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Research Article

Identification of Genetic Variation of Melatonin Receptor 1A (MTNR1A) Gene in Kıvırcık Breed Ewes by *Mnl*I and *Rsa*I Restriction Enzymes [1] [2]

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Abstract

Melatonin receptor 1A (MTNR1A) gene encodes melatonin hormone which regulates the function of seasonal reproductive activity in sheep. The aim of this study was to make the genetic characterization and identify the variant alleles of MTNR1A gene in Kıvırcık breed. Blood samples of 110 Kıvırcık sheep were collected from five different farms located in Kırklareli and Istanbul. DNA extraction was performed from blood samples. Exon 2, the polymorphic region of Melatonin receptor 1A gene, was amplified and PCR products were genotyped by using *Mnl*I and *Rsa*I enzymes. The observed alleles and genotypes for *Mnl*I enzyme were; M (0.891), m (0.109) and MM (0.782), Mm (0.218) respectively. Kıvırcık sheep was null from mm genotype. Also identified alleles were C (0.682), T (0.318) and genotypes were CC (0.582), CT (0.200), TT (0.218) for *Rsa*I enzyme. The most frequent genotypes were MM (78%) and CC (58%) in Kıvırcık ewes. Since MM and CC genotypes were known with their positive effect on out of season reproductive activities, Kıvırcık ewes with these genotypes might suggested to be used in out of season lambing when demanded.

Keywords: *Kıvırcık, Sheep, Melatonin, Receptor, Genetic variation*

Kıvırcık Irkı Koyunlarda Melatonin Reseptör 1A (MTNR1A) Geninin *Mnl*I ve *Rsa*I Restriksiyon Enzimleri ile Genetik Varyasyonunun Belirlenmesi

Özet

Melatonin reseptör 1A (MTNR1A) geni koyunlarda mevsime bağlı üreme fonksiyonlarını düzenleyen bir hormon olan melatonini kodlamaktadır. Bu çalışmanın amacı Kıvırcık ırkı koyunda MTNR1A geninin genetik varyasyonunun ve allel çeşitliliğini belirlemektir. Kırklareli ve İstanbul illerindeki 5 farklı çiftlikten olmak üzere toplam 110 adet Kıvırcık ırkı koyununa ait kan örnekleri toplanarak DNA izolasyonu yapılmıştır. MTNR1A geninde polimorfik olan ekzon 2 bölgesi PZR ile yükseltgenmiş olup *Mnl*I ve *Rsa*I enzimleri kullanılarak allel ve genotip tespitleri yapılmıştır. Gözlenen alleller ve genotipler *Mnl*I için M (0.891) ve m (0.109) alleleri ile MM (0.782) ve Mm (0.218) genotipleri, *Rsa*I için C (0.682) ve T (0.318) alleleri ile CC (0.582), CT (0.200) ve TT (0.218) genotipleri olmuştur. Kıvırcık koyunlarında mm genotipi gözlenmemiş olup, en yüksek oranda gözlenen genotipler, koyunlarda mevsim dışı üreme faaliyetlerini pozitif olarak etkilediği bilinen MM (%78) ve CC (%58) olarak tespit edilmiştir. Büyük bir çoğunluğu MM ve CC genotiplerine sahip olan Kıvırcık ırkı koyunların mevsim dışı kuzulatmada yaygın olarak kullanılması yetiştiricilere önerilebilir.

