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Nucleotide Sequence, SNPs and Haplotypes among Breeds of Goat Melatonin Receptor *1A* Gene

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Abstract: Melatonin Receptor 1A (MTNR1A) is a potential candidate gene that may affect seasonal reproduction in goats. The aim of this study was to search for Single-Nucleotide Polymorphisms (SNPs) in the goat *MTNR1A* gene. Coding exons (1 and 2) were amplified by PCR and sequenced. Sequencing showed that the entire goat MTNR1A coding region consists of 1101 nucleotides and 366 deduced amino acids. The goat MTNR1A (exon 1) nucleotide sequence is most highly homologous to sheep (99.6%) and most distant to mouse and human (77.1 and 85%, respectively). The MTNR1A amino acid sequence is again most similar to sheep (98.6%) and most distant to human and mouse (83.0 and 83.9%). The goat *MTNR1A* gene differs between Japanese Saanen, Shiba, Boer and three Asian native goats with 18 potential nucleotide substitutions in the coding region and 3 SNPs in the 3' non-coding region. Eleven novel SNPs were detected in this study, adding to 10 SNPs earlir reported. The SNPs were differently distributed among the breeds examined in this study. Haplotype analysis indicated that there are 13 types in the samples and different distributions of any haplotype similar to the SNPs one. Ten SNPs were detected following PCR-RFLP using suitable restriction enzymes. These results provide valuable information on polymorphisms in goat breed genotypes which may be used for further characterization and comparative studies in goat breeds.

Key words: Goat, melatonin receptor, SNP, haplotype, Japan

INTRODUCTION

Globally, goats provide milk, meat and dairy products (Dubeuf and Boyazoglu, 2009). Goat meat and milk are especially valuable for developing countries and production continues to increase (Aziz, 2010). According to the United Nations Food and Agriculture Organization (FAO), global annual production in 2008 for goat milk was 15.2 million metric tons and 4.8 million metric tons of goat meat was produced (Faostat, 2008). Goat meat is considered a healthier meat alternative as it is lower in fat content and cholesterol compared to for example, chicken meat (Glimp, 1995; Banskalieva *et al*., 2000). Fresh goat milk is used to produce dairy products such as cheese, yogurt and butter and is consumed by infants and other people with allergies to cow milk (Klinger and Rosenthal, 1997).

Some goat breeds are bred specifically for their milk. Doe produce 6-8 pounds of milk daily (approximately 3 L) during lactation, around the third and fourth lactation cycle (Gipson and Grossman, 1990; Uehara *et al*., 2007). Although, the total goat population in Japan is very small with the highest population in the Okinawa prefecture, goat milk and its processed dairy products continue to show a steady growth in production as consumers are becoming more aware of the higher protein and lower cholesterol levels in goat products. Recently, goat milk consumers in Japan have been particularly influenced by Japanese middle-class women (Ozawa *et al*., 2009, 2010). Milk is produced for approximately 10 months following kidding but production is dry for 2 months before freshening (Shinojo and Toma, 1984). Goat milk production in Japan all year round is not possible which is problematic because there is a shortage of goat milk during the winter season.

Goat reproduction is popularly described as seasonal. The onset and length of the breeding season is dependent on various factors such as latitude, breed and

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photoperiod (Fatet *et al*., 2010). Seasonal reproductive activity for goat species living in temperate latitudes is characterized by alternating ovulatory and anovulatory periods which are triggered by annual variations in day length. In mammals, photoperiodic information is translated into a daily cycle of melatonin secretion from the pineal gland (Reiter, 1980).

