ISSN 1022-7954, Russian Journal of Genetics, 2016, Vol. 52, No. 9, pp. 977–984. © Pleiades Publishing, Inc., 2016.

ANIMAL GENETICS

Identification of Novel SNPs of Ovine *PRL* Gene and Their Association with Milk Production Traits[1]

O. Ozmen[a] and S. Kul[b]

[a] *Department of Genetics, Ankara University, Ankara, 06110 Turkey*
[b] *Department of Animal Breeding, Firat University, Elazig, 23119 Turkey*
e-mail: ozgeozmen@gmail.com, ozgeozmen@ankara.edu.tr

Received November 19, 2015

Abstract—Prolactin (PRL) is a lactogenic hormone that plays a significant role in milk production; its depletion in sheep provokes a severe reduction of milk secretion. Thus, PRL also could be used as a positional marker gene associated with milk production and composition traits. Therefore, the purpose of the study was to identify genotype frequencies of single nucleotide polymorphisms the intron 2 in ovine PRL gene and its possible association genotypes with milk traits in dairy sheep breeds. The genetic structures of ovine PRL gene were examined by PCR-RFLP and DNA sequencing methods in three sheep populations. Four hundred and fifty blood and milk samples were used in the study, which included 150 samples from each of Sakiz, Akkaraman and Awassi ewes respectively. As a result, PRL genotype *AA* showed a strong association with milk yields content, whereas the animals carrying *BB* genotype had a higher fat percentage value in the three sheep breeds. Haplotype analysis of the obtained sequences showed the presence of 12 haplotypes in the PRL intron 2 region. In the present study, we have reported for the first time 48 SNPs of the PRL gene for intron 2 in dairy sheep breeds. These preliminary results indicate that the identified SNPs lend themselves readily for further research regarding physiological impacts such as milk production and reproductive traits in other dairy sheep populations.

Keywords: prolactin, sheep, milk production traits, SNPs
DOI: 10.1134/S1022795416090118

INTRODUCTION

The dairy sheep industry plays a vital role in many Mediterranean countries, especially for the production of high quality cheese from local dairy breeds [1, 2]. In the last few years, interest concerning the milk from small ruminants has been increasing in order to find new ways of exploiting the potential of local breeds [3]. According to FAO, total sheep milk production of Turkey was 1.101.013 tons in 2013 and the number of sheep was 27.425.233 heads in 2013 [4]. In Turkey, sheep breeds are generally multipurpose, reared for meat and milk production [5] and dairy sheep have been farmed traditionally, almost all milk is used for cheese production and, consequently, milk content traits are very important [2].

For the identification and characterization of genetic variants associated with milk performance traits in various sheep breeds, many research efforts have been based on the candidate gene approach [1, 6–8]. Because of financial and practical restrictions, genome-wide selection is usually unfeasible for most dairy sheep breeds, making the application of selection schemes assisted by molecular information on causal mutations of genes affecting milk traits an attractive alternative in dairy sheep [1, 2]. Polymorphisms within selected candidate genes can be tested for their associations with quantitative traits to better understand their effects and can be used in marker-assisted selection [9]. Most association studies between the ovine milk protein polymorphisms and the milk performance traits have assessed mainly the effects of single gene and some controversial results have been revealed [10–13].

In dairy sheep, several genes (*CSN1S, CSN3, DGAT1, GHR, LGB, LEP, STAT5A* and *PRL*) have been investigated for their relationships with milk traits [8]. Prolactin (PRL) is a lactogenic hormone that plays a significant role in milk production; its depletion in sheep provokes a severe reduction of milk secretion [14], suggesting that PRL is a functional candidate gene that could contribute to variations in milk yield. In addition, the PRL gene is located in a region of the ovine chromosome 20 where putative QTL for fat percentage [15] and milk, fat, and protein yield [12] have been proposed. Thus, PRL also could be used as a positional marker gene associated with milk production and composition traits [16].

[1] The article is published in the original.

Furthermore, typing autochthonous sheep breeds at milk protein loci gives the possibility of increasing productivity and avoiding the loss of genetic variability, thus preserving biodiversity with particular attention to endangered breeds carrying special milk protein variants [3].

Among the limited polymorphisms that have been identified in the ovine PRL gene, 2 variants (A and B) within intron 2 have been associated with milk-related traits [7, 8]. The PRL genotypes significantly affected milk yield, fat and protein content in Serra da Estrela sheep [7]. Staiger et al. [8] suggested that the PRL intron 2 polymorphism significantly affects milk yield in East Friesian sheep and could therefore be used as a potential marker in selection breeding programs [16]. Based on the above considerations, the objective of this study is to detect novel variants in the region of the intron 2 and partial exon 2 region in the ovine PRL genotypes and its possible association with milk production traits in Sakiz, Akkaraman and Awassi dairy sheep breeds.