Anahtar sözcükler: *Kıvırcık, Koyun, Melatonin, Reseptör, Genetik varyasyon*

INTRODUCTION

Kıvırcık is an important red meat source in Turkey and a native sheep breed known with its good meat quality [1]. Kıvırcık breed is raised in Thrace region, southern and eastern provinces in Marmara region and in some Aegean provinces of Turkey [2]. Age, body weight and photoperiod are the most significant factors that effect of puberty in ewes [3]. Small ruminant reproductive activity increases during decreasing photoperiods. Related process

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depends on melatonin hormone which plays an essential role in controlling seasonal reproduction by photoperiodic information. Melatonin is secreted from pineal gland in proportion to the period of darkness [4] and its production is controlled by day/night alteration. The peak level of melatonin secretion is positively correlated with the length of the dark hours [5]. Short photoperiods influence positively on melatonin level, enhance secretion of gonadotropic releasing hormone (GnRH) and correspondingly luteinizing hormone (LH). Melatonin is link with two specific high affinity receptors, melatonin receptor 1A and 1B that are located in hypophyseal pars tuberalis [6]. However Melatonin receptor 1A (MTNR1A) is the main receptor mediating melatonin action to modulate GnRH pulsatile activity [6], therefor it is involved in the regulation of reproductive activity [7]. Futhermore melatonin has a protective effect against aluminum accumulation [8]. Exogenous applications of this hormone during the summer encourage the onset of puberty [3]. To activate out of season reproduction hormonal treatments are used in sheep breeding. Variations in MTNR1A gene have significant effect on melatonin binding sites to pars tuberalis of hypothalamus [7]. Therefor these variations also effect the respond to melatonin treatment [3]. However demands for hormone free products directs to a search for alternative methods [9]. Knowledge of genes and genetic markers that influence on out of season lambing would allow more efficient and intensive selection programs for reproduction [10]. The use of genetic markers for reproduction, especially photoperiod sensitivity, is a promising method in sheep [9]. The variation among animals can be determined at the DNA level with various molecular techniques. Utilizing this information in selection program is a growing interest, especially for the traits that are difficult to improve with conventional methods [10].

MTNR1A gene located on chromosome 26 of sheep genome. Its genomic structure consist of two exons divided by a large intron [11]. Exon 1 encodes the first transmembrane domain and the first intracellular loop and exon 2 codifies for the remaining part of the receptor. Various studies in different sheep breeds were reported two single nucleotide polymorphisms (SNPs) at position 606 (C>T) and 612 (G>A) in exon 2 region which are also identified as silent mutations. Related SNPs can be identified by *Rsa*I and *Mnl*I enzymes respectively. Polymorphic regions in both *Rsa*I and *Mnl*I recognition sites were also reported about their association with the seasonal ovulation and reproductive activity in ewes [7,12]. Related polymorphic sites were studied various sheep breeds such as Columbia [13], Merino d'arles [7,14], small tailed Han sheep [15], Ile de France sheep [16], Prolific Olkuska, Polish Mountain sheep, Suffolk, Merino-Romanov sheep [17], Karakul [18], Awasi [19-21], Mouflon wild sheep [12], Sarda [3,22], crossbred of 50% Dorset, 25% Rambouillet, and 25% Finnsheep ewes [23], Akkaraman, Chios [20,21], Rasa Aragonesa [9], Local Starozagorska, Local Karnobatska, Breznishka and Sofiiska [24], Dağlıç, Gökçeada, Karacabey Merino, Karayaka, Kıvırcık [21], Zandi sheep [25], Dorset [10], Zel, Naeini [26], Indian Chokla [27], Marwari and Magna [28]. Trechel *et al.*[29] provide evidence of a modification in the melatonin signaling pathway by comparing two polymorphic variants which makes MTNR1A gene a potential DNA marker for out of season breeding.

The aim of this study was identify the genetic variation of MTNR1A gene in Kıvırcık which is a noted and desirable native sheep breed with its meat quality in Turkey.

MATERIAL and METHODS

This study was approved by Ethic Committee of the Istanbul University Veterinary Faculty (Approval number: 2010/184).

Animals

Animal samples of this study come from five purebred Kıvırcık flocks. The four of the flocks were located in Kırklareli province. Twenty ewes were selected randomly from each flock. The fifth flock was belong to Research and Education Farm of Istanbul University Faculty of Veterinary Medicine in which thirty ewes were selected randomly. Blood samples of Kıvırcık (n=110) ewes were collected from Vena jugullaris into steril vacuumed EDTA tubes from Kırklareli (n=80) and Istanbul (n=30) provinces.

