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Allele Frequency of c.486A>G Polymorphism of the *AA-NAT* Gene in Iranian Indigenous and Exotic Sheep Populations

Research Article

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ABSTRACT

Typically, lambing percentage is classified as a composite trait and is crucial to profitability in domestic sheep farming. In breeding programs, out-of-season reproduction of sheep is an important tool because seasonal reproduction limits productivity and flexibility. Understanding the complexities of genetic aspects of the none- seasonal reproduction has received significant critical attention in the literature. In this puzzle, the arylalkylamine-N-acetyltransferase (AA-NAT) is a rate-limiting enzyme of the melatonin synthesis pathway and is highlighted as a candidate gene that is responsible for melatonin synthesis and is thus directly associated with out-of-season reproduction in sheep. With this scenario research, we aimed to examine the allele frequency of the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) polymorphism in the AA-NAT gene in 11 native and exotic sheep populations. A total of 220 blood samples were taken from 11 breeds of sheep, including the exotic breeds Romanov (ROMV) and [Awassi (AWAS), Arabi (ARAB), Naaimi (NAIM) Iraqi native sheep] and [Ghezel (GHZL), Makui (MAKU)], Kurdi (KURD), Baluchi (BALCH), Afshari (AFSR) Iranian native sheep] and two Afshari-Booroola and Romanov-Ghezel F1 cross). Here, we describe the Smal-RFLP genotypes and allele frequency patterns of the (c.486A>G) casual single nucleotide polymorphism (SNP) in the exon of AA-NAT within and between the examined sheep breeds. In addition, PCR sequencing methods were used to double-check A/G and confirmation of PCR-RFLP results. This mutation changed the Arg > Gly structure from helix to helix-effective in improving non-seasonal reproduction. Interestingly, the observed variation of G allele ranged from 0.1 to 0.43 in all study breeds. ROMV is a candidate for non-seasonal sheep breeds and its cross expresses the highest G allele frequency among other breeds studied. The frequency of the AA-NAT genotype was significantly different between breeds in this study. Therefore, the exotic 486A>G mutation created a useful mirror to identify genomics aspects of seasonal/non-seasonal reproduction in sheep.

KEY WORDS *AA-NAT* gene, estrus non-breeding season, melatonin, PCR-RFLP.

INTRODUCTION

Traditionally, the domestic sheep is a seasonally polyestrous short-day breeding habitat with a regular reproduction pattern in autumn. A challenging problem that arises in this area is long reproductive intervals and fewer multiple births in the same year. Therefore, profitability depends on the out-of-season and success with multiple estruses during a year (Gündoan *et al.* 2003). In addition, domestication and evolutionary selection pressures also play a critical contribution in the production of sheep with heat from the seasonal variations (e.g. Romanov, Finn, and Dorset breeds) (Bartlewski *et al.* 2011). Genetic improvement of traits related to estrus of the season in domestic sheep is a

remarkable issue because these traits are low in inheritance mood, are generally expressed late in life, are usually classified as a sex-limited trait, and are only measured in limited lambing seasons and breeding systems (Bister *et al*. 1999).

The ability to breed out of season has been demonstrated for some breeds of sheep. Examples of such breeds are in particular Dorset, Merino, and Rambouillet sheep breeds (Hafez, 2013) and partly Swedish Finewool (Gates *et al*. 1998). By changing the frequency of genes, the fertility period of the ewes can be extended and the oestrus behavior occurs in spring (Lewis *et al*. 1996).

This is advantageous because it offers the possibility of more frequent lambing and thus more lambs. An example of this is the photoperiodic program by Cameron *et al*. (2010), which resulted in three lambing times in two years. This can be developed so that up to five lambing in three years (Lewis *et al*. 1996). Inheritance plays a crucial role in off-season breeding and up to 0.23 of the variation in this trait is due to genetic factors (Asadi-Fozi *et al*. 2020).

In this context, genomic approaches have been used to identify candidate genes or genomic regions that affect the ability of ewes to become estrus and lamb out of season. Many researchers have indicated that the melatonin (MLT) rhythm plays a notable role in physiological changes in the reproductive organs in both seasonal and non-seasonal reproduction sheep breeds (Dupre *et al*. 2008). Since the daily MLT secretion is controlled by the enzyme activity of the alkylamine N-acetyltransferase (AA-NAT) (Foulkes *et al*. 1997), the genetic mutation of the AA-NAT of the ovaries has been recommended.