Elongated high melatonin concentration is stimulated by Gonadotropin Releasing Hormone (GnRH) secretion from the hypothalamus in short day breeders such as the goat and sheep (Reppert *et al*., 1994; Carcangiu *et al*., 2005). Two types of melatonin receptor (1a and b) have been found in mammals. However, only Melatonin Receptor 1A (MTNR1A) has been found to be related to seasonal breeding activity and what adjustments or changes may occur in breeding patterns (Reppert *et al*., 1996; Barrett *et al*., 1997). To date, polymorphisms on the large part of exon 2 in the goat *MTNR1A* gene have been examined in a number of breeds (Migaud *et al*., 2002; Chu *et al*., 2007; Carcangiu *et al*., 2009). However, not all the coding regions have been examined, particularly exon 1. Furthermore, major genes affecting seasonal breeding have not yet been identified in the goat.

The objectives of this study were to obtain the nucleotide and amino acid sequences of the goat MTNR1A coding regions to assess sequence differences between the goat and other mammals and to locate sequence differences between goat breeds which may be used to identify reproductive characteristics in the goat.

MATERIALS AND METHODS

Animals: Seventy-six goats were used in this study including 3 domestic breeds and 3 native populations. Fifteen Japanese Saanen and 13 Shiba goats were reared in the Nagano branch of Ibaraki station, National Livestock Breeding Center and 12 Boer goats, reared at the Animal research center in the Okinawa prefecture and introduced from New Zealand in 2010. Researchers also used 12 Bangladeshi native goats from the Chapai Nawabganj and Rajahahi regions, 12 Mongolian native goats from the Hovsgol region and finally, 12 Korean native goats, introduced from Korea in the 1980s which were maintained as a closed colony at the Department of Agriculture, Tokyo University of Agriculture.

Sequence analysis: To detect nucleotide differences in the goat *MTNR1A* gene coding region, researchers determined nucleotide sequences by direct sequencing with genomic DNA. Genomic DNA was isolated from blood samples using a phenol-chloroform DNA extraction method (Sambrook and Russell, 2001). The coding region sequences were determined with 3 primer sets (one pair in exon 1 and two pairs in exon 2, Table 1) which were designed based on the sheep MTNR1A mRNA sequence and the bovine genome sequence, located on chromosome 27 (Gen Bank Accession No. OAU14109 and NC_007328, respectively). PCR was performed in a total volume of 15 μL, containing 20 ng of genomic DNA, 6.25 pmol of each primer, 0.2 mM of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.375 U of BIOTaq HS DNA polymerase (Bioline, Taunton, MA, USA). PCR conditions were as follows: 94°C for 9 min followed by 35 cycles of 94°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec and a final extension at 72°C for 5 min. For the amplification of exon 1 region, PCR was carried out with KOD-FX Taq DNA polymerase (Toyobo, Osaka, Japan) instead of BIOTaq HS DNA polymerase as the target fragment is GC rich and otherwise difficult to amplify. The PCR products were subsequently purified using ExoSAP-IT (GE Healthcare UK Ltd., Buckinghamshire, England) and sequenced with an ABI 3130 xl DNASequencer using the BigDye Terminator Ver. 3.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo, Japan). Sequencing was performed for both sense and antisense complementary strands to confirm nucleotide sequences.

Polymorphism and haplotype analysis: Following DNA sequencing, the nucleotide sequences were assembled using ATGC Software Ver. 4.0.10 (Genetyx, Tokyo, Japan). The assembled sequence data including the partial goat sequence earlier reported (Genbank Accession No. AF419334, Migaud *et al*., 2002) was compared to other known MTNR1A sequences in different mammals,

Table 1: Primer sequences used in this study, target regions and its origin

Primer name	Primer sequence (5'-3')	Amplified region	Product size (bp)	Originated
gM TNRIA-exl-F4	caccggcgccggccctgccagc	Exon 1	380	Sheep cDNA sequence
gM TNRIA-exl-Rl	aagagccagttgacagcccga			Cattle genome sequence
gM TNRIA ex2-1-F1	actgagtgaccgacacttttc	First half of exon 2	653	Cattle genome sequence
gM TNRIA-ex2-1-R2	ggtttgttgtccggtttcacc			Goat sequence
gM TNRIA-ex2-2-F1	tttcatagttccgatgctcgt	Second half of exon 2	562	Goat sequence
gM TNRIA-ex2-2-R1	cccacgttgttttcctaggca			Sheep cDNA sequence

