Short Paper

Novel polymorphism of *AA-NAT* gene in Indian goat breeds differing in reproductive traits

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Summary

This is the first description of the polymorphisms of arylalkylamine-N-acetyltransferase (*AA-NAT*) gene in Indian goats with different reproductive traits (twinning percentage and age of sexual maturity). Based on the important role of *AA-NAT* in reproduction, it is considered as a possible candidate gene for this trait. Two novel synonymous SNPs, C825T (exon2) and C1249T (exon3) were identified. All three possible genotypes (CC, CT and TT) were identified for C825T mutation whereas two genotypes were observed (CC and CT) for C1249T mutation. SNPs C825T and C1249T changed recognition site of restriction enzyme *BtsCI* (GGA***T***G) and *AciI* (C***C***GC) and thus can be genotyped by the relatively simple and cost effective technique of PCR-RFLP for establishing further association with reproductive traits. Present results add to the meager existing knowledge and extend the spectrum of genetic variation of caprine candidate genes of reproductive traits, which is another step towards improvement of goat genetic resources and breeding.

Key words: Arylalkylamine-N-acetyltransferase (*AA-NAT*) gene, Goat, Polymorphism

Introduction

Arylalkylamine-N-acetyltransferase (*AA-NAT*) is a key enzyme associated with melatonin (MLT) biosynthesis. *AA-NAT* is part of the large Gcn5-related acetyltransferase (G-NAT) superfamily (Dyda *et al*., 2000). MLT plays a key role in regulation of the reproductive system of seasonal estrous animals. In ewes, MLT can induce estrous cycle, increase the ovulation rate (Zuniga *et al*., 2002) and litter size (Scott *et al*., 2009), enhance luteal function, improve embryo viability and enhance ovarian response to the ram effect (Abecia *et al*., 2008). In rams, MLT can increase percentage of progressive motile spermatozoa and number of spermatozoa attaching oocytes (Casao *et al*., 2010). Therefore, being the rate-limiting enzyme in MLT biosynthesis, *AA-NAT* is critical for animal reproductive system. Human *AA-NAT* gene is 2.5 kb in length, maps to chromosome 17q25 and has four exons (Steven *et al*., 1996), of which the exon1 remains untranslated, while the other three (238, 155 and 453 bp) code for a 207-amino acid protein. Chu *et al*. (2013) reported the associations between polymorphism of *AA-NAT* gene and litter size for the first time in high-prolificacy Jining Grey goat. More than 20 breeds of goat have been reported in India with wider phenotypic variations and adaptations to different agro-climatic conditions. Differences in prolificacy and sexual maturity have also been recorded (Acharya, 1982). There are breeds such as Black Bengal, displaying significant characteristics of early reproductive maturity and high prolificacy, whereas breeds like Sirohi are late maturing with lower prolificacy.

In view of its biological role, *AA-NAT* is a candidate gene for reproductive traits. Therefore, the objectives of the present study were, firstly, to obtain the status of partial *AA-NAT* gene (exon2 and 3) of Indian goats by generating nucleotide sequence and sequence assembly in a panel of goat breeds differing in reproductive traits and secondly, to identify intra-species polymorphisms for assessment of variability at molecular level.

Materials and Methods

Animal selection, sample collection and genomic DNA isolation

Nine well-recognized breeds with different prolificacy rate (number of kids per kidding) and age of sexual maturity from different geographic regions of India were selected (Table 1). Five unrelated animals of each breed were selected from their breeding tracts. Blood was collected aseptically from the jugular vein in a vacutainer tube containing EDTA and genomic DNA was extracted following phenol-chloroform protocol (Sambrook and Fristch, 1989).

PCR amplification, sequencing and polymorphism detection

Two pairs of primers reported by Chu *et al*. (2013) were utilized for amplification of exon2 and 3 of *AA-*

NAT gene (Table 2). The PCR was carried out in 25 µL reaction volume with about 50-100 ng genomic DNA. The reaction mixture consisted of 250 µM of each dATP, dCTP, dGTP, dTTP, 2.0 mM $MgCl_2$, 50 pmol of each primer, 1 U *Taq* polymerase and corresponding *Taq* buffer. The amplification conditions were: initial denaturation for 3 min at 95°C; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 1 min; and finally extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 1.8% agarose gel in parallel with a 50 bp DNA ladder, enzymatically purified and sequenced using both primers (forward and reverse) by the dideoxynucleotide chain termination reaction (Sanger *et al*., 1977). Sequencing was performed in an automated ABI -3100 sequencer (applied Biosystems) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (applied Biosystems).

