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Polymorphism of Exon V of prolactin gene and its association with Cashmere traits in Changthangi Pashmina goats

S SHANAZ[1], T A S GANAI[2], A A KHAN[3], F D SHEIKH[4], A H SOFI[5] and S T SALAAM[6]

SKUAST of Kashmir, Srinagar, Jammu and Kashmir 192 001 India

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ABSTRACT

Prolactin is one of the potential genes which mediates seasonal cues entraining reproductive and hair follicle growth cycles. Seasonal changes in levels of prolactin hormone mediate the patterns of de-iodinase enzyme activity which influence the follicular activity and thereby may affect Cashmere fibre growth and moult. To study polymorphism of the prolactin gene and associate it with fibre production and quality, Changthangi goat 196 bp fragment of prolactin gene Exon-V region was amplified and restriction digested with *ECO 241(BanII)* and in the study three genotypes were observed. These different genotypes were confirmed by DNA sequencing. The frequencies for the observed genotypes A_1A_1, A_2A_2, A_1A_2 were 0.214, 0.291 and 0.495 respectively. The distribution of the genotypes within the Changthangi goat population studied herein did not deviate from the Hardy Weinberg equilibrium. The mean genetic variability parameters for the gene analyzed were 0.4970 (heterozygosity value), 0.3735 (PIC value), 1.9880 (Effective allele number) and 0.6901 (Shannon index I) . The polymorphism observed in the gene showed a non-significant association ($P>0.05$) with Cashmere quality traits. The results obtained here in, because of low sample size, demand further investigation with sufficiently large number of samples.

Key words: Cashmere trait, Pashmina goat, Polymorphism, Prolactin gene

Prolactin (PRL), also known as luteotropic hormone or luteotropin. Owing to the multiple roles and sources, it was suggested to be renamed as 'Omnipotin' or 'Versatilin' by Bernand Nicoll in 1968. Based on its genetic, structural, binding and functional properties, prolactin belongs to the prolactin/growth hormone/placental lactogen family (group I of the helix bundle protein hormones) (Boulay and Paul 1992, Horseman 1994). Genes encoding prolactin is comprised of exons and introns. Prolactin is a polypeptide hormone mainly synthesized in specialized cells of the anterior pituitary gland, the lactotrophs. It is also secreted in central nervous system, the immune system, the uterus and its associated tissues of conception, and even in the mammary gland. It is influential in over 300 separate biological activities in various vertebrates, including humans (Bole *et al.* 1996) including its role in reproduction other than lactation, hair follicle activation and homeostasis.

Present address: [1]Assistant Professor (sshanaz2007 @gmail.com), Division of Animal Genetics and Breeding; [2]Director-Education (tasganai1@gmail.com), [3] Associate Professor (alamkhan1231@yahoo.co.in), Division of Livestock Production and Management; [5]Assistant Professor (sofihassanasif @yahoo.co.in), Division of Livestock Products Technology, Faculty of Veterinary Sciences and Animal Husbandry. [4]Assistant Professor (aizar22@gmail.com), Regional Research Station Leh. [6]Assistant Professor (sheikhtanveersalam@gmail.com), Department of Zoology, Amar Singh College, Srinagar.

Owing to its diverse activities, it appears in a multiplicity of post translational forms ranging from size variants to chemical modifications such as phosphorylation or glycosylation. Prolactin has also been implicated as the principal endocrine regulator in pelage replacement in a diverse array of mammals (Dicks *et al*. 1994, Pearson *et al*. 1996, Thompson *et al*. 1997). All biological functions associated with Prolactin (PRL) are mediated by its specific receptor which is a single-pass membrane-bound protein that belongs to the cytokine receptor family (Kelly *et al*. 2001, Foitzik *et al*. 2003). Seasonal changes in prolactin concentrations determine patterns of Deiodinase enzyme activity which control the rates of synthesis and degradation of tri-iodo-thyronine (T_3), a hormone implicated in the control of hair follicle activity, and so changes in expression with season or prolactin profiles may affect cashmere fibre growth and moult (Rhind *et al*. 2004). Further, the research has shown that peak levels of plasma prolactin are associated with the initiation of Cashmere growth (Kloren *et al*. 1993).

