RESEARCH ARTICLE

Characterization of *MTNR1A* gene in terms of genetic variability in a panel of subtemperate and subtropical Indian sheep breeds

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Abstract

Seasonality of animals is an important adaptive trait for successful survival and production during limited food availability and extreme environmental conditions. Photoperiodic changes in day length are utilized by these seasonal animals as an important environmental cue for regulating their annual rhythms of reproduction cycles. Melatonin is an important hormone which is secreted by the pineal gland in proportion to darkness and its effect is mediated by melatonin receptor subtypes, principally *MTNR1A*. In the present study, polymorphism in the coding sequence at two important SNPs (C606T and G612A), known to be markers for out of season breeding in sheep were studied by PCR-RFLP in a panel of four breeds of sheep from subtemperate and subtropical arid conditions, respectively. The frequencies of 'G' and 'A' alleles with reference to G612A SNP did not differ considerably among all the breeds of sheep. Frequency of 'T' allele of the C606T SNP was found to be dominantly higher in subtemperate sheep breeds in comparison to subtropical sheep breeds. Identified SNPs in the coding region were mostly synonymous and did not lead to any change in conformation of the MTNR1A receptor protein.

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Introduction

Sheep are widely known to display marked seasonality in their breeding activity. The annual cycle of the daily photoperiod is identified as the major determining factor regulating this phenomenon, while environmental temperature, nutritional status, social interactions, lambing date and lactation period are the other factors known to modulate it (Rosa and Bryant 2003). Photoperiodic changes in day length are utilized by the seasonal animals as an important environmental cue, as it does not significantly vary between years, unlike temperature, humidity and average rainfall over a period. In mammals, photoperiodic information is relayed through the secretion of melatonin, secreted by the pineal gland in proportion to the period of darkness (Malpaux *et al.* 2001). Melatonin does regulate the circadian rhythmic cycles and reproductive changes in seasonally reproductive animals (Ortavant *et al.* 1985; Reppert *et al.* 1994; Barrett *et al.* 1997). Melatonin displays its effect in terms of controlling circadian rhythmic cycles by virtue of its binding to melatonin receptors of three different types like *MTNR1A*, *MTNR1B* and *MTNR1C*. In mammals, the *MTNR1A* receptor gene has been documented to be associated with the control of reproduction in animals (Weaver *et al.* 1996; Dubocovich *et al.* 2003). *MTNR1A* are G-coupled protein receptors which are prominently found in the pars tuberalis region of pituitary and prehypothalamic region. The pars tuberalis region, which has a higher density of *MTNR1A* receptors have shown to be associated with the control of prolactin secretion (Dardente 2007; Dupré *et al.* 2008). Premamillary hypothalamic region is chiefly involved in the control of GnRH secretion (Chabot *et al.* 1998; Malpaux *et al.* 1998). MTNR1A receptor protein is coded by *MTNR1A* gene, located on chromosome 26 in sheep and it consists of two exons interrupted by an intron (Reppert *et al.* 1994). The gene has been widely mapped in a number of livestock species like cattle, goat, pig and sheep by Messer *et al.* (1997). Earlier studies on the characterization of *MTNR1A* gene lead to the identification of two important SNPs, C606T and G612A, producing polymorphic restriction sites for *Mnl*I and *Rsa*I enzymes, respectively. These SNPs have been associated with seasonal reproductive pattern in sheep breeds like Merino d'arles (Pelletier *et al.* 2000), small tail Han sheep (Chu *et al.* 2006), Awasi (Faigl *et al.* 2008), Sarda (Carcangiu *et al.* 2009) and Dorset (Mateescu *et al.*

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2009). Therefore, the present study was designed to characterize the *MTNR1A* gene and study the allelic frequency distribution in terms of two SNPs (C606T and G612A) in sheep breeds of two different agro-climatic conditions of India, one belonging to subtropical arid regions (Malpura and Patanwadi breed of sheep) and other from subtemperate climatic conditions (Sandyno and Nilgiris). It was also aimed to identify novel SNPs in the coding sequence of *MTNR1A* and to find out any differences occurring due to the regional distribution of the breeds.