The gene may have an impact on sheep non-seasonal reproductive performance and reproductive traits to improve production (Sharma *et al*. 2015).

During the last decade, the link between the candidate gene approach and seasonal/non-seasonal reproduction of sheep has been at the center of much attention. The literature suggests that the existence of several genes implies non-seasonal reproductive traits in sheep, such as some circadian kaput genes from the locomotor output cycles (CLOCK) (Notter *et al*. 2003). Giantsis *et al*. (2016) pointed association between polymorphism in the melatonin receptor 1A of MNTR1A with seasonal reproduction in indigenous Greek sheep breeds. the PCR - RFLP of the sheep melatonin receptor 1A (*MTNR1A*) gene was analyzed (Rahawy and AL-Timimi, 2016).

Melatonin or darkness hormone production depends on the length of daily light. Short photoperiods have a positive effect on melatonin levels, and this rising melatonin level stimulates to release of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) originating from the pituitary gland. Due to the fundamental role of the AA-NAT enzyme in MLT synthesis (Chattoraj *et al*. 2009).

There have been numerous studies to investigate that the genetic mutation of the gene could also be decisive for seasonal reproduction in sheep. The polymorphisms of the *Ovis aries AA-NAT* gene are also found in Boer goats and Jining Grey goats of China (Dupre *et al*. 2008).

The production of melatonin (MLT) requires the enzyme AA-NAT, which regulates seasonal animal breeding (Ding-ping *et al*. 2012). AA-NAT is an enzyme that is encoded by the AA-NAT gene. It is the central control of the day-night cycle (circadian rhythm) and occurs in all animals. It is present on chromosome 17 in humans and chimpanzees (Coon *et al*. 1996), on chromosome 11 in mice and sheep, on chromosome 10 in rats, and on chromosome 18 in chickens (Cipolla-Neto and Amaral, 2018). *AA-NAT* gene has four exons comprising three introns, all presented in a genomic region of 2.5 kilobases, exon I is untranslated while the other three are 238, 155, and 453 bp long amino acid protein (Addin *et al*. 2016). The cDNA sequences of the *Ovis aries AA-NAT* gene vary between humans, rats, cattle and sheep with homology in the range from 77% to 96.1% (Klein, 2006).

Previous studies have emphasized that biomarkers within the *AA-NAT* gene may be associated with seasonal and non-seasonal reproduction. Researchers have also suggested that an exonic A-G transition is responsible to control seasonal and non-seasonal lambing in different sheep breeds. For example, a study has shown that the GG genotype is more prevalent in non-seasonal breeds, and a series of studies have found that the GA genotype is more prevalent in breeds with seasonal estrus (Ding-ping *et al*. 2012). Investigate the allele frequency of the PCR-RFLP polymorphism in the *AA-NAT* gene in 11 native and exotic sheep populations. To the best of our knowledge, no study of the AA-NAT gene has been carried out in Iranian indigenous sheep and with this motivation, we aimed to examine the allele frequency of the c.486A>G polymorphism in the *AA-NAT* gene in 11 native and exotic sheep populations.

MATERIALS AND METHODS

Animals

A total of 220 blood samples were taken from 11 breeds and each breed of 20 animals of sheep, including exotic ROMV breeds and [AWAS, ARAB, NAIM Iraqi native sheep] and [GHZL, MAKU, KURD, BALCH, AFSR Iranian native sheep] and two F1-AFSR- BOORL and ROMV- GHZ cross). Table 1 and Figure 1 show the morphological distribution and some predominant geographical distribution of the sheep populations studied.