As seasonal changes in PRL may determine patterns of enzyme activity and may affect cashmere fibre growth and moult (Rhind *et al*. 2004), the *PRL* gene is a potential candidate gene for cashmere traits in marker assisted selection (Lan *et al*. 2009).The present study was conducted on Pashmina producing goats with the objective of validating the polymorphisms, in exon V of the prolactin

gene and associate it with cashmere quality traits, as reported earlier (Rhind *et al*. 2004, Lan *et al*. 2009).

MATERIALS AND METHODS

The experimental material for the present study comprised of 103 Changthangi goats of either sex from Pashmina goat farm Upshi, Leh. The animals were randomly selected on the basis of production records available in the farm. The Pashmina and guard hair samples were collected and analyzed for fibre length, diameter and guard hair percentage. The data for the down fibre production/yield/animal from each group was collected from the farm records.

Blood samples were collected from 103 (41 males and 62 females) Pashmina goats. Genomic DNA was isolated as per Sambrook and Russel (2001) by phenol-chloroform extraction method with some modifications. The genomic DNA was checked for its quality, purity and concentration. DNA samples of good quality, purity and concentration were used for further analysis. Primers as used by Lan *et al*. (2009) were used to amplify 196-bp fragment corresponding to exon-V of the goat PRL gene. The forward and reverse primers used were 5'-ATTCCTGGAGCCAAAGAG-3' and 5'-TGTGGGCTTAGCAGTTGT-3' respectively. The standardized combination of various reaction components for the amplification in 25 µl PCR reaction mix contained 200 µM dNTP mixtures, 10 pmol of each forward and reverse primer, 1.5 mM $MgCl_2$, 2.5 µl 10 × buffer, 0.2 µl *Taq* DNA polymerase and 17.1 µl DEPC treated water. The fragment of 196 bp got amplified.

PCR conditions for amplification of prolactin gene: To optimize the PCR conditions for getting the required products, different annealing temperatures were tried for the set of primers. Initial denaturation was performed at 94°C for 5 min, followed by cyclic denaturation, annealing, and extension at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, respectively. The number of cycles of amplification utilized was 32. Final extension was allowed at 72°C for 7 min. The amplification was weak with some spurious bands when visualized in the ethidium bromide stained 2% (w/v) agarose gel seen under UV light after running it in 0.5× TBE. Therefore, for arriving at an appropriate annealing temperature, a gradient from 56°C to 60°C with an increment of 2°C was carried out while keeping the other reaction conditions unchanged. Good results were achieved at 58°C, however with some smearing. The PCR product of desirable quality was achieved after titrating $MgCl_2$ to find appropriate concentration for amplification. Electrophoresis of the PCR product after this modification was carried out in 2% (w/v) agarose gel prepared in 0.5× TBE. The gel was stained with ethidium bromide and its visualization under UV light and amplification of desirable intensity was obtained. Finally the optimized PCR conditions were arrived at and used to get amplification of desired prolactin gene fragment in goat which included, initial denaturation at 94°C for 5 min, annealing at 58°C for 30 sec, extension at 72°C for 30 sec for 32 cycles and the final extension at 72°C for 7 min.

The specific bands of goat prolactin gene fragment (exon-5) of 196 bp length were visualized after electrophoreses through 2% agarose gels (w/v) along with 100 bp DNA marker ladder (Genei).The amplicons were visualized under UV light after ethidium bromide staining and were documented in gel documentation system.

