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ISOLATION AND SEQUENCING OF *Myf5* GENE (PROMOTER REGION) FROM BEHEIRY EGYPTIAN WATER BUFFALOES

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ABSTRACT

Myogenic factor 5 (Myf5) is a member in the myogenic regulatory factor (MRF) family of basic helix-loop-helix transcription factors, which is involved in the differentiation and maturation of myotubes and is highly expressed postnatally. The sequences of this gene are well known in cattle and small ruminants but in buffalo no available sequences were previously recorded. Therefore, the current investigation threw the light on the isolation and sequencing the promoter region of this gene, which is the most appropriate portion of Myf5 that contains single nucleotide polymorphisms (SNPs), in Egyptian buffalo. To achieve this task, PCR was performed in which gene-specific primers flanking to promoter region of Myf5 were utilized. A PCR products of 700bp, of Myf5 gene corresponding to the expected product size, was successfully amplified. Sequencing of the purified PCR products. A novel A309T SNP was detected in the promoter sequences of the Myf5 locus (at nucleotide number 309 of the promoter) among Beheiry Egyptian water buffaloes. Three different single-stranded conformation polymorphism patterns were observed in the Myf5 locus: TT, AT, and AA with frequencies of 0.26, 0.23 and 0.51 respectively. Statistical analysis revealed that, the homozygous TT genotype was significantly associated with the average daily gain than AT and AA genotypes from birth to 9 mo of age. The results of this study could be used as a basis for further investigations to associate the detected SNPs with meat quality traits in Egyptian water buffaloes.

Keywords: Myogenic factor 5 (Myf5), SSCP, sequencing, PCR, Egyptian water buffaloes, SNP.

INTRODUCTION

Myogenic factor 5 *(Myf5)* gene belongs to the myogenic regulatory factor (MRF) family of basic helix-loop-helix transcription factors that regulates myogenesis ***(Urbański et al., 2006)***. *Myf5* is expressed during proliferation of myoblasts ***(Hughes and Schiaffino, 1999; Koishi et al., 1995; Pas and Visscher, 1994; Te Pas and Soumillion, 2001)***, and responsible for directing cells into the skeletal muscle programme ***(Braun et al., 1992; Tajbakhsh et al., 1996a)***. Postnatal expression of the *Myf5* gene is characteristic of satellite cell ***(Koishi et al., 1995)***. Satellite cells proliferate and differentiate, thereby enabling postnatal muscle growth ***(Beilharz et al., 1992)***.Without functional Myf5 protein, cells that would normally undergo myogenic differentiation can adopt non-muscle fates, before the onset of MyoD ***(Tajbakhsh et al., 1996b)***. Its roles in muscle growth and development make this gene candidate for molecular markers of meat production in livestock ***(Maak et al., 2006; Robakowska-Hyzorek et al., 2010; Verner et al., 2007).***

Some SNPs were detected in bovine *Myf5* and their association analysis showed a significant correlation with growth and meat production traits ***(Robakowska-Hyzorek et al., 2010)***. In pig, *Myf5* was considered as candidate gene for growth rate and carcass traits ***(Urbański and Pierzchała, 2005)***. Significant associations between genotypes *Myf5* and carcass quality traits were observed, but simultaneously these associations indicated that, the gene mutations analysed were not causal mutations and further studies are necessary ***(Cieślak et al., 2002)***.

The aforementioned studies on *Myf5* gene were conducted on animals other than Egyptian buffaloes. Therefore, the aim of this study was to identify the polymorphisms of Myf5 (promoter) in Beheiry Egyptian water buffalo.

MATERIALS AND METHODS

Sampling and DNA extraction:

The present study comprised 200 pure Beheiry Egyptian water buffalo-bulls (*Bubalus bubalis*) kept in a farm located at Nucleus Herd, Nataff Gedeed station of Mahalet Mousa farm, Kafr el-sheikh Governorate. All used equipments were autoclaved and sterilized before collection the samples as well as during all procedures in the laboratory. Blood samples were collected from the jugular veins, each sample was kept in EDTA- tube (kept in ice box). The genomic DNA was extracted from white blood cells using Gene JET genomic DNA extraction kit following the manufacturer protocol (Fermentas , #K0721/USA).