Table 1 Summary of studied sheep, country origin, Geographical distribution[1]

Breeds	Country origin	Geographical area	Latitude and longatiud
ROMV	Exotic	Canada, France, Russia	
AWAS	Iraq	Karbala	32°36'57.71N, 44°1'29.57 E
ARAB	Iraq	Babel	32°27'49.21 N,44°25'10.67 E
NAIM	Iraq	Najaf	32°1'33.38 N, 44°20'46.5 E
GHZL	Iran	East Azerbaijan	37° 50' 3.9912 N,46° 25' 48.0'E
MAKU	Iran	Westt Azerbaijan	37.4550° N, 45.0000° E
KURD	Iran	Mohabad	36.7684° N, 45.7337° E
BALCH	Iran	Khorasan	35.1020° N, 59.1042° E
AFSR	Iran	Zanjan	36.6830° N, 48.5087° E
AFSR × Booroola	Iran	East Azarbyjan	37° 50' 3.9912 N,46° 25' 48.0'E
ROMV × GHZL	Iran	Tabriz	38.0114° N, 46.4501° E

[1] Exotic breeds Romanov (ROMV) and [Awassi (AWAS), Arabi (ARAB), Naaimi (NAIM) Iraqi native sheep] and [Ghezel (GHZL), Makui (MAKU)), Kurdi (KURD), Baluchi (BALCH), Afshari (AFSR) Iranian native sheep] and two Afshari-Booroola and Romanov-Ghezel F1 crosses).

Figure 1 Morphological characteristics of examined sheep for genotyping the AA-NAT gene polymorphism
Romanov (ROMV) and [Awassi (AWAS), Arabi (ARAB), Naaimi (NAIM) Iraqi native sheep] and [Ghezel (GHZL), Makui (MAKU), Kurdi (KURD), Baluchi (BALCH), Afshari (AFSR) Iranian native sheep] and two Afshari-Booroola and Romanov-Ghezel F1 crosses)

Blood sampling and DNA extraction

In this experiment, we collected whole blood from the jugular vein using K_2-EDTA vacuum tubes and immediately transferred it to the genomic laboratory. Next, we extracted genomic DNA from white according to the purification kit (Rocheh Company, Iran) according to the manufacturer's instructions. The amount and purity of DNA for each sample was determined using a Nanodrop machine (Thermo Fisher Scientific, USA). The samples with low quality and quality were excluded from further analysis.

PCR-RFLP methods for *AA-NAT* gene

Our approach can be viewed as a two-step process. First is the amplification of 1142 bp PCR products. The PCR product included the end part of exon 1 till the beginning area of exon 3 and the sequence of specific primers was obtained from previous research by Ding-ping *et al.* (2012) with the following sequence details: forward 5-AGCGTCCACTGCCTGAAAC-3 and backward 5-GGGATGGAAGCCAAACCTC-3 (Genescript, China).

The amplifications were carried out with a thermal cycler (Biometra Thermocycler T-Gradient, Germany) in a total

volume of 25 L with 1 L DNA (50-100 ng)and 2 master mix (aq DNA Polymerase Master Mix RED, Denmark), 10. performed pmol-1. performed with each primer and nuclease-free water with the following temperature profiles: initial denaturation at 94 ˚C for 7 mins; followed by 35 cycles of denaturation at 95 ˚C for 55 seconds, annealing at 60 ˚C for 50 seconds, and extension at 72 ˚C for 1.5 mins; and final stretching at 72 ˚C for 10 mins. The PCR products were detected by electrophoresis on 1.5% ethidium bromide-stained agarose gels.

Second, restriction fragment length polymorphism (RFLP) analysis was performed with the SmaI Fast Digest type enzyme (New England Biolabs, UK) according to the manufacturer's instructions. This enzyme was (6 nucleotide cleavage) and its palindromic position was GGGCCC within the genome. The digestion results were visualized by vertical 2.5% agarose gel electrophoresis and staining with Ethidium bromide.

PCR-Sequencing methods for *AA-NAT* gene

Following the purification, sequencing was performed by BMG company service, Iran(https://www.bmgtechno.com/) After delivery of sequence raw data, a quality control (QC) test and alignment of the sequence with reference gene was done using online MAFFT multiple alignment tools and MEGA software.

Statistical analysis

Estimates of genotypic and allelic frequencies, Hardy Weinberg equilibrium tests for each breed population were performed by the POPGENE software, version 4.2, (Yeh *et al*. 1997). The chi-square test was used to test significant allele frequencies of the A / G mutation in the population studied (SAS, 2002).