PCR products of caprine prolactin gene were subjected to restriction enzyme digestion by restriction enzyme *ECO 241 (BanII)*. The restriction site of the enzymes used was 5'-GRGCY$^{\downarrow}$C-3'. The restriction enzyme digestion was carried out at 37°C for 14 h. The reaction mixture comprising 18 µl of DEPC treated water, 2 µl 10× R-buffer Tango, 1 µl restriction enzyme *ECO 241* (10 U/µl) and 10 µl PCR product. Digestion was stopped by heating the mixture at 60°C for 30 min and the samples were stored at 4°C. Restriction enzyme digested products were resolved in 2.5% (w/v) agarose gel in 0.5× TBE, that was visualized under UV light on a trans-illuminator to detect the banding pattern of the RE digested samples.

The sequences of different alleles of different fragments of PRL gene were analyzed using the Clustal W subprogram of MegAlign programme of DNASTAR Software (Lasergene, USA) to generate sequence alignment reports.

Statistical analysis: Different genotypes were scored on the basis of RFLP banding patterns they showed. The frequency of different genotypes and alleles were calculated using the standard procedure given by Falconer and Mackay (1996). The data obtained were used to calculate genetic variability parameters, allele counts and frequencies, expected number of alleles for each locus. The exact test for deviations from Hardy-Weinberg equilibrium (HWE) was also performed using software Popgene version 1.31 (Yeh *et al.*1999). The data on fibre diameter, length and guard hair percentage were subjected to least square analysis using least squares maximum likelihood 91 (LSML 91) programme of Harvey (1990).

RESULTS AND DISCUSSION

The amplification product of 196 bp length of the prolactin gene fragment (exon-5) is depicted in Fig.1. The amplified fragment upon digestion with restriction enzyme *ECO241* (*Ban II*) resulted in a single restriction pattern in the animals studied and that consisted of two fragments of 169 bp and 27 bp (Fig. 2). On analysing all the amplified fragments from 103 animals, three patterns of 196 bp (with no restriction site for the enzyme), 196 -169- 27 bp and 169-27bp (with one restriction site for the enzyme) were observed. The observed genotypes were designated as A_1A_1, A_1A_2 and A_2A_2, for the patterns 196 bp (homozygous), 196 -169- 27 bp (heterozygous) and 169-27 bp (homozygous) patterns, respectively (Fig. 2).

Sequencing of both strands of designated alleles confirmed that goat prolactin gene exon –V has a size of 196 bp and there was neither any deletion nor any insertion with respect to already published report for this exon of prolactin gene in the goats. Comparison of sequence

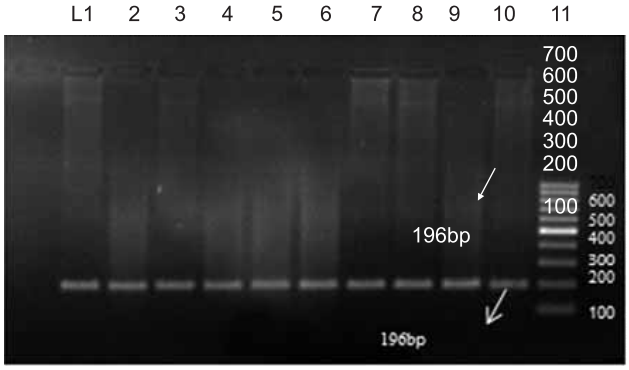

Fig. 1. Gel electrophoresis picture of amplified product of 196 bp region of exon-V fragment of prolactin gene of Changthangi goat. Lane 1–10, 196 bp amplicon; lane 11, 100 bp DNA marker.

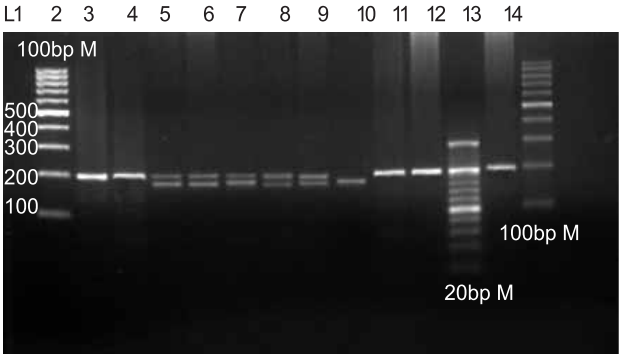

Fig. 2. Restriction fragment analysis of 196 bp amplified products of Exon-V of prolactin gene of different genotypes of Changthangi goat. Lane 1,100 bp DNA marker; lanes 2 and 3, 196 bp undigested PCR product; lanes 4, 5, 6, 7, 8, genotype A_1A_2 (196 and 169 bp present); lane 9, genotype A_2A_2 (169 bp present); lane 12, genotype A_1A_1 (196 bp present only).The 27bp band could not be seen on agarose gel.