Material and methods

Animals and management

Blood samples were collected from two places displaying contrasting climatic conditions, i.e. subtemperate and subtropical arid conditions. The first region was at Sheep Breeding Research Station (SBRS), Sandynallah in the Nilgiri district of Tamil Nadu near Ooty (11.41°N, 76.7°E), where Sandyno and Nilgiri sheep breeds are being maintained. Ooty has a subtemperate climate, with an average rainfall of 140 cm, most of which is received during southwest monsoon, with frosty nights from November to February. The maximum and minimum temperatures are 28 and 0°C, respectively. The entire area consists of rolling hills with an average elevation of 2230 m above mean sea level. The ewes in this station are maintained under semi-intensive system of management, with 8–9 h of grazing. The ewes were provided with concentrate feed during the last month of pregnancy and during lactation. The second place of study was an experimental form of Central Sheep and Wool Research Station (CSWRI), Avikanagar, Malpura, Rajasthan, where Malpura and Patanwadi sheep breeds were being maintained. It is located in the northeastern part of the Rajasthan, between 75.19′ and 76.16′ east longitude and 25.41′ and 26.24′ north latitude. The climatic condition of the area is subtropical semiarid type. The normal annual rainfall at this place (1979–2008) was 622 mm, as recorded by meteorological department. Here, the sheep were fed on natural pasture integrated with concentrated feed. A total of 259 sheep (ewes) (102 Malpura, 56 Patanwadi, 52 Sandyno and 49 Nilgiri) were used in this study. All the sheep used in the study were in the homogenous age group, which is about 2–4 years old.

Blood sample collection

Blood samples (2 mL) were collected from each animal's jugular vein using disposable syringes with 18G needles. The blood samples were kept in microcentrifuge tube containing 200 μL of ACD (citric acid : sodium acetate : dextrose solution) which is an anticoagulant. After collecting, each sample was mixed gently with anticoagulant to prevent the clotting and transported within ice pack to the research laboratory at the Division of Physiology and Biochemistry, Central Sheep and Wool Research Institute, Avikanagar, for the study.

Genomic DNA preparation and qualitative analysis

Genomic DNAs were extracted from whole blood samples using phenol–chloroform method (Sambrook *et al.* 2001). They were subjected to quantitative as well as qualitative estimation using UV nano-spectrophotometer (Dynamica^{TM}, HALLO DNA master) to estimate the purity (260/280 ratio) as well as concentration. Analysed samples which qualified optimum concentration and purity test were used for further study. The DNA samples were then kept at −20°C until use.

Primer sequences

Published primers of Messer *et al.* (1997) were employed for PCR reactions which correspond to position 285–304 sense primer (forward: 5′-TGT GTT TGT GGT GAG CCT GG-3′ 20 mer) and 1108–1089 anti-sense primer (reverse: 5′-ATG GAG AGG GTT TGC GTT TA-3′ 20 mer) of the sequence (GeneBank U14109) of exon-II of ovine *MTNR1A* gene from Reppert *et al.* (1994). The expected amplification fragment size was 824 bp. Primers were synthesized by Sigma Aldrich Chemicals (Bengaluru, India).

PCR amplification

Polymerase chain reaction of all the samples was carried out in 25 μL volume containing 2.5 μL of 10× DreamTaq^{TM} buffer with 20 mM $MgCl_2$ (Fermentas), 0.5 μL of 0.2 mM each dNTPs, 0.5 μL of 10 μM of each primer, 0.2 μL of 5 U/μL Taq DNA polymerase (Fermentas), 100–150 ng genomic DNA as a template and making up volume to 25 μL using nuclease free water. The reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation, annealing and extension (denaturation at 95°C for 1 min, annealing at 62°C for 45 s, extension at 72°C for 1 min), with final extension at 72°C for 10 min on Peltier Thermal Cycler (PTC-200, MJ Research, USA). The resultant PCR products were kept at 4°C until further use. The PCR products were detected by electrophoresis on 2% agarose gel (Axygen Biosciences) in parallel with 100 bp DNA marker (qARTA.Bio) ladder.

