

Polymorphism of sheep *POU1F1* gene exon 6 and 3'UTR region and their association with milk production traits

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(Received 10 Oct, 2013; revised version 20 Jan, 2014; accepted 26 Jan, 2014)

Summary

The purpose of the study was to detect the *AluI* and *DdeI* polymorphisms within *POU1F1* gene exon 6 and 3'UTR region in Turkish sheep breeds, and their association with milk productive traits. Jugular blood samples were collected from 50 Sakiz, 50 White Karaman, and 50 Awassi sheep using EDTA as an anticoagulant. PCR-RFLP and sequencing analysis were performed to investigate possible polymorphisms in the exon 6 and 3' flanking region of the sheep *POU1F1* gene. The PCR products were digested with restriction endonuclease *AluI* and *DdeI*, and biallelic polymorphism was found with restriction endonuclease *AluI* and two genotypes (*TT* (296 bp and 173 bp) and *CC* (235 bp, 173 bp and 61 bp)) were detected. White Karaman and Awassi breeds did not show polymorphisms for *AluI* restriction sites. No polymorphism at the *DdeI* cleavage sites was detected in the three sheep breeds. Significant statistical results were found in milk yield (***P*<0.001), fat (***P*<0.001) and lactose (**P*<0.05) values with *TT* and *CC* genotypes, however no significant association of *TT* and *CC* genotypes with protein values was detected (*P*>0.05) and individuals with genotype *TT* had a superior milk yield in Sakiz sheep breeds. As sequence results, seven variation points were determined for exon 6 (g.185T>C) and 3'UTR (g.220G>A, g.229C>T, g.248C>T, g.250A>T, g.255T>C, g.258C>T) of the sheep *POU1F1* gene. We have reported here for the first time single nucleotide polymorphisms of the *POU1F1* gene for both exon 6 and 3'UTR and its effects on milk traits in Turkish sheep breeds were evaluated.

Key words: Milk traits, *POU1F1* gene, Polymorphism, Turkish sheep breeds

Introduction

POU class 1 homeobox 1 (*POU1F1*) is a member of the tissue-specific POU-containing transcription factor family. It contains a POU DNA-binding domain which consists of two regions, one is POU homoeodomain required for DNA binding and the other is POU-specific domain, necessary for DNA-binding specificity and dimerization of *POU1F1*. This protein has an important role in the transcriptional regulation of growth hormone (*GH*) and prolactin (*PRL*) genes. Studies showed that the *POU1F1* protein is expressed exclusively in somatotrophs, lactotrophs and thyrotrophs and is required for the establishment and maintenance of these differentiated cell types, as well as for cell proliferation of somatotrophs and lactotrophs (Li *et al.*, 1990; Tatsumi and Amino, 1999; Bastos *et al.*, 2006). Sheep *POU1F1* gene is located on chromosome 1q21-22 and consists of six exons which encode a protein of 33 kDa. Given the importance of *POU1F1* gene in the control of gene expression of important hormones is directly associated with growth process and milk production, it is reasonable consider it as a good candidate gene for marker assisted selection (Bastos *et al.*, 2006). Indeed, *POU1F1* gene polymorphisms have been significantly associated with body weight, birth weight, litter size and milk production in cattle, goat and pig species (Yu *et al.*, 1995; Renaville *et al.*, 1997; Lan *et al.*, 2007a, b; Daga *et al.*, 2013).

Sakiz is a high milk yield local sheep breed in Turkey. Milk production varies from 120-250 kg of per lactation depending on management and husbandry conditions and lactation length is 175 days. White Karaman is another local sheep breed in Turkey and bred for both meat and milk production, milk production varies from 50-60 kg. per lactation and lactation length is 140 days. Awassi is principally a milk breed, and milk production varies from 120-160 kg, and lactation length is 165 days. This breed is reared in southeast Turkey (Akcapinar, 2000).