Genotyping

DNA isolation was performed from blood samples by using DNA Pure Kit (Geneaid Biotech™, Taiwan). The region of the MTNR1A gene in sheep was amplified by using PCR with the forward 5'TGTGTTTGTGGTGAGCCTGG3' and reverse 5'ATGGAGAGGGTTTGCGTTTA3'primers [30], which captured a fragment that has a length of 824bp from exon 2 (HQ658144.1). PCR amplification was performed in total volume of 25µl consist from 5 µl Taq PCR Master Mix (200 U/ml Ultra-Pure Taq DNA Polymerase, 1.25 mM dNTPs, 10 mM $MgCl_2$; Geneaid Biotech™, Taiwan), 0.5 µl 20 pmol each primer, 3 µl genomic DNA (100 ng) and 16 µl dH_2O (AccuGENE™, Lonza, Belgium). PCR was performed with the following conditions; denaturing at 94°C in 5 min, 34 cycles of 94°C in 1 min, 62°C in 1 min, 72°C in 1 min and final extension at 72°C in 10 min (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA).

PCR products were digested with both *Mnl*I and *Rsa*I enzymes (MBI Fermentas). Incubation was performed at 37°C by overnight for both *Mnl*I and *Rsa*I cleavage. After performing the digestions, band patterns were visualized on 4% agarose gel stained with ethidium bromide.

The ovine MTNR1A nucleotide data HQ658145.1 and HQ658147.1 which include C606T and G612A SNPs respectively, was aligned with HQ658144.1 nucleotide which includes wild type alleles (C and M). Alignment was performed with nucleotide BLAST tool (http://blast.ncbi.

nlm.nih.gov/Blast.cgi) in order to compare and confirm restriction sites among related nucleotides.

Statistical Analysis

Allele and genotype frequencies, observed and expected heterozygosity values and chi square (X^2) for Hardy-Wienberg equilibrium (HWE) was estimated with PopGene32 program [31].

RESULTS

Two alleles were identified for *Mnl*I (M and m) and *Rsa*I (C and T) digestions of ovine MTNR1A locus. Observed genotypes with *Mnl*I enzyme restriction were MM (78%) and Mm (22%), no mm genotype was determined. With *Rsa*I enzyme restriction observed genotypes were CC (58%), CT (20%) and TT (22%). MTNR1A locus had seven restriction sites for *Mnl*I and four for *Rsa*I enzyme. Band pattern sizes for M allele were; 220bp, 218bp, 135bp, 83bp, 82bp, 36bp, 28bp, 22bp and for C alleles were 411bp, 267bp, 70bp, 53bp, 23bp. However existence of G>A transition in *Mnl*I recognition site (GAGG-AAGG) was result to divergence in the band patterns (303bp, 218bp, 135bp, 82bp, 36bp, 28bp, 22bp) thus it causes to m allele. Also existence of C>T transition in *Rsa*I recognition site (GTAC-GTAT) results to T allele (411, 290, 70, 53 bp) *(Fig. 1)*.

Band patterns for *Mnl*I (M and m) and *Rsa*I (C and T) were visualized on 4% agarose gel *(Fig. 2 A,B)*. However all DNA fragments resulted after *Mnl*I and *Rsa*I digestions could not been observed on agarose gel. Observable DNA fragments for M allele were 303bp, 218bp, 135bp and for m allele were 220bp, 218bp, 135bp. Also visualized band patterns for C allele were 411bp, 267bp and for T allele were 411bp, 290bp.

Allele and genotype frequencies, observed and expected heterozygosity and chi square (X^2) values resulted from both *Mnl*I and *Rsa*I enzyme digestions of ovine MTNR1A locus were given in *Table 1*. Kıvırcık breed ewes were found in HWE at *Mnl*I locus. However deviation from HWE was found significant at *Rsa*I locus ($P<0.01$).