RFLP of amplified product

RFLP analysis of amplified product was conducted by digestion with *Mnl*I and *Rsa*I enzymes separately. 5 U of each enzyme was used to perform restriction digestion. The digestion reaction was carried out in 30 μL reaction volume, containing 10 μL of PCR product, 18 μL of nuclease free water and 2 μL of buffer 'G' (Fermentas) for *Mnl*I, while 2 μL of Tango Buffer^{TM} (Fermentas) for *Rsa*I enzyme respectively, which provide optimum digestion conditions to enzymes for complete digestion of PCR product under study. All the samples were incubated overnight at 37°C followed by a deactivation process conducted at 65°C for 20 min. The resulting digested products were resolved according to their sizes by

electrophoresis on 4% agarose gel (Axygen Biosciences), in parallel with GeneRuler^{TM} 50 bp DNA marker (Fermentas) ladder.

SNPs identification

Thirty animals displaying maximum phenotypic diversity were chosen for sequence characterization from each of the four breeds. For sequence characterization, amplified products were purified from the gel by using a gel extraction kit (AxyPrep^{TM} DNA gel extraction kit, Axygen Biosciences) according to the manufacturer's instruction and sequenced in both forward and reverse directions using Applied Biosystem 3730 DNA analyzer. The obtained sequence was aligned using Megalign algorithm of LASERGENE software. The BLAST analysis was performed for all the sequences to obtain its sequence similarity. Presence of polymorphism and SNPs were analysed using LASERGENE software (DNASTAR) taking the sequence (U14109) as reference.

Results

Genomic DNA isolation and PCR amplification of exon II gene

Genomic DNA of all the samples was analysed by UV spectrophotometric qualitative analysis in terms of their 260/280 ratio and all of them were found to be in satisfactory range. PCR amplification of all the samples was carried out using sense and antisense primer sequences and it resulted in an 824 bp single band corresponding to exon II of the *MTNR1A* melatonin receptor gene (figure 1).

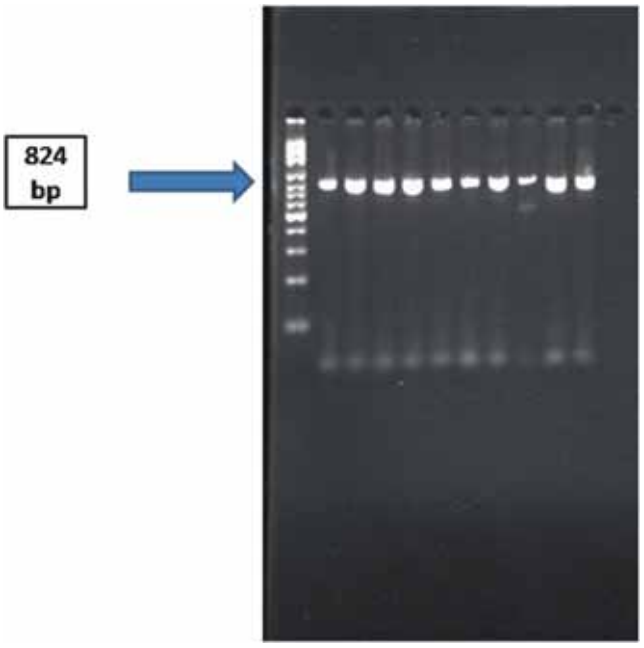

Figure 1. PCR Amplification of *MTNR1A* gene depicted with 100 bp ladder (1st lane).

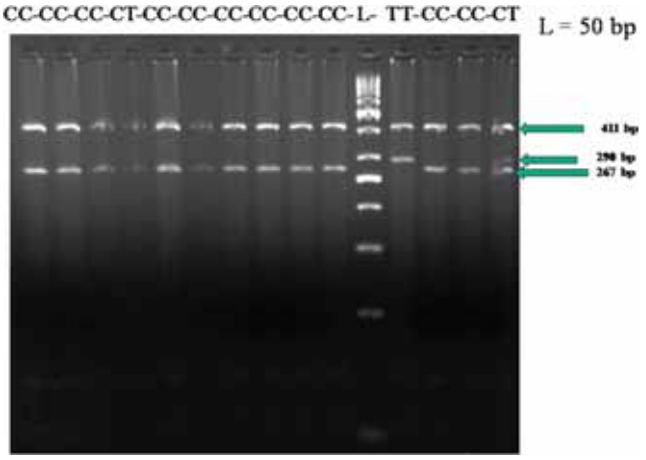

Figure 2. Genotyping of exon-II of *MTNR1A* with *Rsa*I.