including sheep, bovine, horse, human and mouse. Following the comparative species analysis, the assembled data for the coding regions was also compared among individual goats and the breeds used in this study using software for multiple sequence assembly (ATGC Software). Haplotypes for each study animal were identified using the PHASE computer program Version 2.1 (Stephens *et al*., 2001). Finally, several SNPs were analyzed using suitable restriction enzymes. For example, with regards to position 52 (exon 2), earlier reported, the PCR product for the upper half of exon 2 was digested using 2 U of the RsaI restriction enzyme (New England Biolabs Japan, Tokyo, Japan) (Chu *et al*., 2007; Carcangiu *et al*., 2009). Digested fragments were separated by electrophoresis on 7% polyacrylamide gel (Nippon eido, Tokyo, Japan) in parallel with a DNA size marker (pBR322 DNA-MspI digest).

RESULTS AND DISCUSSION

Nucleotide and amino acid sequences of the goat *MTNR1A* gene and homologies with other mammals: The total goat *MTNR1A* gene coding region consists of two exons (exon 1 is 232 bp and exon 2 is 869 bp). The coding regions were successfully amplified using three primer pairs. The amplified fragments were used for sequencing and RFLP analysis. Figure 1 shows the nucleotide sequence and deduced amino acid sequence for the MTNR1A exon 1 coding region (Genbank Accession No. AB716763). Exon 2 sequence contains 1118 nucleotides and was used as a reference (Genbank Accession No. AB716764) (Fig. 2). Sequencing results indicate that the goat MTNR1A coding region consists of 1101 nucleotides and 366 corresponding amino acids (Fig. 1 and 2). The goat MTNR1A (exon 1) is most homologous with sheep followed by cattle, horse, human and mouse (99.6, 97.0, 87.5, 85.0 and 77.1%, respectively). The goat MTNR1A amino acid sequence is most homologous to sheep followed by cattle, horse, mouse and human (98.6, 97.3, 83.9, 83.9 and 83.0%, respectively) (Table 2).

Differences in the goat MTNR1A nucleotide and amino acid sequences among breeds: Comparison between the nucleotide sequences indicates the presence of at least 21 SNPs (Table 3). Researchers renumbered the goat MTNR1A nucleotide sequences from a new start codon as all coding regions were determined for this study. For example, earlier numbered g52a is renumbered g288a in this study. Comparison between coding sequences indicated two amino acid substitutions: a non-synonymous SNP at position 59 in exon 1 (alanine changed to glutamine) and another at position 469 in exon 2 (glycine to arginine). Eleven novel SNPs were found in the samples adding to the 10 SNPs earlier reported. The majority of SNPs earlier observed were found to be differently distributed among populations examined in this study. Two SNPs, earlier reported called g307t and t559c were not observed in the samples (Migaud *et al*., 2002; Chu *et al*., 2007, respectively). Three SNPs, c59a, g469c and g645a were found within a breed and other SNPs were present in two and more breeds.

The goat *MTNR1A* gene was genotyped to determine if its function is in some way associated with seasonal

