samples and DNA isolation: Tilapia tissue samples (*Oreochromis aureus* (n = 25), *Oreochromis niloticus* (n = 25)) were obtained fresh from Çukurova University, Fisheries Faculty, Fresh Water Fishes Research and Production Station in Adana (Turkey). The total genomic DNA was extracted according to Asahida *et al.* [28] procedures.The purified DNA was electrophoresed on 2% agarose gels and stained with ethidium bromide.

PCR: The pairs of primers (Table 1) were designed for each of the exon 1, 2 and 3 regions. The 100 ng of genomic DNA was amplified in the total volume of a 23.875-µl PCRmix. The PCR mix consisted of 16.25 µl Master mix (Thermo, K0171), 2.5 µl forward and reverse primers (20 pmol/µl), 0.125 µl (2.5 U/µl)Taq DNA polymerase and 5-µl ddH_2O. The amplification conditions are shown in Table 2. Negative controls were used in all experiments. Assays were performed in a thermal cycler (Techne), and the amplicons were analysed with 2% agarose gel electrophoresis. The ethidium bromide stained gels were visualised under ultraviolet light.

Table 1. Region, methods, primer's sequence (5!3) and length of PCR products of the myostatin gene.

Region	Using method	Primer's sequence (5→3)	Length of fragment (bp)
Exon 1	PCR-RFLP	F:GACAATGCATCTGTCTCAGATCGTG R: CACTTACGTTCAGTTGCCATCATTAC	390
Exon 2	PCR-RFLP	F:TTCCCATGTCTCCGCAGCTGATTCCG R:CTTAACCACAAGCTCCCTGTTAAAT	435
Exon 3	PCR-RFLP	F:CTAGACAGCTGTGGCTAACCAACCATC R:TCCAGTCCCAGCCAAAGTCCTCGAAGT	342

F: forward and R: reverse

Table 2. PCR conditions.

Location	Primary denaturation in 1st cycle	Denaturation		Annealing		Elongation		Final extension	Number of cycles
	ºC/s	ºC	s	ºC	s	ºC	s	ºC/s	n
Ekzon1	94/120	94	30	58	45	72	120	72/600	30
Ekzon2	94/120	94	30	58	45	72	120	72/600	30
Ekzon3	94/120	94	30	58	45	72	120	72/600	30

Digestion reaction: In this experiment, 10-µl of each PCR product was incubated for 16 h at 37ºC with 1-µl (10 units) of Msp I, Hinc II, and Bsp120 I enzymes separately for the myostatin gene, respectively, for the exon1, exon 2 and exon 3 regions. Digestion products were separated using 2% agarose gel. All DNA samples in gels were stained with ethidium bromide.

Results and Discussion

PCR-RFLP analysis of the exon 1 DNA region of the MSTN gene: A 390 bp fragment for the exon 1 region of the MSTN gene locus was amplified (Fig. 1a). An Msp I (142 bp cutting base position) restriction enzyme was used to digest the PCR products. Digestion of the exon 1 DNA region produced two fragments of 142 and 248 bp. The samples were digested by Msp I and showed one genotype pattern. Consequently, all samples were found to be monomorphic with respect to the digestion of PCR products using the Msp I enzyme (Fig. 1b).

PCR-RFLP analysis of exon 2 DNA region of the MSTN gene: A 435 bp fragment for exon 2 of theMSTN gene locus was amplified (Fig. 2a). The Hinc II (276 cutting base position) restriction enzyme was used to digest the PCR products. Digestion of exon 2 PCR products produced two DNA fragments of 159 and 276 bp. The samples were digested by Hinc II and showed one genotype pattern. Consequently, all samples were found to be monomorphic with respect to the digestion of PCR productsusing the Hinc II enzyme (Fig. 2b).