amplified region of exon -V of designated A_1 and A_2 alleles (Fig. 3) revealed that at position 561, there is presence of "A" nucleotide in Changthangi goat in place of nucleotide "C" reported in other goats. The code present in the prolactin gene of Changthangi goats is CCA as well as CCC. At this position, restriction site (GRGCY/C) of *Eco 241*(*BanII*) is present that differentiates designated $A_1$and A_2 alleles. However, there is no change in coded amino acid proline across the species in spite of this mutation.

The frequencies of designated A_1A_1, A_2A_2 and A_1A_2 genotypes based on the observed polymorphic patterns of Exon-V (196 bp) of prolactin gene of Changthangi goats were 0.214, 0.291 and 0.495, respectively. And the designated allelic frequencies of A_1 and A_2 alleles in Changthangi goats for this fragment were 0.461 and 0.539, respectively. The χ^2 test showed that the genotypic distribution of the designated A_1A_1, A_2A_2 and A_1A_2 genotypes of 196 bp amplified prolactin gene were non-significantly (P>0.05) varying from Hardy-Weinberg Equilibrium.

The average fibre length, diameter of down, guard fibres percentage raw and fine yield in *Changthangi* goats are presented in Table 1. The effect of sex of the animal in *Changthangi* goats on mean yield (raw) and fine percentage, fibre length of down fibres, was statistically significant (P<0.05). From the statistical analysis, it was evident that the prolactin genotypes did not affect any of the cashmere traits significantly (P>0.05), though the effect of the sex was significant (P<0.05).

Sequence analysis revealed presence of the SNP at the nucleotide position 561 of the reference sequence. This created the site for *Eco 241* (*BanII*) *and* has resulted in a silent/synonymous mutation. The codon (CCC) changed to (CCA) and however, the amino acid proline remained unchanged in the final mature protein of prolactin traits.

Lan *et al*. (2009) observed a novel SNP at g.576C>A(X76049: g.576C>A). This transversion destroyed an *Eco241* (GRGCY$^{\downarrow}$C) restriction site which resulted in an amino acid exchange from proline to threonine at position 176 of the protein sequence, i.e. proline (CCC) to threonine (ACC). The mutations observed were missense in nature. They observed the SNP in their study on 1367 Chinese indigenous goats including the female Inner Mongolian White Cashmere goats. The association of SNP with fibre quality traits was non-significant (P<0.05) in our study. The results, however, were in contradiction to Lan *et al*. (2009) wherein they observed a strong association with the prolactin genotypes with regard to this SNP.

The frequencies of the two alleles of prolactin gene in the present study were 0.461 for A_1 and 0.539 for A_2. Lan

Table 1. Effect of designated genotypes of exon -5 of prolactin gene and sex on mean fibre length, mean fibre diameter, yield and guard hair percentage

Factor	N	Avg yield (g)	Fine %	Yield fine (g)	Guard %	Fibre length (mm)	Fibre diameter (µ)
Population mean (µ)	103	276.959±6.214	191.389±5.087	69.559±1.122	19.282±0.918	64.92±2.47	14.095±0.145
				Sex			
Male	41	307.610^{b}±9.311	207.145^{b}±7.622	67.794±1.682	20.487±61.37	74.48±3.69^{b}	14.094±0.217
Female	62	246.308^{a}±7.704	175.633^{a}±6.306	71.325±1.391	18.076±1.139	55.35±3.06^{a}	14.095±0.179
				Genotype			
A_1A_1	22	266.569±12.604	172.437±10.318	65.827±2.276	21.658±1.863	59.33±5.00	14.142±0.293
A_1A_2	51	283.758±8.180	199.796±6.697	70.918 ±1.477	18.276±1.209	68.59±3.25	14.264 ±0.190
A_2A_2	30	280.550±10.702	201.934±8.761	71.932 ±1.933	17.910±1.582	66.829±4.246	13.878 ±0.249

The means for sexes and genotypes bearing different superscripts differ significantly (P<0.05).