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Amino acid    1                                  M A  G R L  W G S  P G G T
Nucleotide    1 caccggcgcc ggccctgcca gcgcgatggc ggggcggctg tggggctcgc cgggcgggac
Amino acid   13 P K G  N G S  S A L  L N V  S Q P  A P G  A G
Nucleotide   61 ccccaagggc aacggcagca gcgcgctgct caacgtctcg cagccggcgc ccggcgccgg
Amino acid   33 D G V  R P R  P S W  L A A  T L A  S I L  I F
Nucleotide  121 ggacggtgtg cggccgcggc cctcgtggct ggccgccacc ctcgcctcca tcctcatctt
Amino acid   53 T I V  V D I  V G N  L L V  V L S  V Y R  N K
Nucleotide  181 caccatcgtg gtggacatcg tgggcaacct cctggtggtc ctgtccgtgt atcggaacaa
Amino acid   73 K L R  N A
Nucleotide  241 gaagctgagg aacgcag/gta gggactctca ggacccggca ggctggacag gcaagccccc
Nucleotide  301 agctttcccc gacggcaacc tcttccccgt tcggccacct ttgcaagcac gcagctgcct
Nucleotide  361 cgggctgtca actggctctt
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Fig. 1: Goat MTNR1A nucleotide sequence and amino acid sequence (single code letter) of the exon 1 region. Underlines indicate forward and reverse primers. Positions of SNP and amino acid changes are shaded in grey. Square box represents the start codon position. A slash is the position of an exon-intron boundary, inferred from the bovine genome

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Nucleotide    1 actgagtgac cgacactttt cactgtcttc cggtaactgt gatgtcaggc catccgagca
Nucleotide   61 gccgacacag ctctttcttg aatacctggg aaagaaaacc catgcaaccc gtgtgagacg
Amino acid   78                                            G  N   V F  V  V S  L
Nucleotide  121 ggccctaacc catgttttct aaacctttct ctttag/ggaa tgtgtttgtg gtgagcctgg
Amino acid   86 A  V  A  D   L  L  V   A  V  Y   P  Y  P  L   A  L  A   S  I  V
Nucleotide  181 cagttgcaga cctgctggtg gccgtgtatc cgtacccctt ggcactggcg tctatagtta
Amino acid  106 N  N  G  W   S  L  S   S  L  H   C  Q  L  S   G  F  L   M  G  L
Nucleotide  241 acaatgggtg gagcctgagc tccctgcatt gccaacttag tggcttcctg atgggcttga
Amino acid  126 S  V  I  G   S  V  F   N  I  T   G  I  A  I   N  R  Y   C  C  I
Nucleotide  301 gcgtcatcgg gtctgttttc aatatcacgg gaattgccat caaccgctac tgctgcatct
Amino acid  146 C  H  S  L   R  Y  D   K  L  Y   S  G  T  N   S  L  C   Y  V  F
Nucleotide  361 gccacagcct cagatacgac aagctgtata gcggcacgaa ttccctctgc tacgtgttcc
Amino acid  166 L  I  W  M   L  T  L   V  A  I   V  P  N  L   C  V  G   T  L  Q
Nucleotide  421 tgatctggat gctgacgctc gtggcgatcg tgcccaacct gtgtgtgggg accctgcagt
Amino acid  186 Y  D  P  R   I  Y  S   C  T  F   T  Q  S  V   S  S  A   Y  T  I
Nucleotide  481 acgacccgag gatctattcc tgtaccttca cgcagtcggt cagctcagcc tacacgatcg
Amino acid  206 A  V  V  V   F  H  F   I  V  P   M  L  V  V   V  F  C   Y  L  R
Nucleotide  541 ccgtggtggt gttccatttc atagttccga tgctcgtagt tgtcttctgt tatctgagaa
Amino acid  226 I  W  A  L   V  L  Q   V  R  W   K  V  K  P   D  N  K   P  K  L
Nucleotide  601 tctgggccct ggttcttcag gtcagatgga aggtgaaacc ggacaacaaa ccgaaactga
Amino acid  246 K  P  Q  D   F  R  N   F  V  T   M  F  V  V   F  V  L   F  A  I
Nucleotide  661 agccccagga cttcaggaat tttgtcacca tgtttgtggt ttttgtcctc tttgccattt
Amino acid  266 C  W  A  P   L  N  F   I  G  L   V  V  A  S   D  P  T   S  M  A
Nucleotide  721 gctgggctcc tctgaacttc attggtctcg ttgtggcctc ggacccccacc agcatggcac
Amino acid  286 P  R  I  P   E  W  L   F  V  A   S  Y  Y  M   A  Y  F   N  S  C
Nucleotide  781 ccaggatccc cgagtggctg tttgtggcta gttactatat ggcatatttc aacagctgcc
Amino acid  306 L  N  A  I   I  Y  G   L  L  N   Q  N  F  R   Q  E  Y   R  K  I
Nucleotide  841 tcaatgcaat catatatgga ctactgaacc aaaatttcag gcaggaatac agaaaaatta
Amino acid  326 I  V  S  L   C  T  T   K  M  F   F  V  D  S   S  N  H   V  A  D
Nucleotide  901 tagtctcatt gtgtaccacc aagatgttct ttgtggatag ctccaatcat gtagcagata
Amino acid  346 R  I  K  R   K  P  S   P  L  I   A  N  H  N   L  I  K   V  D  S
Nucleotide  961 gaattaaacg caaaccttct ccattaatag ccaaccataa cctaataaag gtggactctg
Amino acid  366 V
Nucleotide 1021 tttaaaaatg tggagcatga atggcaagat ctgaacacta ctccaagcct tactcatttt
Nucleotide 1081 cattccctct tggtagatgc ctaggaaaac aacgtggg
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Fig. 2: Goat MTNR1A nucleotide sequence and amino acid sequence (single code letter) of the exon 2 region. Underlines indicate forward (2-1) and reverse (2-2) primers. Positions of SNP and amino acid changes are shaded in grey. Square box represents the stop codon position. A slash is the position of an exon-intron boundary, inferred from the bovine genome