DISCUSSION

Through conventional breeding program, genetic improvement in out of season fertility trait is challenging. For reproductive traits using genetic markers in selection programs will be useful since the trait has low heritability, furthermore it is expressed late in life; observed in one gender; exhibited only in some environmental conditions or management systems [10,23]. The unproductive time period that passes between birth and first lambing is one of the biggest problems in management of sheep breeding [3]. Sezenler *et al.*[32] performed a study to determine some reproductive characteristics of Kıvırcık, Chios and Imroz indigenous sheep breeds of Turkey. Mating season duration

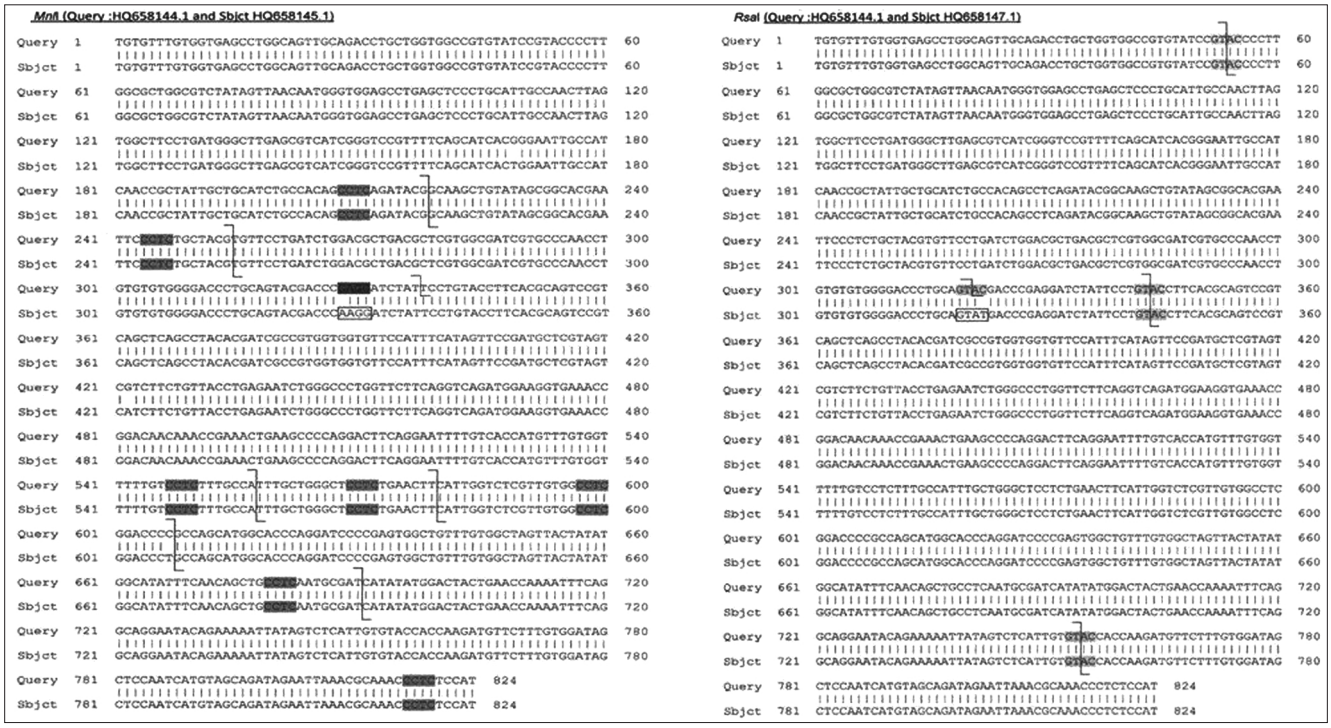

Fig 1. Restriction sites of *Mnl*I (Query; HQ658144.1; M allele and Sbjct; HQ658145.1; m allele) and *Rsa*I (Query; HQ658144.1; C allele and Sbjct; HQ658147.1; T allele) enzymes within ovine MTNR1A gene