Polymerase chain reaction (PCR):

A partial sequence of *Myf5* promoter was amplified by PCR using primers (inserted below) designed by Primer 3.0 software based on the published sequences of water buffalo in india (GenBank accession number, NC_007303).

The PCR was carried out in a reaction volume of 50 µL, containing 4.0 µL DNA template (approximately 100 ng), 1.0 µL (0.20 mM) dNTP, 5.0 µL buffer, 3.0 µL (2.5 mM) MgCl2, 2.0 µL 10µmol/L forward primer, 2.0µL 10µmol/L reverse primer, 1.0 µL 10X Taq DNA polymerase (5 U/µL, Fermentas, #K1071, European Union), and 32µL nuclease free water. Thermal cycling parameters were performed as follows: initial denaturation at 94°C for 5 min, 35 cycles of amplification (94°C for 40 s for DNA denaturation, annealing 56°C for 1 min, extension at 72°C for 1 min) and final extension at 72°C for 10 min. The samples were held at 4°C. PCR products were resolved by electrophoresis on 2% agarose gel in 1X TAE, stained with ethidium bromide and visualized with UV light of gel documentation system (Biometra Biomedizinische Analytik, GmbH).

Localization	Ta (°c)	Size (bp)	Reverse primer (/5 - /3)	Forward primer (/5- /3)	Locus
Promoter region	55	700	CTCCCTTCTCCCTCTTT	CATTCTCCTACCCCTGATTT	Myf5

Single stranded conformation polymorphism(SSCP) and sequencing:

For performing SSCP analysis, the PCR products (5 µL) were mixed with an equal volume of denaturing solution (25 mM EDTA, 95% formamide, 0.025% xylene-cyanol and 0.025% bromophenol blue), heated for 5 min at 94 °C, rapidly chilled on ice and then loaded on a non denaturing 12% polyacrylamide gel (39:1 acrylamide to bis-acrylamide). SSCP gel was run in 1× TBE buffer at 200 V for 10 h at 4 °C. To visualize the DNA fragments, the gel was stained with a 0.5 µg/ml solution of ethidium bromide in 1× TBE buffer for 20 min, and then destained in distilled water for 15 min. SSCP genotypes were identified by differential migration due to fragment conformation. After the polymorphism was detected, the PCR products of different electrophoresis patterns were purified, and then sent to MacroGen Company (South Korea) to sequence in both directions using ABI 3730XL DNA sequencer (Applied Biosystem, USA). The sequences were analyzed with Geneious 4.8.4 software.

Statistical analysis:

Allele and genotype frequencies of A309T SNP at nucleotide number 309 of promoter region in Egyptian buffalo *Myf5* were estimated by direct counting. Hardy–Weinberg equilibrium (HWE), He (gene heterozygosity), Ho (gene homozygosity), and Ne (effective allele numbers; reciprocal of homozygosity) were computed by POPGENE software (Version 1.31; ***Yeh et al., 1999***). Association analyses were

conducted using least square means (LSM) estimates procedure by using SPSS (version 16.0) software to analyze the relationship between the three genotypes (TT, AT and AA) of A309T SNP and growth traits in buffalo according to the following linear model: $Y_{klm} = \mu + A_k + Gl + E_{klm}$, where Y_{klm} was the trait measured on each of the klmth animals, μ was the overall population mean, A_k was fixed effect due to the kth age (in days), Gl was the fixed effect associated with lth genotype and E_{klm} was the random error.

RESULTS

The promoter region of the Myf5 gene was determined using PCR (Fig.1) and its genotyping was identified in 200 Beheiry Egyptian water buffaloes using SSCP method. Three SSCP banding patterns (TT/AT/AA) were detected in the promoter region of Myf5 locus (Fig.2). The sequence of this Myf5 locus that combined with other loci of Myf5 gene in Beheiry Egyptian water buffaloes were submitted to GenBank with accession numbers KC107773 which showed a novel A309T SNP at nucleotide number 309 of promoter of Myf5 locus among the Beheiry Egyptian water buffaloes (Fig.3). The obtained sequence was examined against previously known sequences published in GenBank data base using Blast search. The results of this step confirmed that, the sequence as expected is highly similar to a bovine *Myf5* (*Bos taurus* accession no. NC_007303) except in four positions. These four SNPs were; C33A, G46T, C50T and T71C.

