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Association between AA-NAT gene polymorphism and reproductive performance in sheep

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Abstract Arylalkylamine N-acetyltransferase (AA-NAT) is critical enzyme in Melatonin (MLT) biosynthesis for MLT regulating the animal seasonal breeding. In this study, DNA sequencing methods were applied to detect the polymorphisms of the AA-NAT gene in 179 Chinese sheep belonging to two non-seasonal reproduction breeds and two seasonal reproduction breeds. One mutation at exon 3 (NM_001009461:c.486A > G) was firstly described at the sheep AA-NAT locus. Hence, we described the *Sma*I PCR-RFLP method for detecting EX3 486A > G mutation, frequencies of the AA-NAT-G allele varied from 0.871 to 0.908 in two non-seasonal reproduction breeds and 0.517 to 0.578 in two seasonal reproduction breeds. The associations of *Sma*I polymorphism with estrus traits was analyzed in non-seasonal reproduction breeds sheep and seasonal reproduction breeds sheep, the significant statistical results were found between them, the GG genotype frequencies was higher in non-seasonal reproduction breeds ($p < 0.001$), while, the GA genotype frequencies was higher in seasonal reproduction breeds ($p < 0.05$). Hence, the EX3 486A > G mutation could facilitate association analysis and serve as a genetic marker for Chinese sheep breeding and genetics.

Keywords: AA-NAT gene, polymorphism, reproduction, seasonality, sheep

INTRODUCTION

Breeding non-seasonal reproduction sheep is a major goal to increasing the intensity of sheep production, because mutton and wool is an important economic staple to fulfill consumer demands throughout the year. Therefore, there is a need for reliable method that can be used as selection tools in non-seasonal reproduction sheep breeding schemes. An effective approach is establishing the genetic basis on non-seasonal reproduction breed for improving production in sheep. There is a variety of genes affecting non-seasonal reproduction traits in sheep have been successfully identified such as the melatonin 1A receptor (Chu et al. 2003; Hernandez et al. 2005; Faigl et al. 2008; Mateescu et al. 2009; Carcangiu et al. 2011) and some clock genes that may be involved in control of seasonal breeding (Notter and Cockett, 2005). However, there were also failed to detect any association between these gene and reproductive seasonality in some ewes (Hernandez et al. 2005). Therefore, the discovery of other genes with non-seasonal reproduction is necessary in sheep. The Melatonin is produced in the pineal gland, which is involved an important role in several physiological and pathological processes (Reiter et al. 1995), and its oscillatory production synchronizes in seasonally estrus responsive animals such as sheep and hamsters (Wayne et al. 1988). While the production of melatonin was determined by arylalkylamine-N-acetyl-transferase (AA-NAT) activity (Klein and Berg, 1970). Therefore, it has been suggested that genetic mutation of the AA-NAT gene may potentially influence seasonally estrus responsive in the sheep population.

The present study investigates the association between AA-NAT polymorphisms and reproduction in 179 Chinese sheep belonging to two non-seasonal reproduction breeds (Chu et al. 2006; Liu et al. 2006) and two seasonal reproduction breeds (Yu, 1988) which managed under the same feeding conditions. In the exon 3 region, a mutation (NM_001009461:c.486A > G) single nucleotide polymorphism (SNP) was firstly described at the sheep AA-NAT locus. The EX3 486A > G SNP may therefore be an important determinant of the non-seasonal reproduction in sheep.

MATERIALS AND METHODS

DNA samples

Genomic DNA samples were obtained from 179 female ewes belonging to four sheep breeds: Xinjiang Fine Wool sheep (XF, 58), Small Tail Han sheep (ST, 60), Altay Fat-rumped sheep (AF, 30) and Dolang sheep (DL, 31), which were reared in Xinjiang province (P.R. China). DNA extraction from blood samples was conducted as is recommended by the E.Z.N.A®. Blood Genomic DNA Isolation System (Omega, Inc.).

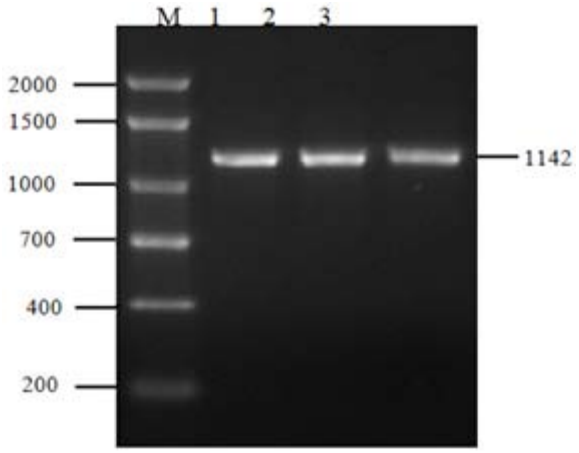

Fig. 1 Analysis on sheep AA-NAT gene PCR production by gel electrophoresis M: Marker; 1, 2, 3: PCR production.