MATERIALS AND METHODS

Animal Resources and DNA Isolation

Sakiz is a high milk yield local dairy sheep breed in Turkey and is well known for its early sexual maturity and outstanding prolificacy. Milk production varies from 120 to 250 kg for 175 days lactation length depending on management and husbandry conditions. Akkaraman is the largest local sheep population bred in Turkey. It's fat tailed sheep and bred as a dual purpose breed with milk production varying from 50 to 60 kg per 140 days lactation. Awassi is principally a milk breed reared in southeastern in Turkey. Its milk production varies from 120 to 160 kg per 165 days lactation [17].

Four hundred and fifty blood and milk samples were used in in the study which included a set of three 150 samples from Sakiz, Akkaraman and Awassi ewes respectively. The sample selected randomly consisted of animals that were 4 years old, multiparous and lactating. Akkaraman – Awassi and Sakiz breeds were reared, respectively, in Elazig and Balikesir county at the commercial herds of three farms. Animals were fed 250 g/head/day concentrate commercial food (crude protein 20% and 2500 ME kcal/kg) as supplement and raised in semi-intensive conditions. Measurements of milk yield were initiated on the 14th day of lactation to exclude the risk of contamination with colostrum. Animals were milked twice a day at constant intervals and 20 ml milk sample was collected for milk analysis. Individual milk yield was recorded every day during lactation for each individual and each breed. Milk sample was analysed for milk composition (fat, protein, lactose, density and non-fat solids) using Lactoscan milk analyser (Milktronic Company, Nova Zagora, Bulgaria). Jugular blood samples (2 mL/ewe) were collected aseptically from each of the animals, using EDTA as an anticoagulant. Genomic DNA was extracted from the whole blood using the phenol chloroform method [18]. All samples were delivered back to the laboratory in icebox.

For this study, PCR amplification for PRL gene were amplified and thereafter digested with *Hae*III restriction enzyme as described by Vincent and Rothschild [19]. Allele *A* contained 3 restriction sites for *Hae*III and resulted in 4 fragments of 1400, 530, 360 and 150 bp whereas the presence of an additional restriction site in the *B* allele resulted in 5 fragments of 1400, 510, 360, 150, and 20 bp. PCR products of digestion were resolved by electrophoresis on a 4% agarose gel stained with ethidium bromide. Then, fifteen randomly chosen PCR samples of each genotype were sequenced from both directions to confirm the results obtained with PCR-RFLP technique. Direct sequencing was performed on 3100 ABI PRISM sequencer (Applied Biosystems, USA) and sequenced by commercial services. Sequences were obtained with the same primers used for PCR amplification. Obtained sequences were aligned with the KC764410 for PRL sequence of the GenBank.

Bioinformatics Analysis

The genotype, allelic frequencies and the observed and expected heterozygosity and Hardy-Weinberg equilibrium were calculated using Arlequin ver. 3.5.1.3 package program [20]. Sequences were analyzed using the BioEdit ver. 7.2.5 software [21] for sequence alignment.

The DnaSP software 5.10.01 [22] was used to calculate polymorphic sites, average number of nucleotide differences (k), number of haplotypes (h), nucleotide diversity (π) haplotype diversity (H_d) within and among breeds; Watterson's theta estimator for the studied species separately using a haplotype sequence was obtained. Pi (π) is based on the average number of nucleotide differences between the sequence, and theta is based on the total number of segregating sites in the sequence [23].

Network software 4.6.1.2. [24] was used to build the network of haplotype groups using the median joining algorithm. MEGA ver. 6.0 [25] was used for the phylogenetic sequence analyses of haplotypes by the Neighbour-Joining method based on Kimura-2P model and the reliability of the inferred tree was assessed by bootstrap (1000 replicates) (data not shown).

Association Analysis

Statistical analysis was performed using R-Project software (R Core Team, 2013) and general linear mixed model (GLMM) was applied to analyse association between PRL genotypes and milk yield, fat, pro-