The frequencies of the TT, AT, and AA genotypes in the A309T SNP of the Myf5 locus 0.51 (102) for AA, 0.23 (46) for AT, and 0.26 (52) for TT with allele frequencies of 0.625 for A and 0.375 for T. Chi-Square (χ^2) equals 52.4315 (P value < 0.05) which means that, there was

a significant differences between expected and observed frequencies, indicating a Hardy-Weinberg disequilibrium (Table 1). The values of Ne were approaching 2 (Table 1). Association of TT, AT, and AA genotypes at A309T SNP at nucleotide number 309 of promoter of Myf5 with the growth traits (bodyweight and average daily gain, ADG) was analyzed in Beheiry Egyptian water buffaloes at birth and then at 3, 6, 9, 12, 18, and 24 mo of age. All data concerning growth traits were obtained from farm records. There was a significant association between ADG and A309T from 6 to 9 mo of age ($P \leq 0.05$), with a significant high ADG of the TT individuals (Table 2). Therefore the homozygous TT genotype is more favorable than the heterozygous AT or homozygous AA genotype.

DISCUSSION

The postnatal muscle growth and differentiation is characterized by many cellular and metabolic events related to myogenesis (meat synthesis) and controlled by different genes ***(Pierzchała et al., 2011).*** These myogenic genes are considered as candidate genes for meat production traits ***(Te Pas et al., 1999; Verner et al., 2007; Wyszynska-Koko et al., 2006)***. *Myf5* comes on the top of these myogenic genes. Previous studies have shown that *Myf5* SNPs are associated with meat traits in cattle ***(Robakowska-Hyzorek et al., 2010)***.

Comparing to bovine *Myf5,* there were four SNPs; C33A, G46T, C50T and T71C. This result indicated that, the sequence of *Myf5* is highly conserved between cattle and buffalo. Similarly, four SNPs were detected in the promoter region of bovine *Myf5*: G723T SNP, A1307G (RFLP-AvaII) SNP, C1026T (RFLP-TaqI) SNP, and C969T (RFLP-NsiI) SNP ***(Robakowska-Hyzorek et al., 2010)***.

SNPs in the coding region especially those change the amino acid sequences of the protein, are crucial for genotype based selection and marker assisted selection (MAS). For example, a non-synonymous A1553C SNP in exon 2 of bovine *Myf5* gene causes an amino acid substitution (glycine / proline) was significantly associated with live weight, loin eye height, loin eye area and water holding capacity, while no effect of genotype on back fat thickness, marbling, meat tenderness and rib area ***(Ujan et al., 2011)***. In addition, four SNPs were found in exon 1 of bovine *Myf5*: T236C (RFLP-HphI) SNP, C209G (RFLP–BanII) SNP, C233G (RFLP-AvaI) SNP, and G234C (RFLP-BsiEI) SNP. T236C SNP is synonymous as it does not change the amino acid sequence (Phe/Phe), whereas the other three nucleotide substitutions are non-synonymous: the C209G SNP results in a change from Asp to Glu at position 17, C233G SNP changes the amino acid at position 25 from Asp to Glu; and the G234C SNP changes Gly to Arg at position 26. A1911G SNP in intron 2, C2434T and G2630A in the 3′-UTR region were detected in bovine *Myf5* ***(Bhuiyan et al., 2009)***.

On the other hand our results indicated that there was A309T SNP between Beheiry Egyptian water buffaloes and a consequent significant association between ADG and A309T from 6 to 9 mo of age ($P \leq 0.05$), with a significant high ADG of the TT individuals. Therefore the homozygous TT genotype is more favorable than the heterozygous AT or homozygous AA genotype. The majority of detected SNPs are usually present in the non-coding region of the genes, in promoters. These SNPs in promoter may lead to change in the binding sites for transcription factors necessary for transcription of this gene or other related genes. It is therefore possible that the detected SNPs in promoter of *Myf5* gene in

Beheiry Egyptian water buffaloes might have indirect effect on the function of the growth hormone. Further investigations including gene expression as well as statistical analysis of a large population size can shed the light on the significance of these SNPs and whether they can affect Myf5 function or not.