Table 2: Nucleotide and amino acid sequences similarity of goat MTNRIA to other mammalian species

Mammalian species	Nucleotide (%) Coding region of exon 1	Coding region of exon 2	Amino acid (%) (All coding region)	Genbank accession No.
Sheep	99.6	98.5	98.6	U14109.1
Cattle	97.0	96.8	97.3	XM-002698656.1
Horse	87.5	85.0	83.9	XM-001490171.1
Human	85.0	84.2	83.0*	NM-005958.3
Mouse	77.1	80.8	83.9*	NT-039460.7

*Calculated without first 32 amino acid residues

reproduction. Researchers were able to examine 10 out of the total 21 SNPs using PCR-RFLP (Table 3). This study found the presence of a RsaI cleavage site in the amplicon using the exon 2-1 primer pair which produced three fragments of 290, 214 and 149 bp in length (characterized as the R allele). The absence of this site produced only two fragments, 504 and 149 bp (r allele). PCR-RFLP for g288a, earlier called g52a (Chu *et al*., 2007; Carcangiu *et al*., 2009) confirmed the presence or absence of the RsaI restriction site following DNA sequencing. No

Table 3: Position of mutations and nucleotide and amino acid changes in the *MTNR1A* gene compared with the reference sequence

Position of mutations	Position of mutations	Nucleotide change	Amino acid change	Restriction enzyme	References
59	-	C→A	Ala→Glu	HhaI	In this study
288	52	G→A	None	RsaI	Chu *et al.* (2007) and Carcangiu *et al.* (2009)
390	154	T→C	None	-	In this study
426	190	C→T	None	-	Migaud *et al.* (2002), Chu *et al.* (2007) and Carcangiu *et al.* (2009)
468	232	C→T	None	-	Carcangiu *et al.* (2009)
469	233	G→C	Gly→Arg	-	Migaud *et al.* (2002)
474	238	G→A	None	EcoRI	In this study
489	253	C→T	None	HpyCHIV	Carcangiu *et al.* (2009)
513	277	G→A	None	HgaI	In this study
588	352	G→A	None	HpyCHIII	In this study
594	358	G→A	None	-	Carcangiu *et al.* (2009)
645	409	G→A	None	-	In this study
657	421	T→C	None	-	Migaud *et al.* (2002), Chu *et al.* (2007) and Carcangiu *et al.* (2009)
660	424	C→T	None	MboII	Migaud *et al.* (2002), Chu *et al.* (2007) and Carcangiu *et al.* (2009)
729	493	G→A	None	-	In this study
813	577	C→T	None	Tsp509I	Migaud *et al.* (2002) and Carcangiu *et al.* (2009)
825	589	C→A	None	BfaI	Migaud *et al.* (2002), Chu *et al.* (2007) and Carcangiu *et al.* (2009)
1008	772	T→C	None	MboII	In this study
1117	-	T→A	-	-	In this study
1121	-	C→T	-	-	In this study
1171	-	A→G	-	-	In this study