The purpose of the study was to detect the *AluI* and *DdeI* polymorphisms within *POU1F1* gene exon 6 and 3'UTR region in Turkish sheep breeds, and their association with milk productive traits.

Materials and Methods

Animal resources and DNA isolation

Animals were chosen at random and were 4 years old, multiparous and lactating ewes and in their third lactation. During the day animals grazed on pasture and received as supplement 250 g/head/day concentrate commercial food (crude protein 20% and 2500 ME kcal/kg), animals were raised in semi-intensive conditions. Monthly individual milk yield has been recorded from day 30 of lactation until day 140, 165, 175 for White Karaman, Awassi and Sakiz, respectively (for

a total 9, 10, 11 samples for each ewe for White Karaman, Awassi and Sakiz, respectively). Animals were milked once a day in the evening. Individual milk yield was recorded every fifteen days during lactation and each individual milk sample was analyzed for fat, protein and lactose using Lactoscan milk analyser.

Jugular blood samples (2 ml per ewe) were collected from 50 Sakiz, 50 White Karaman, 50 Awassi sheep using EDTA as an anticoagulant. Genomic DNA was extracted from the whole blood using the phenol chloroform method (Sambrook *et al.*, 1989).

The study was approved by the Ethical Committee of Laboratory Animals, Firat University: 09/02, 04/10/2007.

PCR amplification

For this study according to the ovine (AJ549207) sequence of exon 4-6 of *POUIF1* gene, the following PCR primers were designed: Forward: 5'-GTATTGCTGCTAAAGACGCC-3' and Reverse: 5'-GAGGGAAAGATATAGTGAAAGGG-3'. For primer design, Primer 3 software (Rozen and Skaletsky, 2000) was used. PCR reaction was carried out in 50 µL of total volume, containing 10 X PCR buffer (50 mM/L KCl, 10 mM/L Tris-HCl (pH = 8.0), 0.1% Triton X-100), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 10 pM/L of each primer, 50 ng ovine genomic DNA and 1 U Taq DNA polymerase (Eppendorf AG, Hamburg, Germany). PCR conditions were as follows: denaturation at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min, on Mastercycler® (Eppendorf AG, Hamburg, Germany). The PCR products were separated by electrophoresis on 2% agarose gels (Promega, Madison, WI, United States) in parallel with a 100 bp DNA marker.

Genotyping

20 µl of PCR product was digested separately with 10 U *DdeI* and 10 U *AluI* (Fermentas GmbH, St. Leon-Rot, Germany) at 37°C overnight. PCR products of digestion were resolved by electrophoresis on a 4% agarose gel stained with ethidium bromide. Then, ten samples of each genotype were sequenced from both directions to confirm the results obtained with PCR-RFLP technique. Obtained sequence was aligned with the AJ549207 sequence of the GenBank.

Statistical analysis

Direct counting was used to estimate phenotype and allele frequencies of *POUIF1* gene *AluI* genetic variants. ANOVA (one-way) was used to analyse association of *POUIF1* variants with milk yield, fat and protein contents. Minitab statistical software was applied to evaluate the link between genotype and milk traits.

Results

In this study, the primers for the exon 6 and its flanking region of ovine *POUIF1* gene were used for

amplification of genomic DNA of three Turkish sheep breeds and uniform fragment was obtained after 2% agarose gel electrophoresis. The results showed that amplification fragment size (469 bp) was consistent with the target one and had good specificity, which could be directly analyzed by RFLP.