In conclusion, this is a preliminary study that provides the researchers with raw data which can be used as a basis for further traits to relate these SNPs with meat production and quality traits in Beheiry Egyptian water buffaloes.

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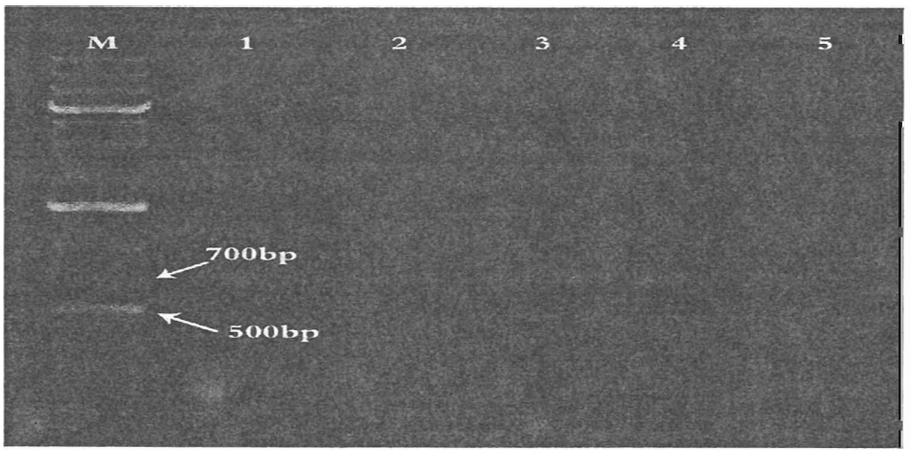

Fig. (1): Ethidium bromide stained agarose gel of PCR products representing amplification of *Myf5* locus with size of 700bp (lanes 1-5) in five Beheiry Egyptian water buffaloes.

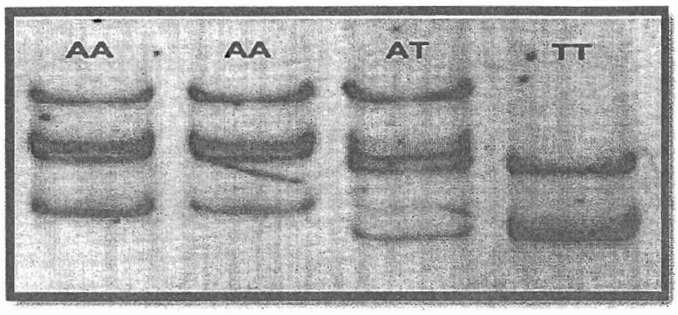

Fig. (2): SSCP patterns of *Myf5* locus of four Beheiry Egyptian water buffaloes showed trimorphic SSCP patterns (AA, AT, TT) in promoter region.

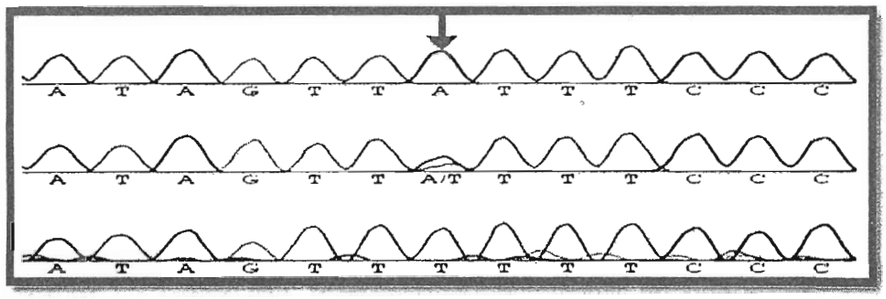

Fig. (3): Nucleotide sequences of *Myf5* locus in three Egyptian water buffaloes showed A309T SNP at nucleotide number 309 of promoter region. The arrow indicates the position of the SNP.

