Original Research

Study on KAP 8.2 Gene Polymorphisms in Nagaland Long Haired Goat of Northeastern India

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Abstract

The study was conducted to detect polymorphism of keratin associated protein 8.2 (KAP 8.2) gene to determine association between the genotype and fibre traits in Nagaland Long Haired goats (NLHG) of Northeastern India. NLGH is an important germplasm which comprises a small population of non-descript indigenous animals having unique long fibres over the body. The fibre traits studied were fibre diameter, fibre yield and fibre length. Twenty five animals were used to detect polymorphisms in hircine KAP 8.2 gene by means of Polymerase chain reaction–restriction length polymorphism (PCR-RFLP). Digestion of the PCR product using Pvu II revealed two genotypes viz. AA and AB. AA genotype yielded one fragment (501bp) and AB genotype yielded two fragments (331 and 170 bp). Allele A and genotype AB were predominant in the breed. The χ^2 test revealed that the genotype frequencies were not in agreement with Hardy-Weinberg equilibrium in the population . The frequencies of A and B alleles were found to be 0.700 and 0.300 respectively and the genotypic frequencies of KAP 8.2 were found to be 0.400 and 0.600 for AA and AB genotypes respectively. Fibre diameter of NLHG was found to differ significantly ($P < 0.05$) according to the genotypes. Goats with AA genotypes had significantly thinner fibre diameter (0.214 ± 0.006 mm) as compared to that of AB genotypes (0.257 ± 0.007 mm). However, fibre yield and fibre length did not differ in respect of the two genotypes. By further analysis, at position 124 bp and 281 bp of the original sequence of KAP 8.2 gene T→C and G→ C nucleotide substitution was found. Presence of polymorphism in KAP 8.2 opens interesting prospects for future selective breeding programme, especially based on marker-assisted selection.

Key words: Association, Fibre Traits, KAP 8.2 Gene, Polymorphism, Nagaland Long Haired Goats

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Introduction

Nagaland Long Haired goat (NLHG) is an unique and important germplasm restricted to highlands of Zunheboto and Tuensang districts in the state of Nagaland in India. Locally known as *Apu-Asu-Ne*. It is a very unique animal because of the unique long hairs over the body. The coat colour is mostly white with a specific black patch on head and neck region (Fig.1).

Fig.1: Adult male NLHG

Sometimes, black patches are also observed below the knee joints in some animals (Zaman *et al*., 2013). They constitute a small population about 7000 in numbers and yet to be recognized as a breed. The NLHG is genetically distinct from the other available goats of this region *viz.* Assam hill goat and Black Bengal (Zaman *et al.*, 2015). These animals play an important role in the traditional and cultural value of Sumi tribes of Nagaland, India. The unique long coarse fibres obtained from these goats are used by the local tribes for making different valuable items for traditional and ritual use like head gears, belts, ear rings, hair bands etc. This valuable germplasm have not been much explored yet. Fibre diameter, fibre length and fibre yield are the important fibre traits. Fibre diameter determines the quality and price of fibre. Keratin Associated Protein (KAP) gene is one of the major candidate gene for wool or fibre traits (China Supakorn , 2009**)**. The fibre is a complex structure composed primarily of proteins from the keratin family. KAP is a major component of the matrix between hair keratins and form intermediate filaments (KIFs) which generate cells of the central hair-forming compartment. KAP form the rigid hair shaft through a cross-linked network with KIF. Hair keratins and their associated proteins are important in the formation of cashmere fiber (Zhao *et al*., 2008). KAP are encoded by a large number of multigene families. In general, the KAP gene consists of a single exon of less than 1,000 bp (Zhang *et al*., 2011). It is **c**lassified into 3 groups on the basis of their amino acid composition: high sulfur (16 - 30 % cysteine content), ultra-high sulfur (> 30 % cysteine content) and high glycine/tyrosine (HGT) protein content (Rogers *et al.*, 2002).The keratin-associated protein 8.2 (KAP 8.2) is one member of the HGT proteins (Aoki *et al*., 1997). The KAP 8.2 gene has a coding sequence of 192 bp, which lacks introns and mainly is

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expressed in the wool follicle cortex. Genes encoding HGT proteins have been located on chromosome 21q22.1 (Rogers *et al.*, 2002) in humans. It has been reported that allelic variation at the KAP8 locus and fibre diameter was significantly associated in sheep (Parsons *et al.*, 1994). References are available suggesting that KAP 8 gene may play important role in determining the phenotypes for different cashmere quality and production traits. Hence, this study was conducted to identify polymorphisms in the KAP 8.2 gene and to evaluate the association of these polymorphisms with fibre traits in NLHG.