The results of this study indicated the presence of two *AluI* cleavage sites (235 and 296) within the *POUIF1* gene exon 6 and its flanking region sequence. According to this, two genotypes were shown. Allele C restriction site resulted in 235 bp, 173 bp and 61 bp fragments, whereas T allele occurred in the absence of the restriction site in the 236 bp, resulting in a three fragments 296 bp and 173 bp (Figs. 1 and 2). Therefore, genotype *CC* and *TT* demonstrated three and two bands, respectively. While genotype *TC* showed four bands however, genotype *TC* was not found in Awassi, White Karaman and Sakiz sheep breeds. Allelic and genotypic frequencies of *POUIF1* gene for *AluI* site in three Turkish sheep breeds were presented in Table 1. As a result, a biallelic polymorphism was found with restriction endonuclease *AluI* and two genotypes were detected, *CC* (235 bp, 173 bp and 61 bp) and *TT* (296 bp and 173 bp). Allelic frequency for Sakiz, White Karaman and Awassi breeds was 0.36, 1 and 1, respectively for allele C; 0.64, 0 and 0, respectively for allele T. The most frequently observed genotype was *TT* in Sakiz sheep breed. White Karaman and Awassi breeds did not show polymorphisms for *AluI* restriction sites.

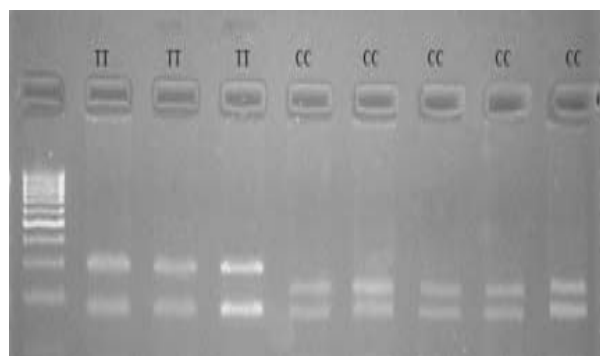


Fig. 1: Agarose gel electrophoresis band patterns after digestion with *AluI* endonuclease within the exon 6 and its flanking region sequence of the sheep *POUIF1* gene. Lane M: 100 bp DNA marker. Lane 2-4: *TT* genotype (296 bp and 173 bp), and Lane 5-9: *CC* genotype (235 bp, 173 bp and 61 bp). As one small band (61 bp) was invisible on 4% agarose gel only two bands were visible for *CC* genotype

Table 1: Gene and genotype frequency of the exon 6 of *POUIF1* gene for *AluI* site in three Turkish sheep breeds

Sheep breeds	n	<i>TT</i>	<i>TC</i>	<i>CC</i>	T	C
Awassi	50	0.00	0.00	1	0.00	1
		0	0	50		
W. Karaman	50	0.00	0.00	1	0.00	1
		0	0	50		
Sakiz	50	0.64	0.00	0.36	0.64	0.36
		32	0	18		
Total	150				0.22	0.78

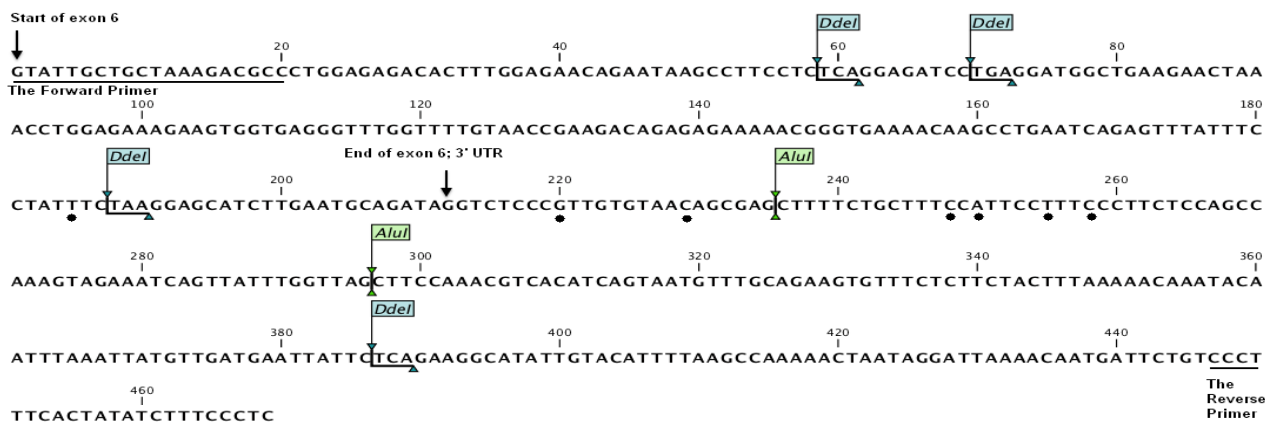


Fig. 2: The structure of sheep *POU1F1* gene and SNPs location for exon 6 and 3'UTR and *AluI*, *DdeI* enzyme restriction sites. Black dots (●) represent mutation points