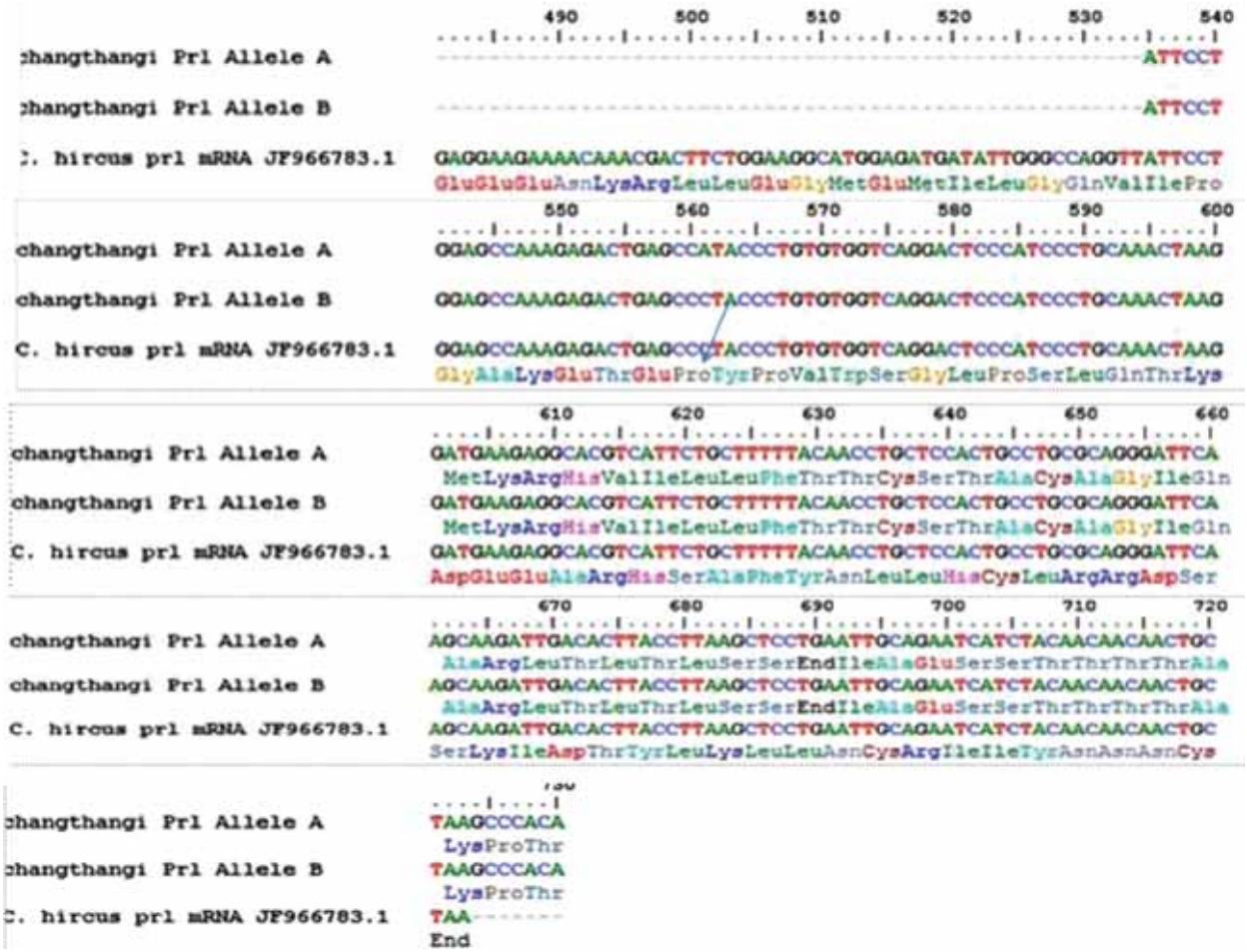

Fig. 3. Nucleotide sequences of amplified fragment of 196 bp of Exon V of prolactin gene of Changthangi goat.