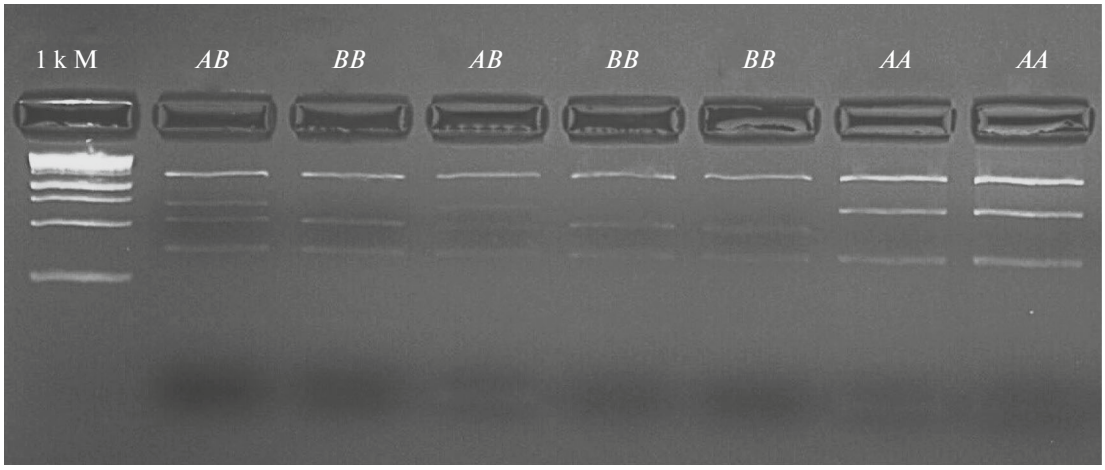

Fig. 1. Agarose gel electrophoresis band patterns after digestion with *Hae*III endonuclease within the intron 2 region sequence of the sheep PRL gene Lane M: 1 kb DNA marker; lane 2,4: *AB* genotype (1400, 530, 510, 360, 150, and 20 bp); lane 3,5,6: *BB* genotype (1400, 510, 360, 150, and 20 bp); lane 7,8: *AA* genotype (1400, 530, 360, and 150 bp).

tein and lactose percentages. Dependent variables in the analysis were milk yield, fat, protein and lactose percentage whereas the PRL genotypes and breed were considered as fixed effects in the model. The model used was as follows:

$$Y_{ijkl} = \mu + B_i + G_j + GxB_k + A_l + e,$$

where Y is the variable being estimated being test day milk yield, fat, protein or lactose; B_i is the fixed effect of the breed; G_j is the fixed effect of the genotype; GxB_k is the fixed interaction effect of breed and genotype; A_l is the random effect of the animal and e is the residual effect. $P < 0.05$ was considered statistically significant.

RESULTS

Genotypic and Allelic Frequencies

After digestion of the 2.5 kb fragment of PRL gene with the *Hae*III endonuclease three genotypes were identified: *AA* (1400, 530, 360, and 150 bp), *AB* (1400, 530, 510, 360, 150, and 20 bp) and *BB* (1400, 510, 360, 150, and 20 bp) (Fig. 1). The results for genotypic and allelic frequencies of the examined populations are reported in Table 1.

The allele frequency was different between Sakiz and Akkaraman-Awassi breeds. Sakiz ewes showed higher frequency of allele *A* (0.77) than Akkaraman and Awassi breeds (0.15, 0.23 respectively). The allele distribution of PRL gene in Akkaraman and Awassi sheep breeds was in agreement with Hardy–Weinberg equilibrium by the Chi-square test ($P > 0.05$), on the contrary Sakiz breed was not agreement with Hardy-Weinberg equilibrium ($P < 0.01$).

Haplotype Frequencies

In this study, the sequence analysis of intron 2 of the PRL gene was shown interesting variations in the examined populations. For intron 2, except for reference sequences (KC764410) twelve different haplotypes were obtained (Table 2). The most common haplotype was haplotype 14 (H_14) for Sakiz breed, haplotype 15 (H_15) for Akkaraman breed and haplotype 6 (H_6) for Awassi breed. However, H_13 and H_14 were observed only in the Sakiz breed. Haplo-

Table 1. Distribution of the observed allele frequencies for PRL loci, expected genotype frequencies in accordance with Hardy–Weinberg equilibrium

Population (n = 150 per each)	Allele frequency		Observed genotypes			P value	H_O	H_E
	A	*B*	*AA*	*AB*	*BB*			
SAK	0.77	0.23	96	39	15	0.0019^{**}	0.2600	0.3554
AKK	0.15	0.85	4	37	109	0.7458^{NS}	0.2467	0.2559
AWS	0.23	0.77	8	53	89	1.000^{NS}	0.3533	0.3554

Note: P value for the agreement to Hardy-Weinberg equilibrium, observed (H_O) and expected heterozygosity (H_E) in Sakiz (SAK), Akkaraman (AKK) and Awassi (AWS) sheep breeds. NS – non significant; n – total number of individuals.
** $P < 0.01$