Table 4: Haplotypes in the *MTNR1A* gene its distribution among the breed

Position reference	c59a (c)	g288a (g)	t390c (t)	c426t (c)	c468t (c)	g469c (g)	g474a (g)	c489t (c)	g513a (g)	g588a (g)	g594t (g)	g645a (g)	t657c (t)	c660t (c)
Hap-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap-2	-	-	-	-	-	-	-	-	-	-	-	a	-	-
Hap-3	-	-	-	-	-	c	-	-	-	-	-	-	-	-
Hap-4	a	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap-5	-	-	-	t	.	-	-	-	-	-	-	-	c	t
Hap-6	-	-	-	t	-	-	-	-	-	-	-	-	c	t
Hap-7	-	-	-	t	-	-	-	-	-	-	-	-	c	t
Hap-8	-	-	-	t	-	-	-	-	-	-	-	-	c	t
Hap-9	-	-	-	t	-	-	-	-	a	-	-	-	c	t
Hap-10	-	-	-	t	-	-	-	-	-	-	-	-	c	-
Hap-11	-	-	-	t	t	-	-	t	-	-	-	-	-	-
Hap-12	-	a	-	t	t	-	-	t	-	-	t	-	c	-
Hap-13	-	-	c	t	-	-	a	-	a	a	-	-	c	-

Position reference	g729a (g)	c813t (c)	c825a (c)	t1008c (t)	t1117a (a)	c1121t (c)	a1171g (a)	Saanen	Shiba	No. of chromosomes Boar	Bangladesh	Mongol	Korea
Hap-1	-	-	-	-	-	-	-	6	-	3	3	1	-
Hap-2	-	-	-	-	-	-	-	3	-	-	-	-	-
Hap-3	-	-	-	-	-	-	-	1	-	-	-	-	-
Hap-4	-	-	-	-	-	-	-	12	-	-	-	-	-
Hap-5	-	-	a	-	t	-	-	5	11	6	8	5	-
Hap-6	-	-	-	-	t	-	-	-	-	4	-	-	-
Hap-7	-	-	-	-	-	-	-	-	-	-	1	9	24
Hap-8	-	t	-	-	-	-	-	-	-	-	1	1	-
Hap-9	-	t	-	-	-	-	-	-	15	-	-	-	-
Hap-10	a	-	-	-	t	-	-	-	-	9	2	-	-
Hap-11	-	-		-	t	-	-	-	-	1	-	-	-
Hap-12	-	-	-	-	t	-	-	-	-	1	-	3	-
Hap-13	-	-	-	c	-	t	g	3	-	-	9	5	-

MnlI site variation was detected in the study. Similarly, 9 of the other SNPs examined with restriction enzymes were completely consistent with earlier reported genotypes.

Haplotype analysis: Analysis of DNA from 76 animals for 21 SNPs including 2 non-synonymous substitutions resulted in 13 haplotypes (Hap_1 to Hap_13).