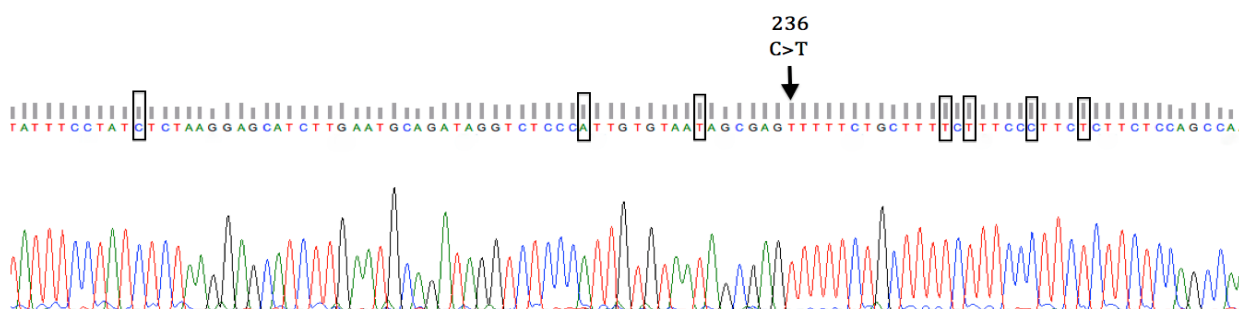


Fig. 3: DNA sequence of genotypes *TT* at the 236 locus and detected variation points in sheep *POU1F1* gene exon 6 and 3'-UTR region

Table 2: Associations of *AluI* PCR-RFLP genotypes with milk traits in Sakiz sheep breeds

Milk traits	Genotype		P-value
	TT (n=32)	CC (n=18)	
Fat (%)	5.78 ± 1.13 ^{***}	8.60 ± 1.20 ^{***}	0.000 ^{***}
Proteins (%)	6.446 ± 0.408	6.286 ± 0.737	0.325 ^{NS}
Lactose (%)	5.482 ± 0.166 [*]	5.313 ± 0.398 [*]	0.040 [*]
Milk yield (kg)	1.233 ± 0.202 ^{***}	0.768 ± 0.160 ^{***}	0.000 ^{**}

^{*}, ^{**}, ^{***} Means of sheep milk traits with different letters are significantly different P<0.05, P<0.01, and P<0.001, respectively

Table 3: Means ± SD of milk traits in White Karaman, Awassi and Sakiz sheep breeds

Milk traits	Sheep breeds			P-value
	Sakiz (n=50)	White Karaman (n=50)	Awassi (n=50)	
Fat (%)	6.792 ± 1.783 ^c	4.756 ± 1.289 ^a	5.597 ± 1.888 ^b	0.000 ^{***}
Proteins (%)	6.3882 ± 0.5476 ^a	4.0468 ± 0.2096 ^b	3.9434 ± 0.1556 ^b	0.000 ^{***}
Lactose (%)	5.4212 ± 0.2814 ^a	6.1256 ± 0.5164 ^b	5.8980 ± 0.2307 ^b	0.000 ^{***}
Milk yield (kg)	1.0653 ± 0.2922 ^a	0.6505 ± 0.1422 ^b	0.9690 ± 0.2876 ^a	0.000 ^{***}

^{a, b, c} Means of sheep milk traits with different letters were significantly different P<0.001

To determine the relationships between genotype *TT* and *CC* and milk traits in Sakiz sheep breed, statistical analyses were used. Significant statistical results were found in milk yield (^{***}P<0.001), fat (^{***}P<0.001) and lactose (^{*}P<0.05) values with *TT* and *CC* genotypes, however, no significant association of *TT* and *CC* genotypes with protein values was detected (P>0.05). Individuals with genotype *TT* had a superior milk yield when compared to genotype *CC*, however, genotype *CC* had a superior fat value (Table 2).