Şekil 1. Koyun MTNR1A geninde *Mnl*I (Query; HQ658144.1; M alleli ve Sbjct; HQ658145.1; m alleli) ve *Rsa*I (Query; HQ658144.1; C alleli ve Sbjct; HQ658147.1; T alleli) enzimleri için kesim bölgeleri

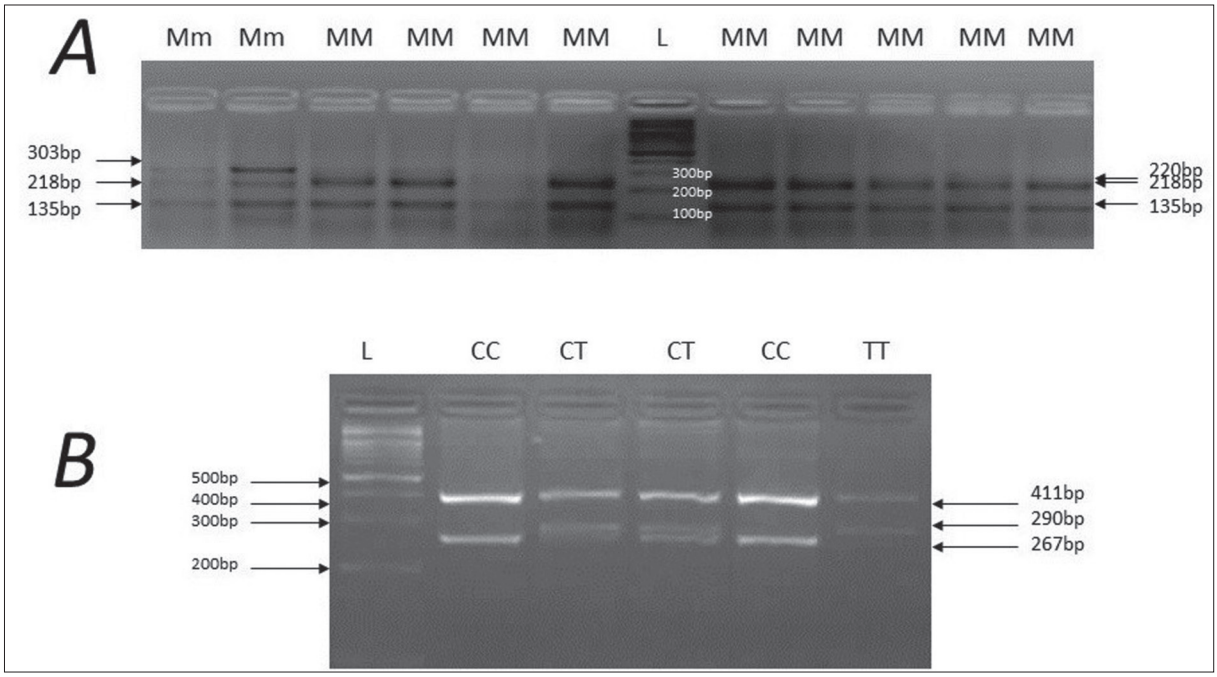

Fig 2. The observed genotypes in Kıvırcık sheep after *Mnl*I (A. Mm; 303bp, 218bp, 135bp in lanes 1, 2 and MM; 218bp, 135 bp in lanes 3, 4, 5, 6, 8, 9, 10, 11, 12) and *Rsa*I (B. CC; 411bp, 267bp in lanes 2, 5, CT; 411bp, 290bp, 267bp in lanes 3, 4, TT; 411bp, 290bp in lane 6) enzyme digestions of MTNR1A gene on 4% agarose gel (L= 100bp ladder)