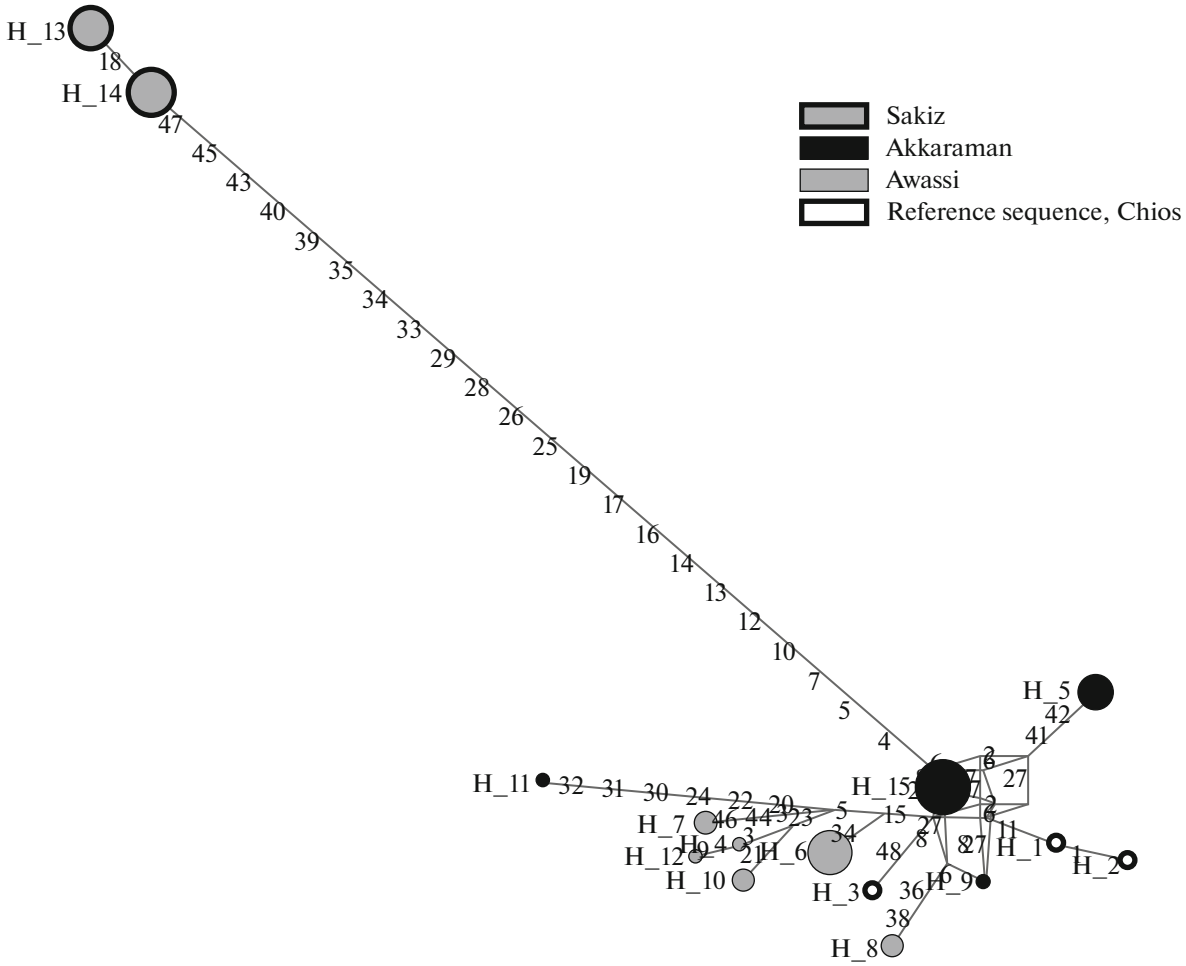

Fig. 2. Median joining network for PRL haplotype observed in examined sheep populations H_1 contains KC764410; H_2 contains HM234397; H_3 contains HM234398 sequence Different colors represent different sheep breeds (H_1 H_2 H_3 contains the reference sequence).

types were determined based on reference sequences KC764410, HM234397 (ovine PRL gene, intron 2 variant A) and HM234398 (ovine PRL gene, intron 2 variant B) and called H_1, H_2 and H_3 in this study, respectively. The PRL haplotype sequences from these sheep breeds have been deposited in the GenBank database under the access number: KP215628–KP215639.

Based on the observed mismatch distributions and the constructed radiation tree (data not shown), two main groups were determined. Neutrality tests were applied separately to these haplotype groups. Neutrality tests at PRL gene, Tajima's *D* value, Fu and Li's *D** and F* values and Fu's *Fs* values are shown in Table 3. Tajima's *D* value, Fu and Li's *D** and *F** values and Fu's *Fs* values for intron 2 group A are positive and the group B negative. Artificially selected populations, like livestock species, do not fulfil the assumptions of random mating and constant population size for the neutrality test, hence positive Tajima's *D* values are likely due to the demographic histories of these species or breeds rather than true balancing selection [23]. Haplotype diversity (H_d), nucleotide diversity (π) and Watterson's theta estimator were calculated separately for the studied species using the haplotype sequences obtained. Since in all groups nucleotide diversities were low but haplotype diversities were high, recent population growth is suggested.