In spite of no polymorphisms being detected in White

Karaman and Awassi sheep breeds, milk traits were analysed. From the milk analysis results, milk yield (^{**}P<0.01), fat (^{**}P<0.01), protein (^{**}P<0.01) and lactose (^{**}P<0.01) values were found as statistically important. Sakiz sheep breed showed the highest milk yield, fat and protein values when compared to other sheep breeds, whereas White Karaman has shown superior lactose value (Table 3).

To confirm the PCR-RFLP results, ten PCR products were sequenced in both directions in an ABI 310 DNA sequencer. The sequence results showed the comparison

between nucleotide sequence of AJ549207 (sheep *POUIF1* gene), and seven variation points (g.185T>C, g.220G>A, g.229C>T, g.248C>T, g.250A>T, g.255T>C, g.258C>T) were detected in four samples, within these, one mutation g.185T>C was found in exon 6 and six other mutations were found in 3' UTR, these variation points cannot be determined to *AluI* restriction enzyme (Figs. 2 and 3).

In this research, *DdeI* restriction enzyme showed four cleavage sites (58, 69, 187 and 386 bp) within the exon 6 and its flanking region sequence (Fig. 2), but none of these were shown to be polymorphic. Consequently, only one genotype was detected in investigated sheep breeds, which is *D1D1* (199 bp, 118 bp, 83 bp, 58 bp and 11 bp) (Fig. 4).

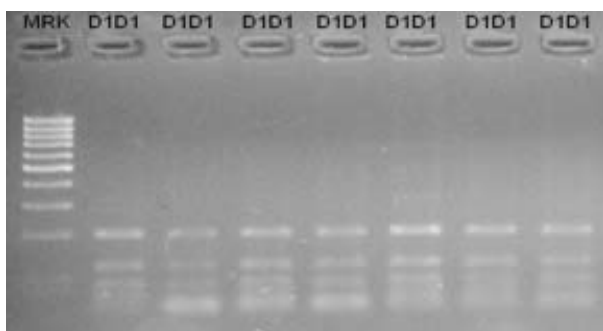


Fig. 4: Agarose gel electrophoresis band patterns after digestion with *DdeI* endonuclease within the exon 6 and its flanking region sequence of the sheep *POUIF1* gene. Lane M: 100 bp DNA marker. Lane 2-7: *D1D1* genotype (199 bp, 118 bp, 83 bp and 58 bp). As one small band (11 bp) was invisible on 4% agarose gel only four bands were visible for *CC* genotype

Discussion

The polymorphisms of sheep *POUIF1* gene were first revealed by Bastos *et al.* (2006). Until now, only a few studies have been carried out on the sheep *POUIF1* gene and their associations with production traits. In various Chinese goat breeds, polymorphisms in exon 6 of the *POUIF1* gene have been shown. Lan *et al.* (2007a) found four mutation points in goat *POUIF1* gene, within these two mutations g.60T>G and g.174T>C were found in exon 6 and two other mutations were found in 3'UTR. This g.174T>C mutation could be detected by *AluI* endonuclease. These authors found that individuals with genotype *TC* (**P*<0.05) had a superior milk yield when compared to genotype *TT* and individuals with genotype *TC* showed significant associations with milk production. Lan *et al.* (2007a) suggested that genotype *TC* could be regarded as a molecular marker for superior milk yield.