Şekil 2. Kıvırcık koyununda %4'lük agaroz jelde MTNR1A geninin *Mnl*I (A. 1, 2 nolu kuyucuklarda Mm: 303bç, 218bç, 135bç; 3, 4, 5, 6, 8, 9, 10, 11, 12 nolu kuyucuklarda MM: 218bç, 135 bç) ve *Rsa*I (B. 2, 5 nolu kuyucuklarda CC: 411bç, 267bç; 3, 4 nolu kuyucuklarda CT: 411bç, 290bç, 267bç; 6 nolu kuyucukta TT: 411bç, 290bç) enzim kesimlerini takiben gözlenen genotipler

Table 1. *Allele and genotype frequencies, observed and expected heterozygosity, chi square (X^2) values of MTNR1A gene in Kıvırcık sheep breed for both MnlI and RsaI enzymes*

Tablo 1. *Kıvırcık koyununda MnlI ve RsaI enzimleri için MTNR1A genine ait allel ve genotip frekansları, gözlenen ve beklenen heterozigotluk ve Ki kare (X^2) değerleri*

Enzyme	Alleles	Allele Frequency	Genotypes	Genotype Frequency	Heterozygosity		X^2
					Ho	He	
*Mnl*I	M	0.891	MM	0.782	0.218	0.195	1.57^{ns}
	m	0.109	Mm	0.218			
			mm	0.000			
*Rsa*I	C	0.682	CC	0.582	0.200	0.436	32.9^{*}
	T	0.318	CT	0.200			
			TT	0.218			

ns: *nonsignificant, *P<0.01*

(225.03, 222.58 and 167.67 days resp.) and anestrus period (139.97, 142.59 and 197.33 days resp.) were reported for Kıvırcık, Chios and Imroz respectively. Kıvırcık had the longest mating duration and the shortest anestrus period among three native breeds. Duration of reproductive season of Kıvırcık was reported approximately up to 8 months. When estrus distribution analysed for months, Sezenler *et al.*[32] found that Kıvırcık show estrus mostly in October. Distribution of reproductive season among the months of a year would be the early summer (June) to winter (January) for Kıvırcık breed.

Pelletier *et al.*[7] reported that M allele has an effect of ovulatory cycling during out of season (in spring) in Merinos d'Arles ewes. Furthermore the homozygous genotype for the absence of a polymorphic *Mnl*I sites (mm) at position 612 of exon 2 was found associated with seasonal anovulatory activity in Merino d'Arles [7]. Moreover M allele was reported with its positive influence on autumn lambing success in Columbia ewes [13]. The mm genotype was more frequent (50%) in wild Mouflon [12] ewes and its reproductive activity was reported as seasonal. Martinez-Royo *et al.*[9] found significant differences in estrous cyclicity among months and genotypes for SNP C606T. The most significant differences between TT and CC genotypes in the percentage of estrous cyclic ewes were reached in May (27.8%, P<0.1), June (29.4%, P<0.05) and July (28.9%, P<0.05). Therefore T allele was reported associated with

a greater percentage of nonseasonal estrous cyclic ewes of Rasa Aragonesa breed. During the anestrus season Rasa Aragonesa ewes with TT genotype showed more estrus activity. C allel is related with a greater percentage of seasonal estrus cyclic ewes in Rasa Aragonesa breed [9]. Sarda sheep that carry one of MM and CC genotypes showed estrus in spring. As a consequence they lambed in autumn (September-December), therefore reproductive activity of Sarda ewes was reported as non-seasonal. Lambs that were born in autumn can be reach puberty by the early summer of the following year. However ewes that were born in spring do not reach puberty until the next autumn, later than those which were born in autumn. Lambs which were born in autumn are being chosen by breeders as replacement ewe lambs and these ewes were probably MM and CC genotype [22]. Small Tail Han [15] and Awassi [19] ewes which were identified to have MM, CC genotypes were reported that they show non-seasonal estrus and ewes with mm, TT genotypes were showed seasonal estrus. However Teyssier *et al.*[14] reported that *Mnl*I site of the MTNR1A gene cannot be used alone as a genetic selection marker for spring (out-of-season) breeding in Merino d'Arles ewes. Furthermore M allele was not found to be related with seasonal reproduction trait in Rasa Aragonesa sheep [9]. Kaczor *et al.*[17] reported that prolific Olkuska ewes with different genotypes did not show significantly different average melatonin concentration during the dark phase (December); an association had not been found between MTNR1A polymorphism and blood melatonin concentration. The effect of related polymorphisms might be determined by the breed and /or environmental conditions.