A median joining network is presented in Fig. 2. The most common haplotype for Sakiz, Akkaraman and Awassi sheep breeds are H_14, H_15 and H_6, respectively. According to the network result H_14 differs from the H_1 by 25 nucleotides; H_15 and H_6 differs from H_1 by four nucleotides. The Sakiz breed showed different haplotype and different variations in comparison with Akkaraman and Awassi.

Genotype Effects on Milk Production Traits

Least squared means of milk traits are represented in Table 4. As milk analyze results, milk yield, fat, protein and lactose percentage were found as statistically

Table 2. Non-coding SNP position at the PRL gene in Sakiz (SAK), Akkaraman (AKK) and Awassi (AWS) sheep breeds. Nucleotides are numbered from 1 to 2500, using KC764410 as the reference sequence

Haplotypes (GenBank no.)	Haplotype frequency			Nucleotide sites																																															
	SAK	AKK	AWS	29	41	42	143	150	201	231	241	270	303	312	336	353	354	365	404	406	436	440	488	518	532	537	574	575	587	614	617	618	619	632	670	673	679	684	689	722	735	736	738	743	762	763	779	787	802	882	953
H_1 (KC764410)				G	C	C	T	G	A	T	A	G	A	G	T	T	G	C	T	C	T	A	C	G	T	C	A	C	C	G	C	A	C	C	A	G	G	C	C	T	C	T	G	G	G	A	T	C	G	G	A
H_2 (HM234397)				A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_3 (HM234398)				.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H_4 (KP215628)	0	0	1	.	G	T	.	A	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_5 (KP215629)	0	5	0	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.
H_6 (KP215630)	0	0	7	.	G	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_7 (KP215631)	0	0	2	.	G	.	.	A	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	A	.	.
H_8 (KP215632)	0	0	2	.	G	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.
H_9 (KP215633)	0	1	0	.	G	.	.	.	G	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_10 (KP215634)	0	0	2	.	G	.	.	A	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_11 (KP215635)	0	1	0	.	G	.	.	A	.	.	.	.	.	A	.	.	.	T	.	.	.	.	G	.	A	.	G	.	.	.	.	.	T	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_12 (KP215636)	0	0	1	.	G	T	.	A	.	.	.	C	.	A	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H_13 (KP215637)	6	0	0	.	G	.	C	A	.	C	.	.	G	A	C	C	A	.	G	T	G	C	.	.	.	.	.	G	T	A	T	G	.	.	.	A	T	T	.	.	.	A	T	.	.	G	.	T	.	T	.
H_14 (KP215638)	7	0	0	.	G	.	C	A	.	C	.	.	G	A	C	C	A	.	G	T	.	C	.	.	.	.	.	G	T	A	T	G	.	.	.	A	T	T	.	.	.	A	T	.	.	G	.	T	.	T	.
H_15 (KP215639)	0	10	0	.	G	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 3. Genetic diversity indexes and neutrality indices in intron 2 PRL gene in the studied sheep breeds

Indexes	Group A	Group B
	Sakiz	Akkaraman, Awassi
Haplotype number (*h*)	2	10
Nucleotide diversity (π)	0.00959	0.00537
Haplotype diversity (H_d)	0.700	0.867
Polymorphic sites	28	27
Theta (θw)	0.00931	0.00723
Tajima's *D*	0.12373	–0.89476
Fu and Li's *F**	1.08119	–0.80739
Fu and Li's *D**	1.25539	–0.57819
Fu's *Fs*	5.804	–1.235

Table 4. Least squares means (± SEM) of milk traits in sampled sheep breeds

Trait	Sakiz	Awassi	Akkaraman	*P* value
Fat, %	$7.066^{a} \pm 0.586$	$5.171^{b} \pm 0.455$	$4.121^{c} \pm 0.381$	0.000***
Lactose, %	$5.419^{c} \pm 0.425$	$5.920^{b} \pm 0.489$	$6.113^{a} \pm 0.491$	0.000***
Protein, %	$6.435^{a} \pm 0.571$	$3.919^{c} \pm 0.327$	$4.029^{b} \pm 0.335$	0.000***
Milk yield, ml	$1.102^{a} \pm 0.083$	$1.051^{a} \pm 0.078$	$0.804^{b} \pm 0.063$	0.000***

[a, b, c] Different superscripts of small letters mean significant difference ($P < 0.001$).

important ($P < 0.001$). Sakiz sheep breed has shown highest milk yield, protein and fat percentage, Akkaraman sheep breed has highest lactose percentage values when compared to among sheep breeds.