et al. (2009), however, observed that the frequency of one of the allele (C) was 0.90 and this allele was correlated with higher fibre length (P=0.014). Shamsalddini *et al*. (2016) investigated the polymorphism in the same exon of prolactin gene and obtained a somewhat similar result as reported by Lan *et al*. (2009). They carried out their study in Raini Cashmere goats of Iran and observed the three different patterns (CC, AC and AA), with the frequencies of 0.39, 0.38, 0.23 in exon 5 region using PCR-SSCP technique. They observed that animals with CC genotype had the higher fibre length compared to those with AA and AC while there was no significant association with fibre diameter. They advocated the use of prolactin gene polymorphism as a molecular marker to improve fibre production without a negative effect on fibre diameter. However, a nonsignificant variation in the allelic frequency of the two alleles in the present study clearly indicated that there was no selection pressure asserted on the fibre traits in the ongoing selection programmes of the Changthangi goats. These observations were contrary to the observations made by Lan *et al*. (2009) and Shamsalddini *et al*. (2016).

The distribution of genotypes within the Changthangi goat population studied herein deviated from the Hardy Weinberg equilibrium (χ^2=0.007) and the observed frequency of 0.214, 0.291, 0.495 were observed for A_1A_1, A_2A_2, A_1A_2 genotypes respectively. Non disturbance in the Hardy Weinberg Equilibrium for these alleles indicate that in selection process for fibre traits in Pashmina, these SNPs are not contributing to change in fibre attributes, because no change in gene frequency of alleles of a gene is indicative of no selection pressure in the alleles. However, the distributions of genotypes within Inner Mongolia White Cashmere (IMWC) population studied by Lan *et al*. (2009) also deviated from Hardy-Weinberg Equilibrium. The distortion in their study was caused by the lacking A_1A_1 genotype. They report that theoretically, the missense mutation in the goat Prolactin gene may decrease milk performance, growth traits, and hair growth traits in the IMWC population. Moreover, the almost complete lack of homozygote A_1A_1 in the analyzed locus was similar to their previous results (Lan *et al*. 2009). They did not put forward any reason for lack of genotype A_1A_1 in these breeding farms. However, they opined that a further investigation is required to explain it.

The comparison of the designated allelic sequences revealed the presence of "A" nucleotide at position 561 in Changthangi goat allele A_1 while as there was nucleotide "C" in A_2 allele. The restriction site 5'-GRGCY$^{\downarrow}$C-3' of Eco *241* (*BanII*) showed clearly the difference of the two alleles A_1 and A_2. The code present in the prolactin gene of

Changthangi goats with allele A_1 was CCA whereas it was CCC with regard to this SNP allele A_2. However, the coded amino acid remained unchanged in spite of this mutation within goat alleles and across species. These results were slightly different from that reported by Lan *et al.* (2009). In their case, mutation was C>A which changed codon CCC to ACC and thereby changing proline to threonine. These results could not be compared with any other report as there is no such information available in public domain.

The Pashmina fibre quality traits recorded had a higher value in males as compared to the females which may be attributed to the effect of male sex hormone and double production in males. However, the effect of prolactin genotypes on cashmere traits was statistically non-significant (P>0.05). As observed from HWE results, the genotypic distribution did not vary significantly (P<0.05) revealing thereby the effect of selection on the alleles was not pronounced in changing the fibre attributes.

From the above discussion it can be concluded that the polymorphism observed in the gene showed a non-significant association (P>0.05) with Cashmere quality traits. The results obtained herein, because of low sample size, demand further investigation with sufficiently large number of samples.

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