PCR-RFLP analysis of exon 3 DNA region of the MSTN gene: A 342 bp DNA fragment for the exon 3 region of the MSTN gene locus was amplified (Fig. 3a). A Bsp120 I (227 cutting base position) restriction enzyme was used to digest the PCR products. Digestion of exon 3 PCR products produced two DNA fragments of 115 and 227 bp. The samples were digested by Bsp120 I and showed one genotype pattern. Consequently, all samples were monomorphic (Fig. 3b).We scanned the restriction fragment length polymorphism (RFLP) of the amplified exon 1, 2 and 3 regions in the myostatin gene in two different tilapia species (*Oreochromis aureus* and *Oreochromis niloticus*). The results showed that exon 1, exon 2 and exon 3 were monomorphic.

Polymorphism of the myostatin gene from several species, including cattle, sheep, chickens, fish and others, were studied. The mutations in the myostatin gene from cattle contributed to phenotype double muscling (DM). In various cattle breeds, several mutations have been found that could be responsible for this phenotype[2, 18].

In other livestock, Dehnavi *et al.* [29] detected that intron 2 was polymorphic but intron 1 and exon 3 were monomorphic in the Zel sheep myostatin gene. Hickford *et al.* [4] reported that variation of the MSTN gene in ovine is correlated with meat production, but growth rate or birth weight were not correlated in New Zealand Romney sheep. Şahin *et al.* [30] found that the exon 3 region of the MSTN locus was monomorphic in both Holstein and Brown Swiss cattle breeds.

Baron *et al.* [31] determined seven SNPs and one deletion in the exon 2 region of the MSTN gene in chicken lines. The studies on the myostatin gene in some fish species were also carried out. Sun *et al.* [26] identified four SNPs in common carp MSTN. They also found that two SNPs in exon 3 were significantly associated with body weight and conditional factors. In another study,

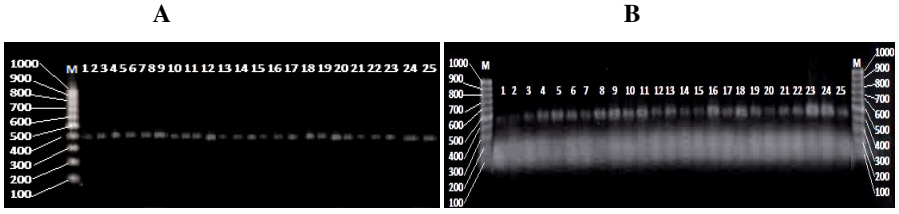


Figure 1a. PCR amplifications of exon 1 in MSTN gene region (390bp, A: *Oreochromis aureus*, B: *Oreochromis niloticus*, 1–25). Lane M, molecular size marker (1000 bp DNA ladder).

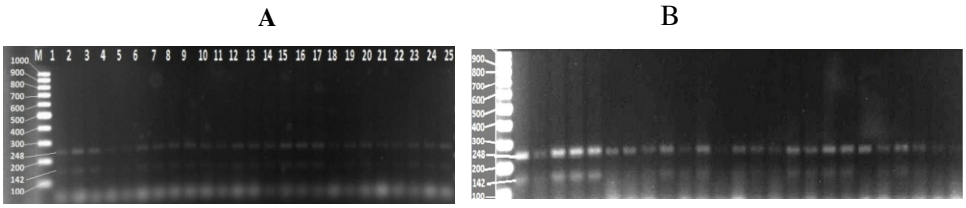


Figure 1b. Photograph of Msp I enzyme digestion products of the exon 1 DNA fragment of MSTN gene on agarose gel. (A: *Oreochromis aureus*(142bp/248bp), B:*Oreochromis niloticus* (142bp/248bp) genotypes, Lane 1–25). Lane M, molecular size marker (1000 bp DNA ladder).

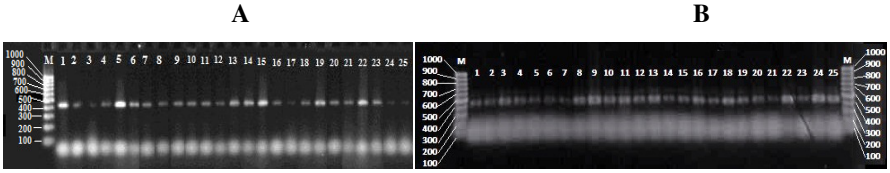


Figure 2a. PCR amplifications of exon 2 in the MSTN gene (435bp, A: *Oreochromis aureus*, B: *Oreochromis niloticus*, 1–25). Lane M, molecular size marker (1000 bp DNA ladder).