Results of the association study of PRL genotypes with milk production traits are shown in Table 5. In Sakiz ewes, allele *A* was higher, while the frequency of allele *B* was more frequent in the Akkarman and Awassi breeds. For PRL genotypes clear differences were observed between Sakiz and Akkaraman-Awassi breeds. In the all studied breeds carrying *AA* genotype had a greater milk yield than those with *AB* and *BB* genotype ($P < 0.001$), while *BB* genotype had a greater milk fat percentage than those with *AB* and *AA* genotype ($P < 0.001$). In Sakiz, heterozygous *AB* ewes higher milk protein percentage when compared homozygous *AA* and *BB* animals. However, there were no statistically significant differences in protein percentage according to genotypes in Akkaraman and Awassi ewes.

DISCUSSION

In this study, the polymorphic *A* allele of ovine PRL was positively associated with milk yield, but it influenced fat and protein percentage negatively. Only few investigators had previously appealed to the PCR-RFLP and DNA sequencing method to study the ovine PRL gene [7, 8, 16].

The two variants in the ovine PRL gene have been determined based on *Hae*III digestions of PCR products, as first described by Vincent and Rothschild [19]. By partial sequencing of the PRL gene intron 2, it was found that the B variant results from a 23 bp deletion of the A variant of the prolactin gene and not from an extra *Hae*III digestion site, as had been reported [16].

Ramos et al. [7] found a significant effect of the PRL genotype on milk yield, fat, and protein content in the Serra da Estrela breed, but no effect was found in the Merino breed. In both breeds, the A allele occurred more frequently than the *B* allele, and Serra da Estrela ewes carrying the *AA* genotype had lesser milk yields compared with ewes with *AB* and *BB* genotypes, which is the opposite of the results presented in this study for Sakiz, Akkaraman and Awassi ewes. On the other hand, the positive effect of the *AA* genotype of milk yield was shown by Staiger et al. [8] and *A* allele was positively associated with milk yield in East Friesian sheep, which is the similar to results presented in this study. Although the possibility of a direct effect of the polymorphism needs to be explored, the association with milk production is more likely due to linkage with a functional mutation. Different alleles being associated with high milk production in different breeds could, therefore, be the result of different linkage phases between the *Hae*III polymorphism and the functional mutation [8]. However, association studies examining individual animal milk production data from different breeds farmed under the same man-

Table 5. Association of PRL genotype with milk production traits in sampled sheep breeds

Trait	PRL genotype			*P* value
	AA	*AB*	*BB*	
Sakiz sheep	$n = 96$	$n = 39$	$n = 15$	
Milk yield, mL	$1.497^{a} \pm 0.013$	$1.034^{b} \pm 0.016$	$0.659^{c} \pm 0.027$	0.000***
Fat, %	$5.362^{c} \pm 0.096$	$7.069^{b} \pm 0.098$	$8.902^{a} \pm 0.127$	0.000***
Lactose, %	$5.470^{a} \pm 0.016$	$5.476^{a} \pm 0.043$	$5.293^{b} \pm 0.110$	0.007***
Protein, %	$6.315^{b} \pm 0.037$	$6.615^{a} \pm 0.071$	$6.279^{b} \pm 0.116$	0.002***
Awassi sheep	$n = 8$	$n = 53$	$n = 89$	
Milk yield, ml	$1.347^{a} \pm 0.024$	$1.023^{b} \pm 0.035$	$0.729^{c} \pm 0.013$	0.000***
Fat ,%	$3.532^{c} \pm 0.127$	$5.123^{b} \pm 0.263$	$6.873^{a} \pm 0.125$	0.000***
Lactose, %	$6.041^{a} \pm 0.047$	$5.925^{ab} \pm 0.034$	$5.843^{b} \pm 0.021$	0.013*
Protein, %	$4.026^{a} \pm 0.031$	$3.958^{a} \pm 0.023$	$3.913^{a} \pm 0.015$	0.051
Akkaraman sheep	$n = 4$	$n = 37$	$n = 109$	
Milk yield, ml	$0.810^{a} \pm 0.023$	$0.679^{b} \pm 0.013$	$0.532^{c} \pm 0.011$	0.000***
Fat, %	$2.942^{c} \pm 0.158$	$4.205^{b} \pm 0.123$	$5.751^{a} \pm 0.102$	0.000***
Lactose, %	$6.045^{ab} \pm 0.155$	$6.195^{a} \pm 0.088$	$6.011^{b} \pm 0.033$	0.062^{NS}
Protein, %	$4.030^{a} \pm 0.170$	$4.076^{a} \pm 0.028$	$4.022^{a} \pm 0.022$	0.437^{NS}

[a, b, c] Different letters indicate significant difference between genotypes. NS – non significant; *n* – total number of individuals.
* $P < 0.05$; *** $P < 0.001$.

agement and nutritional conditions are required to investigate and confirm any putative correlations [16].