PCR conditions

Since there is expected to be high homology between ovine and bovine AA-NAT gene, according to Coon et al. (1995) reported that ovine AA-NAT gene cDNA sequence (GeneBank accession number: NM_001009461) were compared to the orthologous gene sequences from the bovine (GeneBank accession number: NW_177509.2), one pair of primers were designed to amplify the exon 1 and exon 3 of AA-NAT gene. The sequences of these primers were: forward (F) 5'-AGCGTCCACT GCCTGAAAC-3' and reverse (R) 5'-GGGATGGAAG CCAAACCTC-3'. The size of the PCR products was 1142 bp. PCR was performed in a 25 µL reaction volume containing: 80 ng genomic DNA, 0.5 µM of each primer, 1 x buffer (including 1.5 mM $MgCl_2$), 200 µM dNTPs (dATP, dTTP, dCTP and dGTP) and 0.3 units of *Taq* DNA polymerase (TaKaRa). The cycling protocol was 5 min at 95ºC, 34 cycles of denaturing at 95ºC for 40 sec, annealing at 60ºC for 40 sec, extending at 72ºC for 40 sec, with a final extension at 72ºC for 10 min.

Five samples from each breed were randomly selected for PCR amplification. The PCR products were purified for sequencing.

Restriction Fragment Length Polymorphism (RFLP) and detection

The amplified fragment of AA-NAT was digested with *Sma*I (TaKaRa). 5 µl of PCR production with 1 µl 10 x buffer buffer, 1 µl (5U) of *SmaI* and 3 µl ddH_2O up to a total volume of 10 µl, following the manufacturers instruction for 10 hrs at 37ºC. The digestion products were electrophoresed in 2% agarose gel in 1 x TBE and detected by eithdium bromide staining for 1 hr at 85 V.

Statistical analysis

Estimates genotype, alleles frequencies, heterozygosity (He) and polymorphism information content (PIC) were directly calculated, and Hardy-Weinberg equilibrium for each population was analyzed using x^2-test (Cubells et al. 1997), which was performed by SPSS software (version 16.0).

RESULTS

AA-NAT gene PCR amplification

The PCR amplified with 1142 bp sequences obtained were compared against database sequences using BLAST provided by NCBI, and confirmed the amplified sequences were AA-NAT sequences (Figure 1). The amplified AA-NAT gene including the sequences of concluded part of exon1 (152 bp), intron 1 (290 bp), whole exon 2 (155 bp), intron 2 (338 bp), part of exon 3 (207 bp).

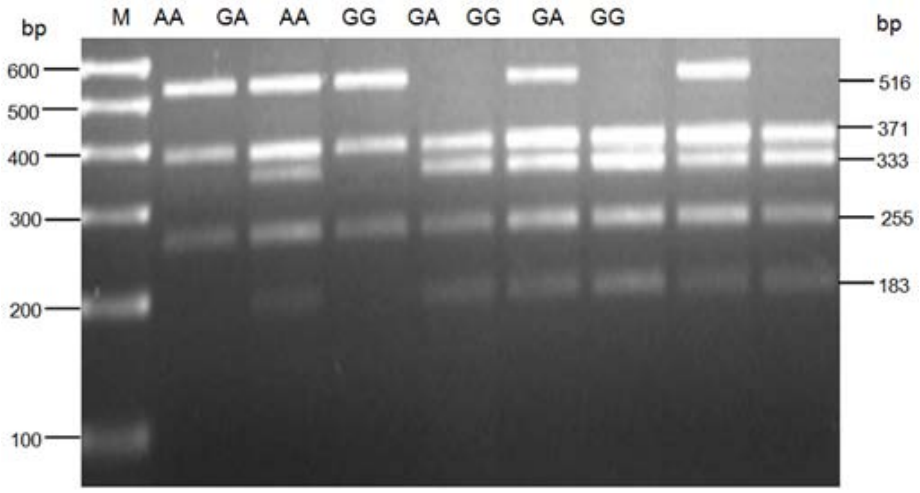

Fig. 2 DNA electrophoretic patterns of PCR products including exon 3 of the sheep AA-NAT gene digested by *Sma*I endonuclease. Lane M: Marker (600, 500, 400, 300, 200, and 100 bp). Lane GA: GA genotype. Lane GG: GG genotype. Lane AA: AA genotype.