RFLP studies

RFLP studies were performed using *Mnl*I and *Rsa*I enzymes to deduce the polymorphism at positions 612 and 606 bp respectively. Digestion with *Mnl*I resulted in cleavage sites at positions 221, 254, 324, 560, 582, 610 and 693 bp, out of which one at position 324 was found to be polymorphic due to G–A transition. GG genotype resulted in bands of 236 and 67 bp due to digestion by *Mnl*I. AA genotype produced a single band of 303 bp due to hampered restriction site due to polymorphism. GA genotype produced bands at positions 303 as well as 236 and 67 bp (figure 2). These two alleles (G and A) were identified in all the four breeds studied.

RFLP was also studied with *Rsa*I for C606T SNP which resulted in cleavage of 824 bp fragment at four positions producing five bands at positions 411, 267, 67, 56 and 23 bp respectively. The restriction site at position 606 bp was found to be polymorphic due to C to T substitution. It resulted in polymorphic fragments of 267 and 23 bp when restriction site is present (allele C) or presence of a single 290-bp fragment when the cleavage site was absent (allele T). Thus, three genotypes CC, CT and TT were detected in all the studied sheep breeds (figure 3).

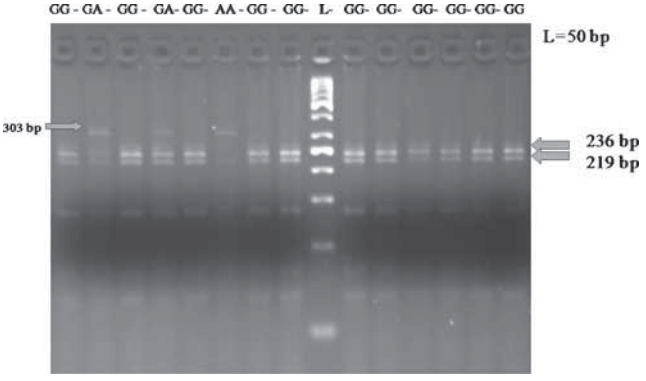

Figure 3. Genotyping of exon-II of *MTNR1A* with *Mnl*I.

Genotypic and allelic frequencies estimation

Population of all the breeds were found to be in Hardy–Weinberg equilibrium (HWE) as tested by web-based tool of online encyclopedia for genetic epidemiology studies (Rodriguez *et al.* 2009). Genotypic and allelic frequencies were estimated for all the breeds by manual calculations and are depicted in tables 1 and 2. The minor allele frequency for 'C606T' was comparatively higher in Sandyno and Nilgiri breeds of sheep (0.538 and 0.439) than Malpura and Patanwadi breeds of sheep (0.065 and 0.169 respectively) (figure 4).

The minor allele frequency for G612A was slightly higher in subtropical sheep breed in comparison to subtemperate sheep breed (figure 5). GG was found to be the major genotype in all the breeds of sheep.

Sequence characterization

Thirty samples of amplified product from each of the breeds were sequenced to study the variability of the gene in subtropical and subtemperate sheep breeds. Sequencing of the samples revealed several SNPs which are presented in table 3. Most of the polymorphisms/mutations did not lead to amino acid changes except at G706A where there was a substitution of valine by isoleucine. We have also observed that mutations at positions G453T, G612A, G706A and C891T seem to be closely linked. We did not observe the mutation at positions 426 and 555 in any of the subtropical sheep breed, while they were found to be present in Nilgiri and Sandyno. Annotated gene sequences were submitted to GenBank, NCBI and following accession numbers were obtained (KJ865679, KJ865680, KJ865681, KJ865682, KM006919, KM006920, KM006921, KM006926 and KM006927).

Discussion

Sheep breeds originating from temperate climate at mid latitudes or high latitudes are seasonal breeders, which use the

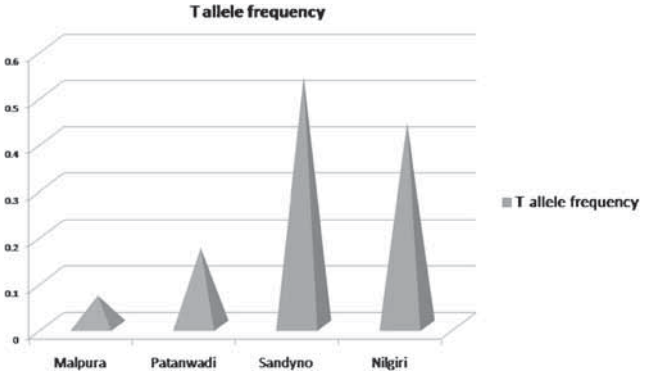

Figure 4. 'T' allele frequency in various breeds of sheep due to C606T SNP.