Sequence data were edited manually using Chromas Ver. 2.33, (http://www.technelysium.com.au/chromas.html). Multiple sequence alignments were performed with MegAlign program of LASERGENE software version 5.07 (DNASTAR Inc., Madison, WI) to identify polymorphisms (mutations or single nucleotide polymorphisms). The coding DNA sequence was conceptually translated to amino acid sequences using ChromasPro software. Nucleotide BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) was used for sequence homology searches in public databases.

Results

The two primer pairs amplified specific regions of the *AA-NAT* gene, with fragment sizes of 163 bp (primer pair AA2) and 175 bp (primer pair AA3), respectively. The amplified fragments of 45 goats in nine different breeds were used for identifying polymorphisms within Indian goats. Since Caprine gene sequence is not yet available, sheep *AA-NAT* gene sequence (GenBank accession No. JX444551.1) was used for comparing the sequence information obtained by sequencing of the PCR fragments (AA2 and AA3) corresponding to expressive regions of *AA-NAT* gene in Indian goat.

AA-NAT sequences of sheep and Indian goat exhibited high similarity (98.5%) with only five variations (Table 3). Majority of nucleotide substitutions were transition changes (80%). Since nucleotide variations were observed in coding region it was interesting to see if they caused any amino acid changes in the corresponding protein. However all substitutions were synonymous and hence no change is expected in the amino acid sequence of the translated protein among sheep and goats. Nine goat breeds of India were explored for prospecting of SNPs in *AA-NAT* gene, as diverse animals can increase the chances of SNP discovery. Two SNPs (C/T) were identified, one each in the region amplified with primer pair AA2 and AA3. Mutation was identified in exon2 at position 825 bp (Fig. 1) and in exon3 at position 1249 bp (Fig. 2) (GenBank accession

Table 1: Distribution and physical characteristics of Indian goat breeds selected for characterization of *AA-NAT* gene

Breed	Geographical distribution	Sexual maturity/age at puberty (in months)	Prolificacy/twinning percentage	Reference
Beetal	Punjab	Medium/11	High/ >50	
Barbari	Uttar Pradesh	Medium-late/11-17	High/ >50	
Black-Bengal	West Bengal, Bihar, Jharkhand	Early/6-8	High/ >50	Acharya, 1982
Malabari	Kerala	Early/8-10	High/ >50	
Osmanabadi	Maharashtra	Medium/11	Medium/ >25	
Sangamneri	Maharashtra	Medium/10	Medium/ >25	
Jakhrana	Rajasthan	Medium/ <12	Medium/ >25	
Ganjam	Orissa	Late/ >15	Low/ <25	
Sirohi	Rajasthan	Late/12-18	Low/ <25	

Table 2: Characteristics of primers used for amplification of the *AA-NAT* gene

Primer	Primer sequence (5′→3′)	Amplicon size (bp)	Nucleotide position* (amplified region)	Annealing temperature
AA2	F: ATGTCCACGCCGAGCATCCACT R: CCTCTCGCTCAATCTCAAACACG	163	694-856 bp (exon2)	59°C
AA3	F: ATCAAACTGAACAGGGCAGA R: AAGTATGACAAGAGATACGGTCAGG	175	1148-1302 bp (exon3)	59°C

* The number corresponds to sequence of sheep *AA-NAT* gene (GenBank accession No. JX444551.1)

Table 3: Location of nucleotide substitutions and SNPs identified in goat *AA-NAT* gene

Primer	Nucleotide position (sheep, JX444551.1)	Sheep (JX444551.1)	Indian goat	Type of change	Genotype frequency	Allele frequency
AA2	792	T	C	Transition		
	822	G	C	Transversion		
	825	C	C/T	Transition (SNP)	CC=0.24 CT=0.50 TT=0.26	C=0.48 T=0.52
AA3	1249	C	C/T	Transition (SNP)	CC=0.76 CT=0.24	C=0.88 T=0.12
	1284	T	C	Transition		