Orford et al. [16] sequenced PRL gene intron 2 region for determined 23 bp deletion from Chios sheep breeds. Chios sheep breed has same genetic background with Sakiz and it is reared in Greece. These researchers were found 2 haplotypes, which is HM234397 and HM234398. In this research, Network analysis was performed with Orford et al. [16] haplotypes. Interestingly, these haplotypes has been found to same group with Akkaraman and Awassi breeds and differ from Sakiz by 24 variation points. The Sakiz breed differs from the Akkaraman, Awassi and Chios breeds (Fig. 2).

The classical view is that intronic mutations do not alter the composition or function of the protein produced by a gene [8]. Nevertheless, introns and the act of their removal by the spliceosome can affect gene expression at many different levels, including transcription, polyadenylation, mRNA export, translational efficiency, and the rate of mRNA decay [26].

The different indices of neutrality at PRL, Tajima's *D* value, Fu and Li's *D** and *F** values and Fu's *Fs* values was found different among to the groups. As a results, this suggests that population expansion can be based on the negative neutrality test values obtained in group B, which is belong to Akkaraman and Awassi sheep breeds. Under positive selection there is an excess of rare polymorphisms and Tajima's *D* value, Fu and Li's *D** and *F** values and Fu's *Fs* values are negative. The population variations for intron 2 of the PRL gene polymorphisms were found in Sakiz, Akkaraman and Awassi ewes.

Haplotype analysis of the obtained sequences showed the presence of 48 SNPs in the PRL intron 2 region. The number of polymorphisms identified showed high variability among breeds. Of the 12 haplotypes six were specific for Awassi, four for the Akkaraman and two for the Sakiz breed. Although Sakiz breed had shown two haplotypes, it presented the 28 polymorphic sites. It was interesting to note that Akkaraman and Awassi sheep were found similar haplotype group, whereas the Sakiz breed had different variations. We propose that this difference is probably the consequence of selection and possibly even QTL or gene(s) regulating milk production traits.

In the present study, we have reported for the first time 48 SNPs of the PRL gene for intron 2 and its association with milk traits in Sakiz, Akkaraman and Awassi sheep breeds. In all examined populations, PRL genotype *AA* were significantly associated with greater milk yield, whereas the animals carrying *BB* genotype had a higher milk fat percentage value. Although, our results suggest that PRL variants effect

of some milk traits, its association with milk production requires further validation from different sheep breeds. We concluded that the identified SNPs lend themselves readily for further research regarding physiological impacts such as milk production and reproductive traits in other dairy sheep populations.

ACKNOWLEDGMENTS

The project was supported by Scientific Research Projects Council of Firat University.