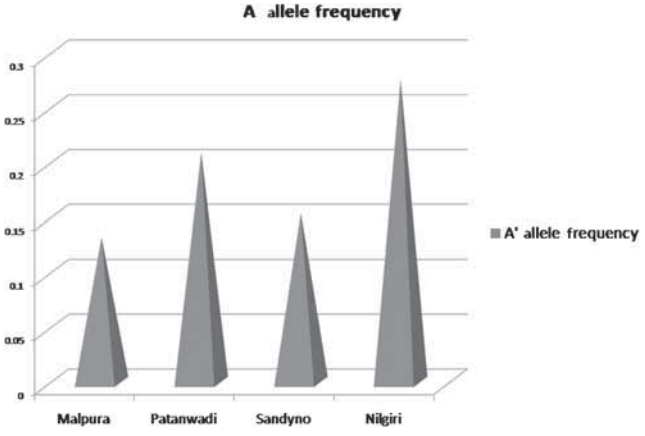

Figure 5. 'A' allele frequency in various breeds of sheep due to G612A SNP.

Table 1. Allele and genotypic frequencies of PCR-RFLP of the *MTNR1A* gene (exon II) in sheep breeds digested with *Rsa*I.

Breed	No. of samples	Allele frequency		Genotype frequency		
		C	T	CC	CT	TT
Malpura	100	0.935	0.065	0.874 (88)	0.121 (11)	0.004 (01)
Patanwadi	56	0.723	0.169	0.522 (38)	0.245 (17)	0.029 (01)
Sandyno	52	0.461	0.538	0.213 (14)	0.497 (20)	0.289 (18)
Nilgiri	49	0.561	0.439	0.314 (17)	0.492 (21)	0.192 (11)

Table 2. Allele and genotypic frequencies of PCR-RFLP of the *MTNR1A* gene (exon II) in sheep breeds digested with *Mnl*I.

Breed	No. of samples	Allele frequency		Genotype frequency		
		G	A	GG	GA	AA
Malpura	102	0.867	0.132	0.752 (79)	0.229 (19)	0.017 (04)
Patanwadi	55	0.790	0.209	0.625 (33)	0.330 (21)	0.044 (01)
Sandyno	52	0.846	0.154	0.716 (38)	0.260 (12)	0.024 (02)
Nilgiri	49	0.724	0.276	0.524 (26)	0.399 (19)	0.076 (04)

Table 3. Polymorphisms/mutations observed in different breeds.

No. of SNP	Polymorphism position	No. of nucleotide substitution	Amino acid change	Breed
1	426	AG**C**/AG**T** (Ser/Ser)	No amino acid change	N, S
2	453	AC**G**/AC**T** (Thr/Thr)	No amino acid change	N, S, M, P
3	555	AC**G**/AC**A** (Thr/Thr)	No amino acid change	N, S
4	606	TA**C**/TA**T** (Tyr/Tyr)	No amino acid change	N, S, M, P
5	612	CC**G**/CC**A** (Pro/Pro)	No amino acid change	N, S, M, P
6	675	GT**G**/GT**A** (Val/Val)	No amino acid change	N
7	706	**G**TC/**A**TC (Val/Ile)	Val/Ile	N, S, M, P
8	783	CT**G**/CT**A** (Leu/Leu)	No amino acid change	S, N
9	801	AG**G**/AG**A** (Arg/Arg)	No amino acid change	S, N
10	891	CC**C**/CC**T** (Pro/Pro)	No amino acid change	S, N

N, Nilgiri; S, Sandyno; M, Malpura; P, Patanwadi. Single-nucleotide polymorphism positions are in bold.