RESULTS AND DISCUSSION

The investigation of AA-NAT will provide insights into the essential synthesis of melatonin in the pineal gland (Chattoraj *et al*. 2009). Many works have suggested the presence of a relationship between the AA-NAT gene and the biological clock in mammals (Soria *et al*. 2010), melatonin production (Ying *et al*. 2004) and sleep patterns (Wang *et al*. 2004), delayed sleep phase syndrome (Pereira *et al*. 2007). Although the effects of the *AA-NAT* gene on the physiological signaling pathway in humans have been extensively investigated, only a few studies have systematically examined the contribution. In this study, we examined the pattern and variability of the *AA-NAT* gene polymorphism in various exotic and indigenous breeds of sheep. We hypothesized that the pattern and variability of the G-favorable allele associated with non-seasonal reproduction would be different between exotic and native breeds.

PCR-RFLP *AA-NAT* gene fragments

The *AA-NAT* gene was successfully amplified, resulting in an expected 1142 bp PCR product amplicon according to previous literature. The results of the SmaI enzyme assay showed three genotypes: AA (516 bp, 371 bp, 255 bp), GG (371 bp, 333 bp, 255 bp, 183 bp), and GA (516 bp, 371 bp, 333 bp, 255 bp, 183 bp) (Figure 2).

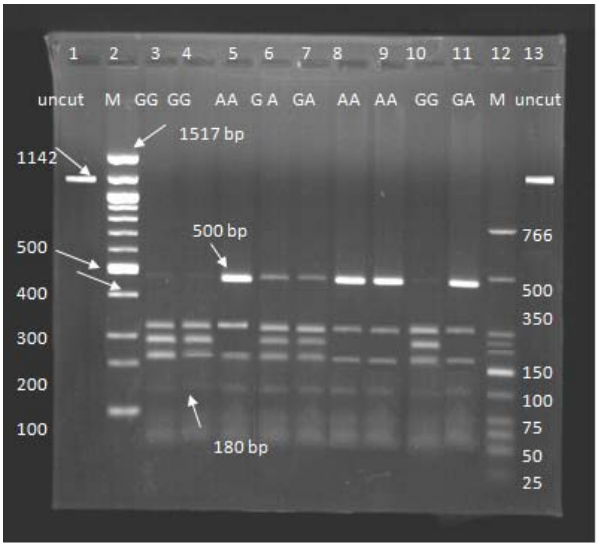

Figure 2 Overview of the PCR-RFLP pattern in the *AA-NAT* gene in the sheep population examined
The *AA-NAT* gene was successfully amplified, resulting in an expected 1142 bp PCR product amplicon according to previous literature. The results of the SmaI enzyme assay showed three genotypes: **AA** (516 bp, 371 bp, 255 bp), **GG** (371 bp, 333 bp, 255 bp, 183 bp), and **GA** (516 bp, 371 bp, 333 bp, 255 bp, 183 bp)

All three genotypes were identified in most of the breeds studied and show adequate polymorphism within the locus studied. This mutation changed the Arg> Gly structure from helix to helix - effective in improving non-seasonal reproduction. Figure 2 presents three RFLP patterns and genotypes of the *AA-NAT* gene across studied breeds.

PCR-sequencing methods for *AA-NAT* gene

Figure 3 provides the location and existence of A/G mutation in the 1142 bp amplicon of the *AA-NAT* gene.

Genotype ad allele frequencies of *AA-NAT* gene in the studied breed

Figure 4 compares the summary molecular statistics for genotype and allele frequencies of A/G mutation across studied breeds in the present study. Interestingly, the frequencies of the G allele varied from 0.1 to 0.43 in all of the breeds examined.