Table (1): Genotype distribution and allelic frequencies at A309T SNP of Beheiry Egyptian water buffaloes *Myf5* locus and the estimated Chi-Square (χ^2), HWE = Hardy-Weinberg equilibrium.

Association analysis	*Ne*	*He*	χ^2/HWE (P value)	Allele frequencies		Genotype frequencies (number)			SNP
Associated with average daily gain with TT>AA, AT.	1.8824	0.4688	52.4315 (0.00) (< 0.05)	T	A	TT 0.26	AT 0.23	AA 0.51	A309T
				0.375	0.625	(52)	(46)	(102)	

χ^2: Chi-Square value. HWE: Hardy-Weinberg equilibrium.

He: gene heterozygosity. Ne: effective allele numbers.

Table (2): Correlation of genotypes at promoter of Myf5 locus with growth traits in Beheiry Egyptian water buffaloes using LSM (Least Square Means) ± SE (Standard Errors).

Genotype			Growth traits	Age
TT	AT	AA		
32.562±0.345	33.712±0.361	31.863±0.235	BW(Kg)	Birth
74.488±0.707	73.734±0.826	75.136 ±0.507	BW(Kg)	3Mo
0.465±0.03	0.444±0.04	0.480±0.03	ADG(Kg)	Birth – 3Mo
129.723±0.484*	102.252±0.632*	101.379±0.396*	BW(Kg)	6Mo
0.613±0.03*	0.316±0.03*	0.291±0.02*	ADG(Kg)	3 – 6Mo
171.294±0.687*	141.61±0.757*	143.796±0.483*	BW(Kg)	9Mo
0.461±0.03	0.437±0.02	0.471±0.03	ADG(Kg)	6 – 9Mo
209.196±0.389*	184.293±0.481*	180.188±0.319*	BW(Kg)	12Mo
0.421±0.02	0.474±0.03	0.404±0.03	ADG(Kg)	9 – 12Mo
260.749±2.544	252.726±2.760	258.129±1.783	BW(Kg)	18Mo
0.286±0.02	0.380±0.03	0.433±0.03	ADG(Kg)	12 – 18Mo
484.442±9.381	476.261±10.328	478.245±7.151	BW(Kg)	24Mo
1.242±0.03	1.242±0.03	1.223±0.02	ADG(Kg)	18 – 24Mo

BW-bodyweight, ADG-average daily gain. Values are least squares means (± SEM).

* Significance at P ≤0.05.

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عزل وتتابع الجين المنظم لنمو العضله من الجاموس المصرى

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يعتبر الجين العامل عضلي 5 (*Myf5*) هو عضو في الأسرة MyoD، التي تشارك في التمايز والنضج من مرحلة myotubes و مرحلة ما بعد الولادة كما أن هذا الجين معروف جيدا في الأبقار والمجترات الصغيرة ولكن في الجاموس لا يوجد تسلسل لهذا الجين العضلي قبل هذه الدراسة . وبالتالي، فإن الهدف الرئيسي من إجراء هذه الدراسة هو محاوله عزل و تسلسل هذا الجين ، الذي يحتوي على الأشكال المتعددة للتركيبات النووية المنفردة (النيوكلوتيدات). لإجراء هذه الدراسة تم القيام بعمل تقنية تفاعل البلمره المتسلسل (PCR) وقد أظهرت النتائج ان الحجم الناتج عن تفاعل البلمره المتسلسل هو 700 زوج من النيوكلوتيدات وبعد التنقيه وعمل تتابع نيوكلوتيدى لها وتم تحديد وجود طفره في الجزء المنشط عند النيكلوتيده رقم 309 والتي أدت إلى تغيير النيوكلوتيده من الادينين الي الثايمين وأظهرت النتائج وجود ان الطراز الجيني المتماثل الثايمين الثايمين (Homozygous genotype TT) في هذه الطفره له ارتباط هام بمعدل النمو اليومي عند عمر الولادة إلى عمر 9 أشهر وهذه الدراسة الأولى من نوعها لعزل هذا الجين فى الجاموس المصري.