Table 4 shows haplotypes compared to reference sequences including the haplotype-a which was reported as a partial sequence (Migaud *et al.*, 2002). Four groups of SNPs were found to be in complete linkage disequilibrium in the samples (g288a and g594t, c426t and t657c, c232 and c489t and t390c, g474a, g588a, t1008c, c1121t and a1171g). Haplotype distribution among breeds is showed in Table 4. The Japanese Saanen, Shiba and Boer goats bred specific haplotypes in the study, Hap_2, 3 and 4, Hap_9 and Hap_6 and 11, respectively. In this study, distribution analysis of the 13 haplotypes showed

that 12 types were observed in more than two animals and/or breeds while Hap_11 was found only in one animal in the study (Boer breed). Japanese Saanen showed 6 haplotypes with Hap_4 showing as the major type which was until this study, the only haplotype observed in this breed. Shiba showed 2 haplotypes, Hap_5 and 8, though Hap_8 was mainly observed in this breed. Boer showed 6 haplotypes with Hap_9 as the major type observed. Bangladeshi native had 6 haplotypes with Hap_5 and 13 as the major types. The Mongolian native had 6 haplotypes and Hap_7 was the major type. Korean native had one haplotype, Hap_7 which was observed in all native populations examined in this study.

Nucleotide and amino acid sequences of the goat *MTNR1A* gene and homologies with other mammals: Goat *MTNR1A* gene coding regions, exon 1 and exon 2, similarly found in the human and bovine genome were successfully sequenced. Exon 1, a 380 nucleotide sequence including flanking regions and primer sequences was thoroughly examined in this study. The exon 1 reference sequence was renumbered from a new start codon. Exon 1 was difficult to amplify as the 4 forward primers and 4 reverse primers designed for the region were based on sheep and bovine sequences. However, the amplification was successful when using a high performance Taq DNA polymerase, KOD FX polymerase. The exon 2 sequence is 1118 nucleotides long including flanking nucleotides and primers and the genomic reference sequence includes a partial sequence previously reported (Migaud *et al*., 2002). The results indicate that the entire goat MTNR1A coding region consists of 1101 nucleotides and 366 deduced amino acids.

As sequences for all putative goat MTNR1A coding regions were determined in this study, researchers could therefore compare the full length of the goat amino acid sequence to other mammals. Nucleotide sequence and deduced amino acid sequence homologies for the *MTNR1A* gene between goat and other mammals were between 77.1-99.6 and 83.0-98.6%, respectively (Table 2). The sheep nucleotide and amino acid sequences were highest (98.5 and 98.6%, respectively). Nucleotide sequence similarities with other mammals following sheep, decreased in order of bovine, horse, human and mouse. These results were also found in part in a earlier report (Migaud *et al*., 2002). Human and mouse sequences revealed low similarities for both nucleotide and amino acid sequences in comparison to the domestic farm animals.

Nucleotide and amino acids sequence differences in goat breeds: Comparison among the goat breeds and populations in this study revealed the presence of at least 21 sequence differences for the goat *MTNR1A* gene. Sequence differences consisted of 18 nucleotide substitutions in the exon 1 and 2 coding regions (c59a, g288a, t390c, c426t, c468t, g469c, g474a, c489t, g513a, g588a, g594t, g645a, t657c, c660t, c729a, c813t, c825a and t1008c) and 3 changes were found in the 3 non-coding region (t1117a, c1121t and a1171g). Eleven novel SNPs were found in the samples adding to the 10 SNPs earlier reported although, 2 SNPs (g307t and t559c earlier called) were not observed in the samples (Migaud *et al*., 2002; Chu *et al*., 2007; Carcangiu *et al*., 2009).

Comparison between coding sequences revealed two amino acid substitutions: Non-synonymous SNPs at position 59 in exon 1, changing alanine to glutamine and at position 469 in exon 2, changing glycine to arginine. These non-synonymous SNPs were detected only in the Japanese Saanen breed. The different reproductive activity between breeds also provided an opportunity to examine what affect the *MTNR1A* gene may have on seasonal breeding or reproduction patterns. Shiba doe are aseasonal and Japanese Saanen and Boer seasonally breed. Examining these three breeds researchers found only two candidate SNPs, g513a and c813t, out of 21 SNPs which may be associated with reproductive status. These 2 SNPs are non-synonymous substitutions however, gene expression and protein function are not affected so, it is unlikely these SNPs have some affected on seasonal breeding. If an allele with non-synonymous SNPs is associated with high or low gene expression in the Shiba breed their aseasonal breeding pattern may be affected via an additional mechanism. Esposito *et al*. (2011) report a piwi-interacting RNA (piRNA), located in the intron 1 of the human *MTNR1A* gene which negatively regulates gene expression by binding to the MTNR1A genomic region. It is possible that an SNP may have some causative affect with regards to the goat MTNR1A promoter or enhancer region.