annual variation in daily photoperiod in timing the annual reproductive cycles, while in tropical and subtropical environment; ewes are completely aseasonal or intermittently polyestrous (Rosa and Bryant 2003). India is a country with multiple agroclimatic zones which provided us a unique opportunity to go for comparative profiling of two SNP markers in terms of their frequency distribution in subtemperate and subtropical environmental conditions. Earlier studies on SNP G612A have found it to be an important marker in influencing seasonal reproduction in sheep, but most of the studies were carried out on temperate sheep breeds which are known to be seasonal. Pelletier *et al.* (2000) showed an association of G allele to ovarian activity in spring in Merino d'arles ewes, whereas Notter *et al.* (2003) reported association of GG genotype to breed out of season. Therefore, they found that animals with greater G allele frequency have a greater tendency to perform out of season breeding or round the year breeding. With reference to the G612A SNP marker, we found that the frequency of A allele was found to be higher in subtemperate sheep breeds (Nilgiri and Sandyno) in comparison to that of subtropical breeds (Malpura and Patanwadi), although GG was found to be the major genotype in all of them.

C606T is another important marker which has been reported to be influencing seasonal reproduction in sheep. Chu *et al.* (2006) performed a descriptive study on the allelic frequency of unseasonal (small tail Han sheep and Hu sheep breeds) and seasonal (Suffolk, Dorset and German mutton Merino sheep) sheep breeds. He reported significantly higher allelic frequency of C allele in unseasonal sheep breeds in comparison to seasonal sheep breeds. Likewise, Carcangiu *et al.* (2009) also reported the association of CC genotype to round the year breeding performance of Sarda sheep breeds. Mura *et al.* (2014) found that the ewes carrying G/G, G/A, C/C and C/T genotypes exhibited higher fertility rates and fewer number of days between the introduction of rams and parturition than the A/A and T/T genotypes. Martínez-Royo *et al.* (2012) demonstrated that the T allele of C606T of *MNTR1A* gene was associated with a greater percentage of oestrous cyclic ewes in the Rasa Aragonesa breed. Luridiana *et al.* (2014) found that the *MTNR1A* gene affects the response of melatonin treatment on reproduction in Sarda sheep and observed CC to be major genotype in the breed. CC genotype was found to be the major genotype in the tropical breeds of sheep (Saxena *et al.* 2014, 2015a, b) from tropical arid environment of India. Present study highlighted the differential prevalence of the alleles in the subtropical and subtemperate climatic conditions, with the TT/CT genotype having a significantly higher prevalence in subtemperate climatic conditions. Generally, breeds of tropical region are known to breed throughout the year. Malpura and Patanwadi breeds when subjected to improved nutritional regimen and adequate concentrate feeding are known to breed throughout the year (Acharya 1982) and even in extreme summer of arid conditions, they are reported to be showing ovarian activity (Naqvi *et al.* 2012). Nilgiri breed is a short-statured wooly breed inhabiting hilly region of Nilgiri district of Tamil Nadu, India. It has a crossbred base and contains unknown inheritance from Coimbatore, Tasmanian Merino, Cheviot and southern breed. Sandyno breed was produced by crossing Nilgiri (3/8) with Merino or Rambouillet (5/8). On comparing the allele frequency of T allele in two group panels, it was found that subtemperate sheep breeds have higher allele frequency in comparison to subtropical sheep breeds. Based on allelic frequency distribution studies, it can also be concluded that T allele has a much higher prevalence in subtemperate sheep breed group in comparison to A allele.

We have also determined sequence variation in the studied group of breeds by PCR amplification and sequencing of the exon II region of coding sequence. We could find 10 important mutations/polymorphisms, all of which were synonymous, except G706A which leads to substitution of valine by isoleucine. C426T and G555A were only observed in Nilgiri and Sandyno breeds and were not present in Malpura and Patanwadi breeds of sheep, which may be due to crossbred inheritance of these breeds. The mechanism behind the effect of polymorphism at the level of its effect on reproduction

is not clearly established which may be due to the interaction of various *trans*-elements, regulatory sequences, or the epigenetic mechanism that may also need to be tested.

Conclusion

Both the polymorphic loci at positions 606 and 612 have been found to be distributed differently in subtropical and subtemperate breed of sheep. T allele has a much higher prevalence in studied subtemperate sheep breeds in comparison to A allele. Further studies are required to explore the role of epigenetic mechanism, interaction of regulatory sequences or the gene interaction mechanism to explain the effect of these polymorphisms over seasonality in sheep.

Acknowledgements

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