REFERENCES

1. Orford, M., Hadjipavlou, G., Tzamaloukas, O., Chatziplis, D., Koumas, A., Mavrogenis, A., Papachristoforou, C., and Miltiadou, D., A single nucleotide polymorphism in the acetyl-coenzyme A acyltransferase 2 (ACAA2) gene is associated with milk yield in Chios sheep, *J. Dairy. Sci.*, 2012, vol. 95, pp. 3419–3427.
2. Carta, A., Casu, S., and Salaris. S., Invited review: current state of genetic improvement in dairy sheep, *J. Dairy. Sci.*, 2009, vol. 92, pp. 5814–5833.
3. Selvaggi, M., Laudadio, V., Dario, C., and Tufarelli, V., Investigating the genetic polymorphism of sheep milk proteins: a useful tool for dairy production, *J. Sci. Food. Agric.,* 2014, vol. 94, pp. 3090–3099.
4. FAO (Food and Agriculture Organization of the United Nations). Statistical Databases. http://faostat3.fao.org/home/E. Accessed December 2014.
5. Unal, N., Akcapinar, H., Atasoy, F., Yakan, A., and Ugurlu, M., Milk yield and milking traits measured with different methods in Bafra sheep, *Rev. Med. Vet.,* 2008, vol. 159, pp 494–501.
6. Moioli, B., D'Andrea, M., and Pilla, F., Candidate genes affecting sheep and goat milk quality, *Small Ruminant Res.,* 2007, vol. 68, pp. 179–192.
7. Ramos, A.M., Matos, C.A.P., Russo-Almeida, P.A., Bettencourt, C.M.V., Matos, J., Martins, A., Pinheiro, C., and Rangel-Figueiredo, T., Candidate genes for milk production traits in Portuguese dairy sheep, *Small Ruminant Res.,* 2009, vol. 82, pp. 117–121.
8. Staiger, E.A., Thonney, M.L., Buchanan, J.W., Rogers, E.R., Oltenacu, P.A., and Mateescu, R.G., Effect of prolactin β-lactoglobulin. and κ-casein genotype on milk yield in East Friesian sheep, *J. Dairy. Sci.*, 2010, vol. 93, pp. 1736–1742.
9. Wu, X.L., MacNeil, M.D., De. S., Xiao, J.J., Michal, C.T., Gaskins, J.J., Reeves, J.J., Busboom, J.R., Wright, R.W. Jr., and Jiang, Z., Evaluation of candidate gene effects for beef backfat via Bayesian model, *Genetica,* 2005, vol. 125, pp. 103–113.
10. Corral, M., Padilla, J.A., Izquierdo. M., Martínez-Trancón, M., Parejo, J.C., Salazar, J., and Hernández-García, F.I., Detection and genetic characterization of ovine CSN1S2nB polymorphisms and their associations with milk production traits, *Livest. Sci.*, 2013, vol. 153, pp. 10–19.
11. Amigo, L., Recio, I., and Ramos, M., Genetic polymorphism of ovine milk proteins: its influence on technological properties of milk–a review. *Int. Dairy. J.,* 2000, vol. 10, pp. 135–149.
12. Barillet, F., Arranz. J.J., and Carta, A., Mapping quantitative trait loci for milk production and genetic polymorphisms of milk proteins in dairy sheep, *Genet. Sel. Evol.,* 2005, vol. 37, pp. 109–123.
13. Barillet, F., Genetic improvement for dairy production in sheep and goats, *Small Ruminant Res.,* 2007, vol. 70, pp. 60–75.
14. Knight, C.H., Overview of prolactin's role in farm animal lactation, *Livest. Prod. Sci.,* 2001, vol. 70, pp. 87–93.
15. Gutierrez-Gil, B., El-Zarei, M.F., Alvarez, L., et al., Quantitative trait loci underlying milk production traits in sheep, *Anim. Genet.,* 2009, vol. 40, pp. 423–434.
16. Orford, M., Tzamaloukas, O., Papachristoforou, C., and Miltiadou, D., Technical note: a simplified PCR-based assay for the characterization of two prolactin variants that affect milk traits in sheep breeds, *J. Dairy. Sci.,* 2010, vol. 93, pp. 5996–5999.
17. Akcapinar, H., *Sheep Breeding*, Ankara: Ismat, 2000.
18. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning a Laboratory Manual,* New York: Cold Spring Harbor Lab., 1989, 2nd ed.
19. Vincent, A.L. and Rothschild, M.F., Rapid communication: a restriction fragment length polymorphism in the ovine prolactin gene, *J. Anim. Sci.,* 1997, vol. 75, p. 1686.
20. Excoffier, L., Laval, G., and Schneider, S., Arlequin suite ver. 3.5: a new series of programs to perform population genetics analyses under Linux and Windows, *Mol. Ecol. Resour.*, 2010, vol. 10, pp. 564–567.
21. Hall, T., *BioEdit: Biological Sequence Alignment Editor for Win95/98/NT/2K/XP,* Carlsbad, CA: Ibis Biosciences, 2007. http://www.mbio.ncsu.edu./BioEdit/bioedit.html. Accessed December 2014.
22. Rozas, J., Sanchez DelBarrio, J.C., Messeguer, X., and Rozas, R., DnaSP: DNA polymorphism analyses by the coalescent and other methods, *Bioinformatics*, 2003, vol. 19, pp. 2496–2497.
23. Iso-Touru, T., Kantanen, J., Li, M., et al., Divergent evolution in the cytoplasmic domains of PRLR and GHR genes in Artiodactyla, *BMC Evol. Biol.,* 2009, vol. 9, p. 172.
24. Bandelt, H.J., Forster, P., and Röhl, A., Median-joining networks for inferring intraspecific phylogenies, *Mol. Biol. Evol.,* 1999, vol. 16, pp. 37–48.
25. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S., MEGA6: Molecular evolutionary genetics analysis (MEGA) software version 6.0., *Mol. Biol. Evol.,* 2013, vol. 30, pp. 2725–2729.
26. Nott, A., Meislin, S.H., and Moore, M.J., A quantitative analysis of intron effects on mammalian gene expression, *RNA,* 2003, vol. 9, pp. 607–617.