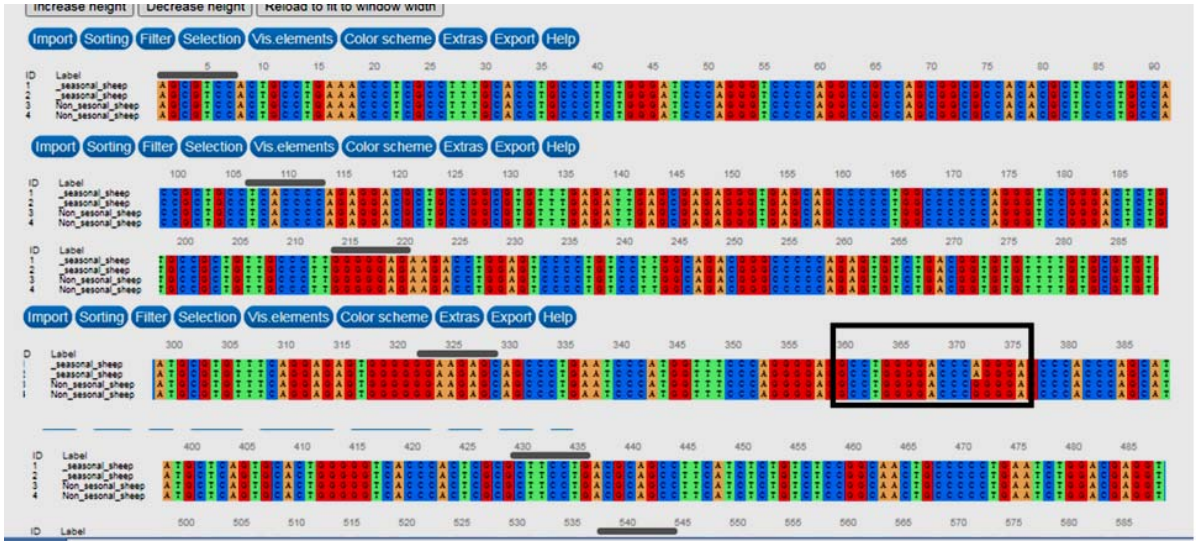

Figure 3 Snapshot for 1142 PCR product of the *AA-NAT* gene and A / G polymorphism in this candidate region

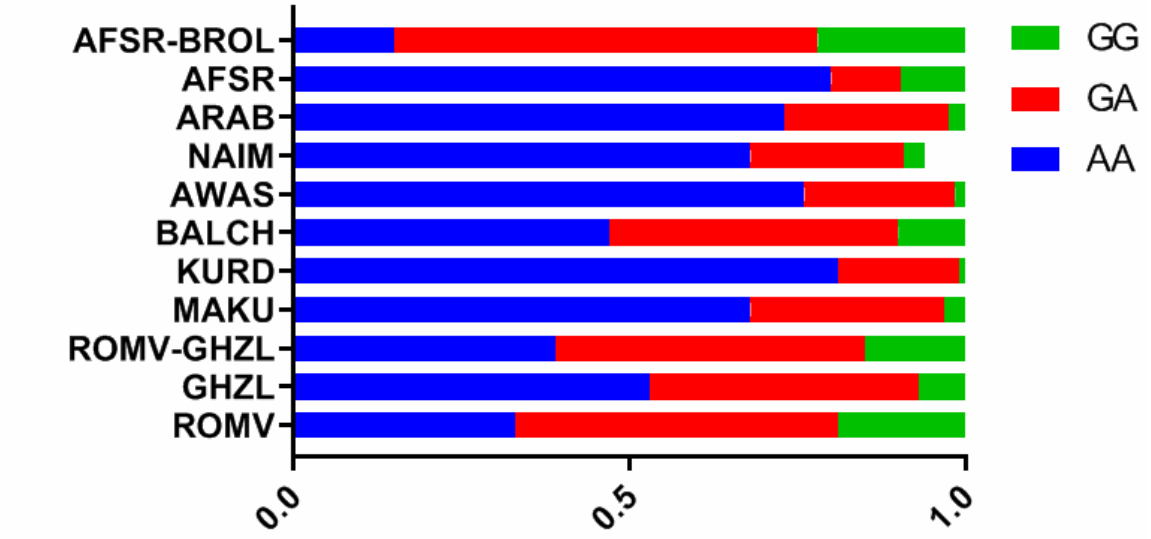

Figure 4 Summary of the identified observed genotype frequencies within the *AA-NAT* gene in all sheep breeds examined
The exotic breeds Romanov (ROMV) and [Awassi (AWAS), Arabi (ARAB), Naaimi (NAIM) Iraqi native sheep] and [Ghezel (GHZL), Makui (MAKU), Kurdi (KURD), Baluchi (BALCH), Afshari (AFSR) Iranian native sheep] and two Afshari-Booroola and Romanov-Ghezel F1 crosses)

ROMV is a candidate for non-seasonal sheep breeds and its cross expresses the highest G allele frequency among other breeds studied. Here, we compare the results of the present report with those of similar literature. For example, reported the same amount of PCR in their work on Egyptian sheep in the first phase. Their PCR-RFLP study (Fathy *et al*. 2018).