Materials and Methods

Extraction of DNA

Blood samples were collected from the 25 numbers (11 males and 14 females) of NLHG from Zunheboto district of Nagaland that constitute the natural habitat of this germplasm. The DNA from the whole blood was extracted using Phenol Chloroform Extraction Procedure (Sambrook and Russell, 2001) with slight modifications using DNAzol reagent instead of SDS and proteinase K.

Yield and Purity of DNA

The purity of the extracted genomic DNA was assessed by UV spectrophotometer (Nanodrop Spectrophotometer, Model- UV/VIS 916). The concentration of genomic DNA was estimated spectrophotometrically taking OD value at 260 nm. Quality of the DNA was checked by agarose gel electrophoresis.

Template DNA preparation for PCR

Based on the observed bands in the agarose gel and concentration determined by spectrophotometer the DNA was diluted using TE buffer to obtain the template DNA concentration approximately 50 to 100 ng per ml and stored at -20 °C till further use.

PCR for Amplifying DNA

The following primer sequences were used for KAP 8.2 gene amplification (Table 1).

Table 1: Primer for PCR-RFLP

Loci	Primer	PCR Product Size(bp)	Reference
KAP 8.2	F : 5' TCCCATCGTAACTCACAT 3' R : 5' AGGTCGGTTGGAAAGAAA 3'	501 bp	Liu *et al.*, 2007

Setting Up of PCR

The PCR reaction was carried out in 0.2 ml PCR tubes in a thermal cycler (Applied Biosystem, USA). PCR was carried out in a final reaction volume of 50 µl. Each reaction volume contained following components for the primer given in Table 2.

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Table 2: Reaction mixture for PCR-RFLP of KAP 8.2 gene

PCR Components	Final Conc.	Volume in µl
DNA Template	100 ng	2
Forward Primer	10 pmol/µl	0.5
Reverse Primer	10 pmol/µl	0.5
Master Mix		25
Nuclease free water		21
$MgCl_2$		1
Total		**50**

PCR Protocol (KAP 8.2)

The PCR protocol used for multiplying the specific areas in the genomic DNA is given in Table 3.

Table 3: PCR protocol

Step	Process	Temperature	Duration
1	Initial denaturation	94 °C	15 sec
2	Denaturation	94 °C	40 sec
3	Annealing	54 °C	35 sec
4	Extension	72 °C	35 sec
5	Repeat steps 2 to 4		35 cycles
6	Final extension	72 °C	10 minutes
7	Hold	4 °C	Until the samples are removed

Agarose Gel Electrophoresis

PCR amplification was confirmed by running 5 µl of PCR product mixed with 2 µl of 6 X gel loading dye on 1.5% agarose gel (depending on the expected size of the amplified product) at a constant voltage of 75 V for 45 minutes in 1X TAE buffer. Ethidium bromide was added @ 0.5µl/100ml gel solution in the gel itself. The amplified product was visualized as a single compact fluorescent band of expected size under UV light and documented by gel documentation system (Gel Logic 100, KODAK).

Restriction Endonuclease Enzyme Digestion

The PCR products (20 µl) were digested with restriction enzymes (Thermo Scientific) as per the manufacturer's protocol. Table 4 depicted the restriction enzyme used. The reaction mixture was vortexed for few seconds for uniform mixing and then incubated at 37 °C for overnight.

Table 4: Restriction endonuclease enzyme and its recognition site in KAP 8.2 Gene

Restriction Enzyme	Source	Recognition Site
PvuII	*Proteus vulgaris*	5'…. CAG↓CTG….. 3' 3'…. GTC↑GAC….. 5'

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Analysis of the PCR–RFLP Products

The enzyme-digested products were loaded @10 µl on 2.5% agarose gel along with 100 bp ladder mixed with 6 X gel loading dye at the rate of 10: 1. Electrophoresis was carried out at 110 V for 1 hour and the bands were visualized and documented using Gel Documentation System. The bands were analysed by comparing with molecular size marker. Genotyping of KAP 8.2 loci was carried out according to the band pattern of respective genotypes. The PCR products were sent for sequencing to Molbiogen (Guwahati, Assam, India).