PCR-RFLP analysis of AA-NAT gene

In order to explore more genetic variations of the AA-NAT gene, the mixture of the PCR products was sequenced. According to NM_001009461 (ovine AA-NAT gene, mRNA sequence), comparisons between amplified AA-NAT gene and NM_001009461, the NM_001009461:c.486A > G mutation at exon 3 of the AA-NAT locus identified a novel single nucleotide polymorphism (SNP). The novel NM_001009461:c.486A > G mutation could be detected by *Sma* I endonuclease (there were also *Sma* Ienzyme restriction sites in 368 bp and 623 bp as known in our amplified sequences); the allele characterized by the presence of G was named AA-NAT-G, and the alternative allele A was called AA-NAT-A. Therefore, the amplified DNA fragment with this endonuclease digestion shows four fragments (183 bp , 255 bp, 333 bp and 371 bp) for the AA-NAT-G allele, three fragment (255 bp, 371 bp and 516

bp) for the AA-NAT-A allele. Correspondingly, GG, GA, and AA genotypes have four bands (183 bp, 255 bp, 333 bp and 371 bp), five bands (183 bp, 255 bp, 333 bp, 371 bp and 516 bp), and three bands (255 bp, 371 bp and 516 bp), respectively (Figure 2). The genotype frequencies, He, and the Hardy-Weinberg equilibrium x^2-test were shown in Table 1, the frequencies of the genotypes and alleles were shown in Figure 3 and Figure 4.

Table 1. Genetic structure of *Sma*I polymorphism in exon 3 at the sheep AA-NAT locusfor different breeds.

Breeds	Observed genotypes			Genotype frequencies			Allele frequencies		He	PIC	Equilibrium x^2-test
	GG	GA	AA	GG	GA	AA	G	A			
ST	49	11	0	0.817	0.183	0	0.908	0.092	0.167	0.153	2.107
XF	19	29	10	0.328	0.500	0.172	0.578	0.422	0.489	0.369	0.035
AL	9	13	8	0.300	0.433	0.267	0.57	0.483	0.499	0.375	0.526
DL	23	8	0	0.742	0.258	0	0.871	0.129	0.225	0.120	2.165

Notes: ST: Small Tail Han sheep; XF: Xinjiang Fine-wool sheep: AL: Altay Fat-rumped sheep; DL: Dolang sheep.

Genotypic frequencies for the various genotypes at the NM_001009461:c.486A > G locus were found to be significantly different among four Chinese sheep breeds, GG and GA genotypes were found in the four sheep breeds, AA genotypes were found in Xinjiang Fine-wool sheep and Altay Fat-rumped sheep. However, AA genotypes were not appearance in Small Tail Han sheep and Dolang sheep. Moreover, significant differences among allelic frequencies of four Chinese sheep breeds were also found, the frequency of the G allele in the Small Tail Han sheep and Dolang sheep was higher when compared to those of the Xinjiang Fine-wool sheep and Altay Fat-rumped sheep ($p < 0.05$). The NM_001009461:c.486A > G locus were in Hardy-Weinberg equilibrium in four Chinese sheep breeds, respectively ($p > 0.05$).

The associations between *Sma*I polymorphism with non-seasonal reproduction and seasonal reproduction traits were analyzed in four Chinese sheep breeds (n = 179) (Table 2). The statistical results showed significant relationships between genotypes (GG and GA) and seasonality, the GG genotype frequencies was the highest when compared to the GA genotype frequencies in non-seasonal reproduction breeds ($p < 0.001$), while, the GA genotype frequencies was higher in seasonal reproduction breeds ($p < 0.05$).

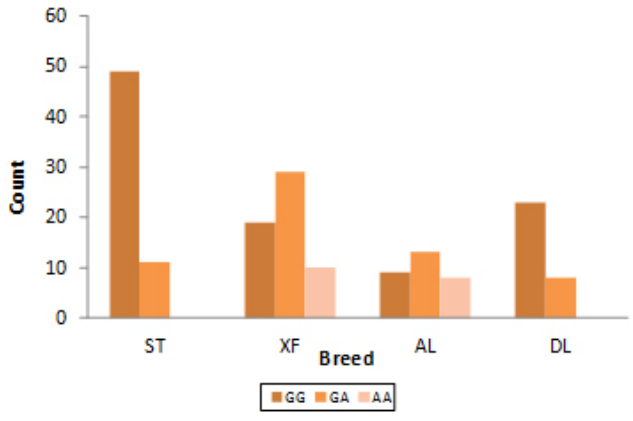

Fig. 3 Genotypic distribution of *Sma*I locus (NM_001009461:c.956A > G) within the ovine AA-NAT gene in four Chinese sheep breeds. ST: Small Tail Han sheep; XF: Xinjiang Fine-wool sheep; AL: Altay Fat-rumped sheep; DL: Dolang sheep. ‘‘Count’’ means the genotype number.