These basic findings are consistent with research showing that G-allele has high frequencies in nonseasonal sheep breeds.

This value is 1142 bp (exon 1), 290 bp (from intron 1), 155 bp (total exon 2), 338 bp (intron 2), and finally 207 bp from exon 3. Egypt, Turkey, China, Iraq, and Bulgaria fully agreed to all reported band sizes of 1142 bp.

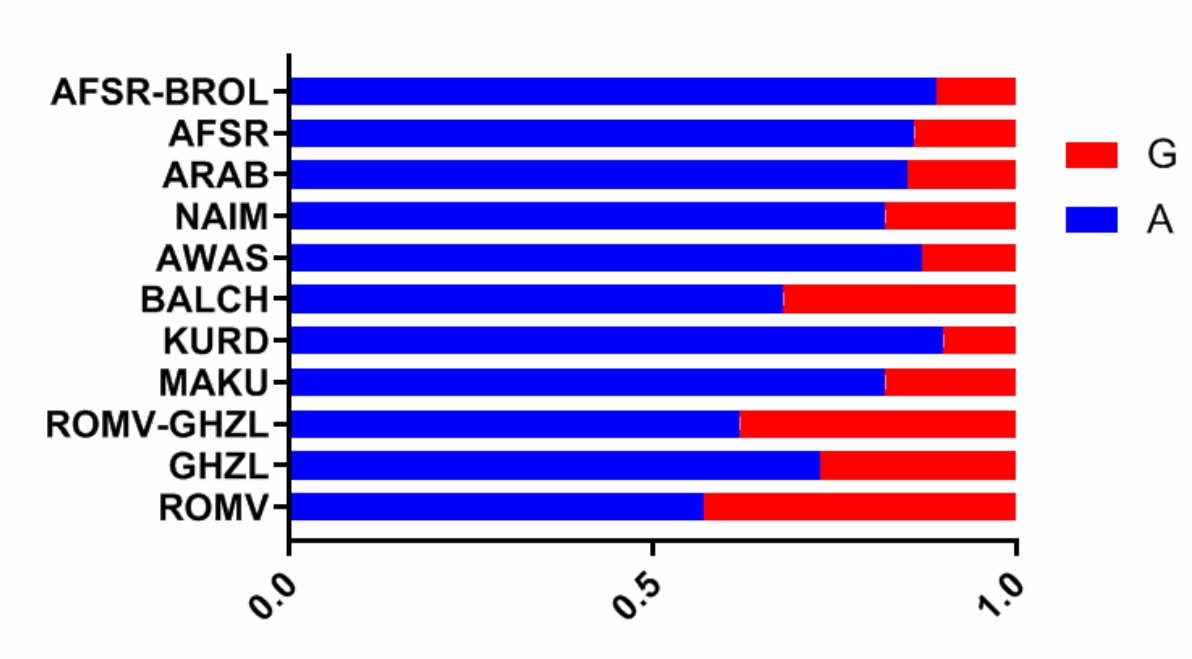

Figure 5 Summary of the identified observed allele frequencies within *AA-NAT* gene across investigated sheep breeds
The exotic breeds Romanov (ROMV) and [Awassi (AWAS), Arabi (ARAB), Naaimi (NAIM) Iraqi native sheep] and [Ghezel (GHZL), Makui (MAKU), Kurdi (KURD), Baluchi (BALCH), Afshari (AFSR) Iranian native sheep] and two Afshari-Booroola and Romanov-Ghezel F1 crosses)