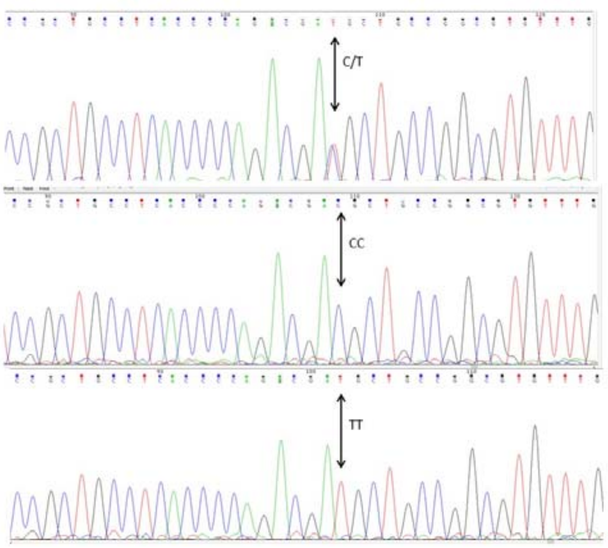

Fig. 1: Sequencing chromatogram of different genotypes at SNP C825T identified in Indian goats (arrow pointed to the mutation site)

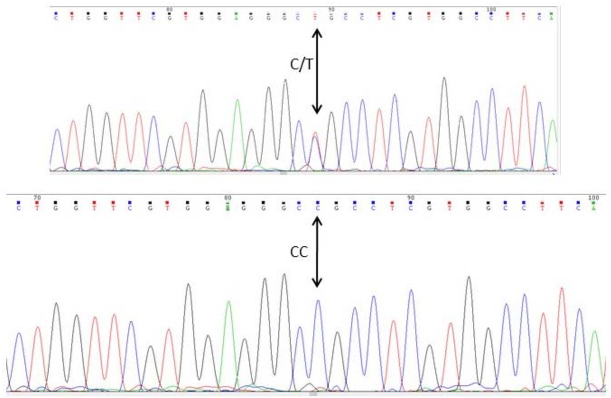

Fig. 2: Sequencing chromatogram of different genotypes at SNP C1249T identified in Indian goats (arrow pointed to the mutation site)

No. JX444551.1) and is being reported for the first time in the caprine *AA-NAT* gene.

Genotype distribution of the mutation did not show obvious difference between sexual precocious and sexual late-maturing goat breeds and there was no consistency with the high or low prolificacy. However, Black Bengal goat was the only monomorphic breed with only one genotype at both the loci, C825T (CT) and C1249T (CC).

Discussion

AA-NAT gene has been reported to have large number of polymorphisms. More than 130 SNPs have been published in NCBI databases including more than twenty in humans and ten in *Bostaurus*. Sekine *et al*. (2001) found initially four SNPs in Japanese people: two mutations (G-542T, C-263G) in the 5' flanking region, one in intron3 (T39A), and one silent mutation in exon4 (C150T). Hohjoh *et al*. (2003) detected another three mutations in exon4 of *AA-NAT* gene in Japanese individuals including G619A, C702T and C756T, of which the former caused an alanine to threonine change at position 129 (Ala129Thr). Ciarleglio *et al*. (2008) identified a C/G mutation in human subjects from various global populations. Four mutations were detected in ovine *AA-NAT* gene (Yu, 2007) including one in exon3 and three in 3′ flanking region. Chu *et al*. (2013) have reported three mutations in goat with one each in exon2, 3 and 4.

However, to date, the association between polymorphism of *AA-NAT* and reproduction in animals remains limited. Jining Grey goat of China has been reported to have a SNP (C265T) that is associated with increased litter size. The goats with genotype CD deliver 0.56 ($P<0.05$) more kids than those of genotype CC (Chu *et al*., 2013). This locus is fixed in Indian goats, though two novel mutations have been identified. All three possible genotypes were observed at C825T locus (Fig. 1) whereas two possible genotypes (CC and CT) were recognized at C1249T locus (Fig. 2). Mutant allele (T) was predominant at C825T locus with allele frequency of 0.52 whereas wild allele (C) was more frequent at C1249T locus having allele frequency of 0.88. Minor homozygote for the mutation in exon3 (C1249T) was not observed in the present investigation which may be due to the fact that minor allele for the loci is rare in Indian goats or its genotype frequency is low and hence could not be detected in current sample size.

Thus, further replication with the bigger sample size is required to establish the allelic frequency in the goat population. As a first step towards realizing this target, we identified the restriction enzyme site which recognizes the novel SNPs in *AA-NAT* gene by using NEB cutter V2.0 (Vincze *et al*., 2003). Both SNPs, C825T and C1249T can be genotyped by restriction fragment length polymorphism (RFLP), as nucleotide variation changes the recognition site of restriction endonuclease *BtsCI* (GGA***T***G) and *AciI* (C***C***GC), respectively. Simple PCR-RFLP based method can be utilized by researchers to associate novel SNPs noticed in present study with the reproductive traits, which may lead to the identification of markers for caprine fecundity and sexual precocity.

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