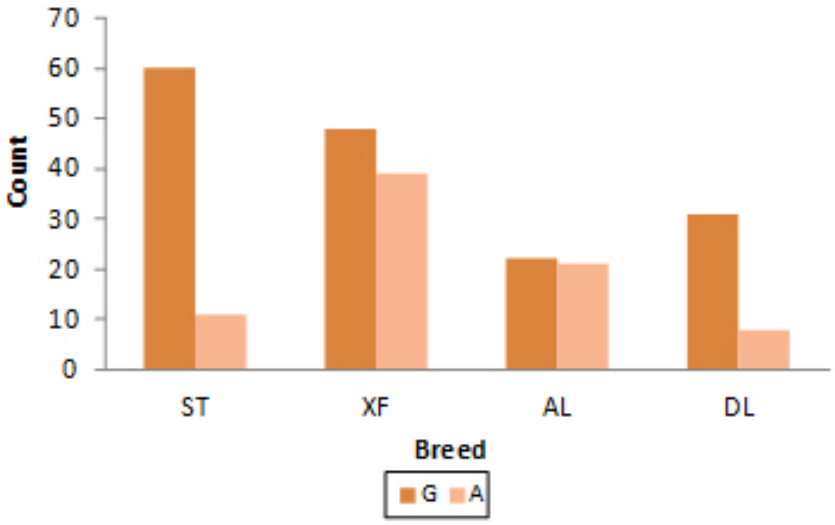

Fig. 4 Allelic distribution of *Sma*I locus (NM_001009461:c.956A > G) within the ovine AA-NAT gene in four Chinese sheep breeds. ST: Small Tail Han sheep; XF: Xinjiang Finewool sheep; AL: Altay Fat-rumped sheep; DL: Dolang sheep. ''Count'' means the genotype number.

Table 2. Association of *Sma*I polymorphism with non-seasonal reproduction and seasonal reproduction traits in exon 3 of the AA-NAT locus in four Chinese sheep breeds.

		Genotype frequencies		
		GG	**GA**	**P value**
Non-seasonal reproduction breeds	ST	0.817	0.183	< 0.001
	DL	0.742	0.258	< 0.001
Seasonal reproduction breeds	XF	0.328	0.500	< 0.05
	AL	0.300	0.433	< 0.05

Notes: ST: Small Tail Han sheep; XF: Xinjiang Fine-wool sheep: AL: Altay Fat-rumped sheep; DL: Dolang sheep.

DISCUSSION

AA-NAT is a key rhythm-generating enzyme of the melatonin synthesis in the pineal gland (Chattoraj et al. 2009). Genetic basis related to polymorphisms in the gene of AA-NAT have been reported more focus on major depression (Soria et al. 2010), melatonin production (Ying et al. 2004), sleep pattern (Wang et al. 2004), delayed sleep phase syndrome (Hohjoh et al. 2003; Pereira et al. 2007), and adolescent idiopathic scoliosis (Wang et al. 2008) in human, but little was known about the polymorphisms association with useasonal and seasonal reproduction in sheep.

The polymorphisms evaluated in our study found that a novel SNP (NM_001009461:c.486A > G) in the exon 3 region of the AA-NAT ovine gene in 179 Chinese sheep belonging to two useasonal reproduction breeds and two seasonal reproduction by PCR-RFLP and DNA sequencing methods. The present study revealed that polymorphisms of the AA-NAT gene are significantly associated with useasonal and seasonal reproduction in four Chinese sheep breeds. The results of this study suggest that the genotype GG might be associated with superior useasonal reproduction, while the genotype GA might be associated with better seasonal reproduction. The novel 486 A > G mutation in exon 3 lead to the changes of amino acids (Arg > Gly), the change in which the α-helical content decreases

while the amount of β-sheets increases dramatically, which probably has effects on AA-NAT protein structure, which exerts better biological function and might affect the useasonal reproduction of sheep, but further verification is needed. In this study, our results suggest that GG genotype had noteworthy useasonal reproduction, and some of those with better performance could be used for the breeding of new useasonal reproduction breeds of sheep. Furthermore, the result could facilitate association analysis and serve as a genetic marker for Chinese sheep breeding and genetics.

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