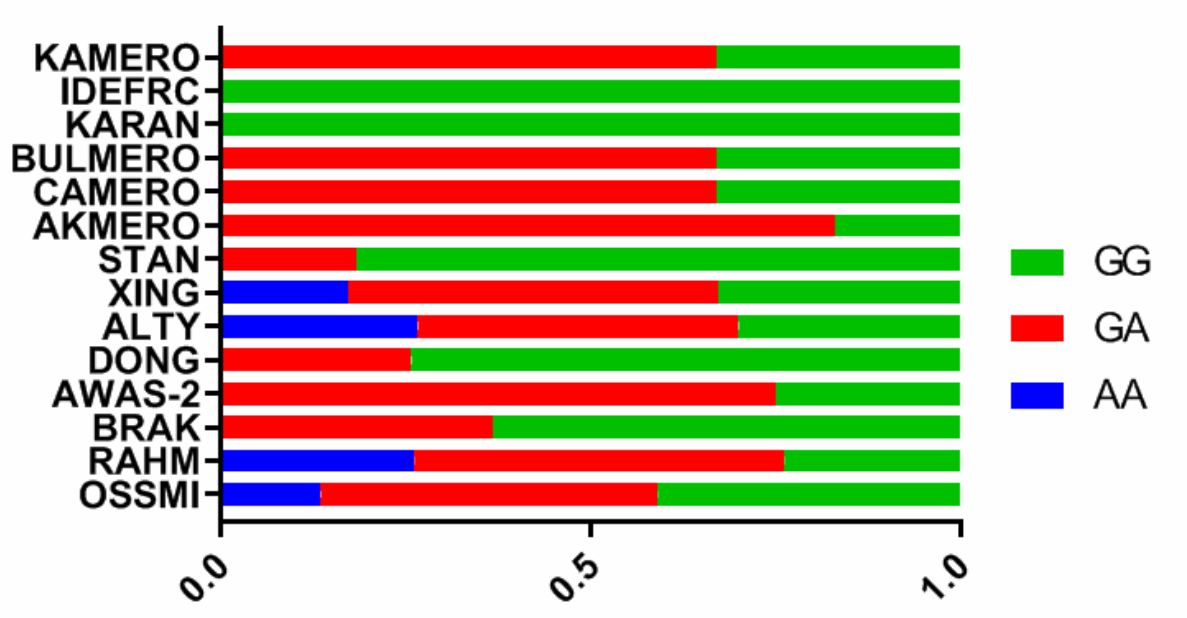

Figure 6 Brief description of the variation in genotype frequency in similar literature on different breeds of sheep
OSSMI: Ossimi sheep; RAHM: Rahmani sheep; BRAK: Baraki; AWAS 2: Awasi; DONG: Dolang; ALTY: Altay; XING: Xinjang sheep; STAN: Small tail han; AKMERO: Askanian Merino; CAMERO: Caucasian Merino; BULMERO: Northeast Bulgarian Merino; KARAN: Karakachan; IDEFRC: II de France and KAMERO: Karnobat Merino

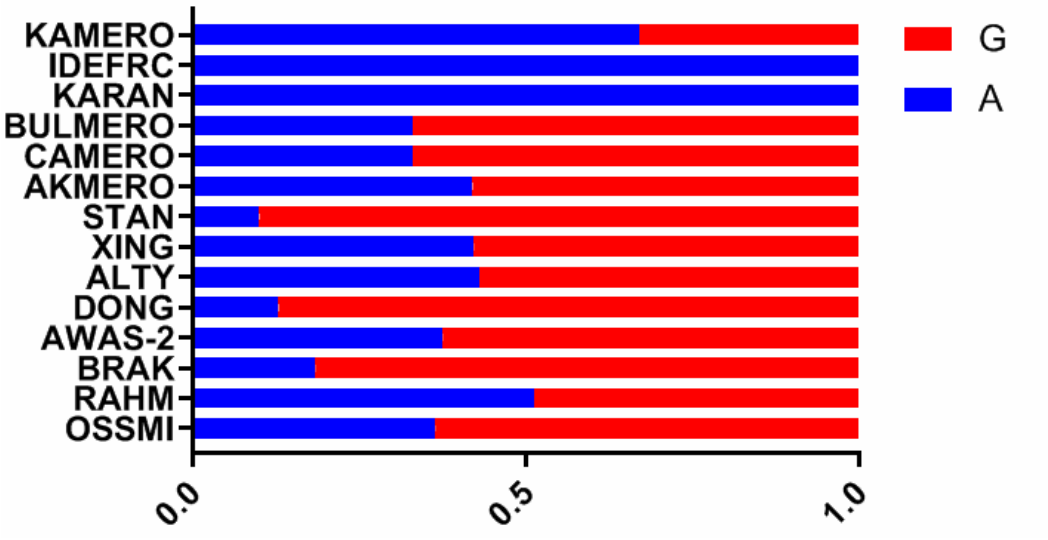

Figure 7 Overview of the variation in genotype frequencies in similar references in different breeds of sheep
OSSMI: Ossimi sheep; RAHM: Rahmani sheep; BRAK: Baraki; AWAS 2: Awasi; DONG: Dolang; ALTY: Altay; XING: Xinjang sheep; STAN: Small tail han; AKMERO: Askanian Merino; CAMERO: Caucasian Merino; BULMERO: Northeast Bulgarian Merino; KARAN: Karakachan; IDEFRC: II de France and KAMERO: Karnobat Merino