This study genotyped the goat *MTNR1A* gene from different breeds to determine whether the gene is associated with seasonal reproduction. Researchers were able to analyze 10 out of 21 SNPs using PCR-RFLP (Table 2). Resarcher located a number of enzyme restriction sites in the *MTNR1A* gene sequence. Only one of these sites had earlier been examined, characterized by a low frequency polymorphism (Chu *et al*., 2007; Carcangiu *et al*., 2009). The r allele was widely distributed in Chinese native breeds (Chu *et al*., 2007). Following

PCR-RFLP, g288a (earlier called g52a) confirmed the presence or absence of the RsaI restriction site. The present study showed that the R/r genotype was observed in Boer and Mongolian native breeds while the r/r genotype was absent in any breed. No r allele was detected by PCR-RFLP analysis in any of the 76 Japanese Saanen or 9 Shiba goats. It is unlikely that seasonal breeding in the Shiba goats is caused even in part by the r allele. Pelletier *et al.* (2000) found new alleles for the ovine *MTNR1A* gene which is characterized by the absence of an MnlI restriction site at position 605 of the coding sequence. No MnlI site variation has been detected previously or in this study (Migaud *et al.*, 2002; Chu *et al.*, 2007; Carcangiu *et al.*, 2009).

Therefore, the goat *MTNR1A* gene may not affect seasonal breeding which is no different to what has been reported for sheep. Nine of the 21 SNPs, examined with a suitable restriction enzyme were found to be completely consistent with genotypes sequenced in this study. In the European breeds, a SNP at position t657c (earlier called t421c) was characterized as one of 27 SNPs analyzed as part of a biodiversity assessment using TaqMan technology (Cappuccio *et al.*, 2006; Pariset *et al.*, 2009). The alleles at the SNP t421c are widely distributed across the breeds examined in this study and in other studies. The results provide valuable information on polymorphisms in the goat *MTNR1A* gene and specific genotypes for each breed may be used for further breed characterization.

Haplotype analysis: DNA analysis from 76 animals for 21 SNPs including 2 non-synonymous substitutions, resulted in the discovery of 13 haplotypes (Hap_1 to Hap_13) which includes a partially sequenced haplotype, earlier reported as haplotype-a by Migaud *et al.* (2002). The results include finding complete linkage disequilibrium which was also found in earlier reports (Migaud *et al.*, 2002; Carcangiu *et al.*, 2009). Migaud *et al.* (2002) reported that Alpine and Creole breeds have 6 haplotypes and 7 SNPs. Hap_1 is identical to haplotype-a as Hap_8 is to haplotype-c and Hap_5 is to haplotype-d. The r allele is found in Hap_12 which was determined using the RsaI enzyme. Nucleotide changes at c468t, c489t and g594t are only present in R/r goats (Carcangiu *et al.*, 2009). No evidence of association between haplotype and seasonal breeding was found that may scientifically explain breeding status.

All Korean native goats have haplotype, Hap_7 and Shiba goats have two types, Hap_5 and Hap_9. Since, these goats were maintained as a closed population for long time, researchers presumed these results would reveal limited genomic diversities. Bangladeshi and Mongolian native goats reveal relatively high diversities based on their haplotype distribution. The Japanese Saanen, Shiba and Boer breeds showed breed specific haplotypes in the study.

CONCLUSION

These results provide valuable information on polymorphisms for further association studies with regard to specific goat genotypes and breed characterization.

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