Lan *et al.* (2007b) described a *DdeI* PCR-RFLP method for detecting a silent allele in goat *POUIF1* gene, according to this method g.102T>C mutation point was detected. The g.102T>G mutation of the exon 6 region removes a *DdeI* endonuclease restriction site (CTNAG). Their results indicated that, the amplified DNA fragment with *DdeI* endonuclease digestion showed five fragments (200 bp, 118 bp, 102 bp, 20 bp

and 11 bp) for *POUIF1-D1* allele and four fragments (200 bp, 118 bp, 113 bp and 20 bp) for *POUIF1-D2* allele. According to Lan *et al.*'s. (2007b) results, the most observed genotype was *D1D1* whereas *D2D2* genotype was not detected and significant associations were found in milk yield between individuals with genotype *D1D1* (**P*<0.05). Genotype *D1D1* has been recommended as a molecular marker for better performance in goat industry by Lan *et al.* (2007b).

Mura *et al.* (2012) performed PCR, SCCP and sequencing methods in order to examine polymorphism within *POUIF1* gene in relation to milk production traits in Sarda sheep breed. Their results indicated that, only the 5'UTR of the fourth intron (g.121C>T) and 3'UTR of the six exon (g.249G>A) fragments displayed two novel SNPs, however, these polymorphisms were not found by Bastos *et al.* (2006). The statistical analysis showed no association between milk productive traits (milk yield, fat, proteins and lactose) and the found polymorphisms.

Bastos *et al.* (2006) found four substitutions in *POUIF1* gene in Churra da Terra Quente sheep breed, one in the second exon in position g.150G>A, two substitutions in the third exon in position g.89G>A and g.105G>A, and one in the fourth intron in position g.93A>G.

In the present study, exon 6 and 3'UTR of the ovine *POUIF1* gene polymorphisms were screened by PCR-RFLP and the sequencing technique. We have reported here for the first time single nucleotide polymorphisms of the *POUIF1* gene for both exon 6 and 3'UTR in Turkish sheep breeds.

We observed that biallelic polymorphism was found with restriction endonuclease *AluI*, whereas no polymorphism was found in *DdeI*. The *AluI* polymorphisms was only shown in Sakiz sheep breeds and *TC* genotype was not detected in genotype analysis in the three sheep breeds. We suggest that this difference is probably the consequence of different environmental conditions, selection and possibly even QTL or gene(s) regulating milk traits. The statistical results revealed significant associations between milk traits and genotypes in Sakiz sheep breeds. The individuals with genotype *TT* had better milk yield than individuals with genotype *CC*, on the contrary, individuals with genotype *CC* showed a higher fat value. Although no polymorphisms were detected in White Karaman and Awassi sheep breeds, milk traits were analysed. The milk analysis results show milk yield, fat, protein and lactose values were found to be statistically important between species. Sakiz sheep breed showed the highest milk yield, fat and protein values when compared to other sheep breeds whereas White Karaman has shown superior lactose value.

To confirm the PCR-RFLP results, ten PCR products were sequenced in both directions in an ABI 310 DNA sequencer. The sequence results show the comparison between nucleotide sequence of AJ549207 (sheep *POUIF1* gene) revealed seven variation points detected in four samples; g.185T>C, g.220G>A, g.229C>T, g.248C>T, g.250A>T, g.255T>C, g.258C>T, within these, one mutation g.185T>C was found in exon 6 and

six other mutations were found in 3'UTR. These detected polymorphisms are different from Bastos *et al.* (2006) and Mura *et al.* (2012). These sequences from Turkish sheep breeds have been deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>) under the accession No.: KC959950.

As a result, Sakiz sheep breed has a different genotype and has shown different mutation points in exon 6 and 3'UTR of the *POUIF1* gene. The next step will be to analyse these SNPs and 3' flanking region of the *POUIF1* gene in a larger sample of Sakiz sheep breeds and evaluate their association with productive traits. According to sequence results six mutation points were determined for 3'UTR, further analysis about 3' flanking region of the *POUIF1* gene could clarify the role of this gene in regulating the productive traits.

Acknowledgement

The project was supported by Scientific Research Projects Council of Firat University (project code 2110).

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