Ding-ping described the EX3 486A> G mutation in Chinese sheep and reported that the G allele varied from 0.871 to 0.908 in two non-seasonal reproductive breeds and from 0.517 to 0.578 in two seasonal reproductive breeds, and also concluded that the AA genotypes do not occur in Small Tail Han sheep and Dolang sheep and the frequency of the G allele in Small Tail Han sheep and Dolang sheep was higher than in Xinjiang sheep with fine wool and Altay Fat Pseux Sleep.

Recent evidence suggests that AA-NATSNP showed significant correlations in Ossimi with the age at the first lambing and the litter size in Egyptian Ossimi sheep (Fathy *et al*. 2018).

Moreover, this polymorphism expresses a significant relationship with lamb interval in Rahmani another Egyptian sheep. Therefore, in the literature on the G allele, the relative importance of lower age at first lambing has been subject to considerable discussion.

Interestingly, similar to our study and Fathy *et al*. (2018) report, other investigators have argued similar patterns for Chinese Altay Fat-Rumped sheep breeds (Ding-ping *et al*. 2012). The evidence reviewed here seems to suggest that the GG genotype might be associated with non-seasonal reproduction, while the GA genotype might be associated with seasonal reproduction. In contrast to our finding, the AA genotype pattern was predominant in Turkish sheep breeds (Oner *et al*. 2014; Addin *et al*. 2016).

It is noteworthy, however, that cycling activity in Egyptian sheep breeds decreases significantly during the spring and summer months (Aboul-Ela and Chemineau, 1990).

Younis and Hatif (2017), showed in Iraqi Awasi sheep that the G allele is 0.625 and the A allele is 0.375.

Additional investigation on AA-NAT would further our understanding of the role of the G allele in estrus out of season in sheep. For example, in (Bozhilova *et al*. 2019), a Similar RFLP-AA-NAT was applied to Bulgarian sheep. however, the locus expressed a monomorphic pattern in animals of the races Il de France and Karakachan, the G allele and the GG genotype were detected. On the other hand, Il de France and Karakachan only showed the GG genotype in the electrophoresis pattern and the results showed inconsistency with our results (Bozhilova *et al*. 2019). In Bulgarian sheep, however, the G allele frequencies were higher than the A allele in various breeds. Therefore, the results of Bozhilova *et al*. (2019) somehow agreed with the results of some Chinese sheep breeds.

The study results provided some interesting findings regarding the correlations between the G allele and gait deviations. The study of Chinese sheep breeds conducted on these Chinese sheep breeds found that the frequencies of the non-seasonal breeds were higher while they tended to be lower.

Our results are in agreement with the results of seasonal reproductive breeds. Bring more evidence, another report emphasizes, the different behavior of G allele frequencies in Kvrck populations, which was lower than the allele frequencies of G allele frequencies obtained in Chinese seasonal reproductive breeds. Figure 5 shows an overview of genotype and allele frequencies pattern across pieces of literature.

The present studies naturally have limitations, such as the study of only one candidate gene, the small sample size, and the lack of information on the reproductive history of the participants. Future investigations using sophisticated tools such as GWAS and whole-genome sequencing tools are necessary to validate the kinds of conclusions that can be drawn from this study.

CONCLUSION

Research on seasonal / non-seasonal reproduction has a long history in sheep farming. These problems are difficult to handle because of the sex-limited nature, low heritability, and large contribution of environmental effects on the phenotypic variation of these features. The key contribution of this work is crossbreed offspring of exotic and native sheep, which tend to show higher G allele frequencies in the *AA-NAT* gene. In summary, the frequencies of the G allele varied from 0.1 to 0.43 in all study breeds. ROMV is a candidate for the non-seasonal sheep breed and its cross expresses the highest G allele frequency among other breeds studied. The frequency of the *AA-NAT* genotype was significantly different between breeds in this study.

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