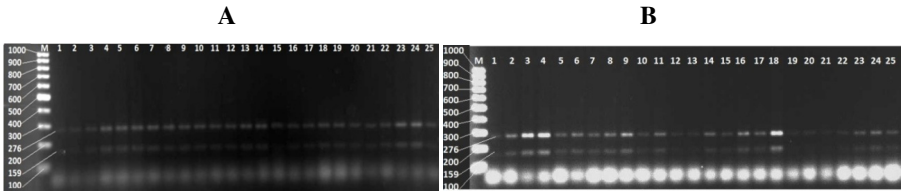


Figure 2b.Photograph of Hinc II enzyme digestion products of the exon 2 DNA fragment from the MSTN gene on agarose gel. (A: *Oreochromis aureus* (276bp/159bp), B:*Oreochromis niloticus* (276bp/159bp) genotypes, Lane 1–25). Lane M, molecular size marker (1000 bp DNA ladder).

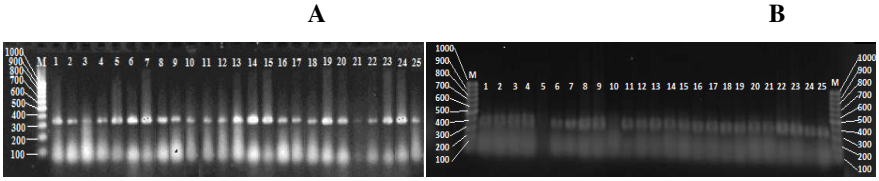


Figure 3a. PCR amplifications of exon 3 in the MSTN gene (342 bp, A: *Oreochromis aureus*, B:*Oreochromis niloticus*). Lane M, molecular size marker (1000 bp DNA ladder).

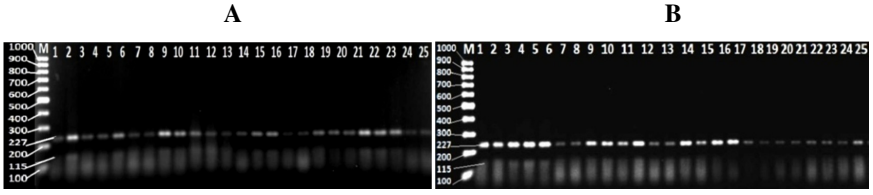


Figure 3b. Photograph of Bsp120 Ienzyme digestion products of the exon 3 fragment in the MSTN gene on agarose gel. (A: *Oreochromis aureus*(227bp/115bp), B:*Oreochromis niloticus* (227bp/115bp) genotypes, Lane 1–25). Lane M, molecular size marker (1000 bp DNA ladder).

Kocabaş *et al.*[16] characterised the myostatin locus and its expression in channel catfish (*Ictalurus punctatus*). They showed that the myostatin locus was highly polymorphic in channel catfish because of the presence of several microsatellites and single nucleotide polymorphic sites.

Polymorphism on the myostatin gene of *Oreochromis aureus* and *Oreochromis niloticus* species in our research has not been previously studied. Because of this, we investigated the polymorphism of the myostatin gene in exons 1, 2 and 3 using the PCR-RFLP technique. We amplified the exon 1, 2 and 3 regions of the myostatin genes from both species. After amplification, the PCR products of exons 1, 2 and 3 were digested with Msp I, Hinc II and Bsp120 I, respectively.

Conclusions

In this study, all samples were monomorphic. It can be concluded that although myostatin polymorphism was not observed in the *Oreochromis aureus* and *Oreochromis niloticus* exon 1–3 MSTN gene with Msp I, Hinc II, and Bsp120 I enzymes, further analysis needs to be conducted to research the SNPs of the exon 1, 2 and 3 regions of the related gene regions.

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