In present study we found that MTNR1A gene had two alleles; M and m, and two genotypes; MM and Mm for *Mnl*I enzyme; C and T alleles, CC, CT and TT genotypes for *Rsa*I restriction site in Kıvırcık breed. We observed that M allele (89%) was much more frequent than m allele (11%) in Kıvırcık breed similar with Magna (95%), Chokla (92%), Zandhi (92%) [25], Marwari (90%) [27], Chios (90%), Awasi (84%), White Karaman (80%) [20], Hu (80%), Karakul (79%) [18], Sarda (78%) [22], Small Tail Han (75%) [15] and Naeini (71%) [28] breeds. However Elmacı *et al.*[21] reported that M allele was less frequent (26%) than m allele (74%) in Kıvırcık sheep. Genotype frequencies of MM and Mm genotypes (78%; 22%) in Kıvırcık breed were resemble with the frequencies reported in Chokla (77%; 21%), Marwari (80%; 19%) [27], Zandhi (82%; 18%) [25], Chios (80%; 20%) and Karakul (70%; 30%) [18] sheep breeds. Similar to our results mm genotype was not observed in Zandhi [25], Awasi, White Karaman, Chios [20] and Karakul [18] breeds. Observed heterozygosity for Mm genotype (0.22) in Kıvırcık breed was found similar with Naeini (0.22) and Zel (0.25) breeds [28]. Observed heterozygosity that Elmaci *et al*.[21] reported for Mm genotype in Kıvırcık breed was higher than our results (0.31). Similar to our findings, C allele (68%) was more frequent than T allele (32%) in Magna (95%; 5%), Chokla (87%; 13%), Marwari (89%, 11%) [27], Gokceada (79%; 21%), Awasi (73%; 26%) [20], Local Karnobatska (73%; 27%) [24] and Small Tail Han (71%, 29%) [15] sheep breeds. Elmaci *et al*.[21] found frequency of C allele (53%) closer to T allele (47%). After RsaI digestion genotypes frequencies from the most frequent to less were; CC (58%), CT (20%) and TT (22%) respectively, which were found similar with Sarda (53%; 26%; 21%) [22] sheep breed. In current study observed heterozygosity (0.2) for CT genotype was found similar with Karayaka (0.24) [21] and Local Karnobatska (0.23) [24] breeds. However Elmaci *et al*.[21] reported observed heterozygosity in Kıvırcık breed for CT genotype was much higher than our result (0.54). We found that Kıvırcık sheep was not in HWE for *Rsa*I site of MTNR1A gene, similarly as reported in Zel and Kıvırcık breeds [21,28]. Differences between findings of Elmaci *et al*.[21] in MTNR1A variation in Kıvırcık breed (n=39) and ours may result from sampling size and inbreeding levels of sampled animals.

In conclusion the current study showed that MTNR1A gene varies for both *Mnl*I and *Rsa*I enzymes in Kıvırcık ewes. Since mm genotype was known to be related with seasonal estrus and anovulatory activity in ewes, it can be assumed that selection process may occurred negatively for this genotype in Kıvırcık breed. The desired alleles for out of season cycling; MM (78%) and CC (58%) were found more frequent than Mm (22%), CT (20%) and TT (22%) genotypes. Kıvırcık ewes, that shows MM and CC genotype, can be suggested to use for autumn lambing when demanded. Further studies are needed to clarify the characterization and genotype variation of MTNR1A gene and its impact on out of season reproductive activities. Our next aim is to investigate the association of non-seasonal (autumn) lambing with MM and CC genotypes in Kıvırcık ewes that may help to develop new suggestions in sheep breeding.

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