Statistical Analysis

Gene and genotypic frequencies were calculated as given by (Falconer and Mackay ,1996) Chi-square (χ^2) test was performed to test if the population was in Hardy-Weinberg Equilibrium.

Association of the Identified Polymorphism of the Gene with Fibre Traits

The association of the identified polymorphism of the gene with fibre traits were analysed using RLM (Reduced Linear Model) method, which was performed by SPSS software (version 16.0). The linear model was:

$$Yijklm = \mu + S_i + D_{ij} + A_k + G_l + (SG)_{il} + e_{ijklm}$$

where, Y_{ijklm} = trait measured on each of the ijklmth animal , µ = is the overall population mean , S_i = is the fixed effect of ith genotype i = 1 (AA), 2(AB), D_{ij} = is the fixed effect of jth year (j = 1, 2,3….n) , A_k = is the final effect of kth age (k = 1, 2, 3…8), G_l = is the fixed effect associated with lth genotype, $(SG)_{il}$ = was the interaction between the ith ewe and lth genotype and e_{ijklm} = the random residual term.

The allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium was tested by comparing expected and observed frequencies using a Chi-square test.

Results and Discussion

In the present study, the amplification of KAP 8.2 gene resulted in a product size of 501 bp (Fig. 2) in all samples in NLHG and was in good agreement with the results reported by (Liu *et al.* 2007) in Inner Mongolian Cashmere goats who used PCR-RFLP technique to identify the genetic variants of KAP 8.2 gene. The PCR-RFLP technique was used to identify the variants in KAP 8.2 based on the variations produced by digestion of a 501 bp amplified product with restriction enzyme Pvu II. Two types of fragment pattern, arbitrarily designated as AA, AB genotype were revealed. Following digestion of the PCR product, AA genotype yielded one fragment (501bp); AB genotype yielded two fragments (331 and 170bp) as shown in Fig. 3. This finding was in conformity with earlier observation of (Liu *et al*. 2007) in Inner Mongolian Cashmere Goats. The frequencies of A and B alleles were 0.700 and 0.300 and those of AA and AB genotypes were 0.3782 and 0.6218 respectively (Table 5).

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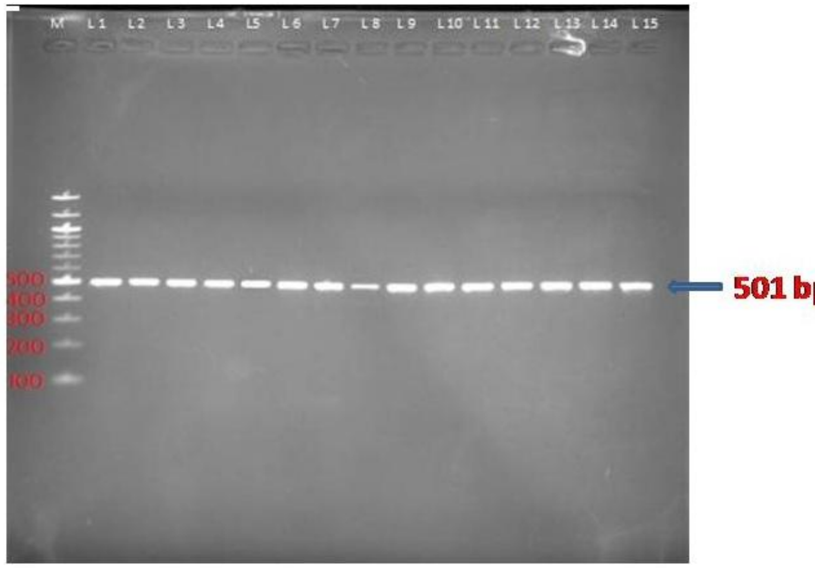

Figure 2: PCR Amplicon (501bp) of KAP 8.2 gene

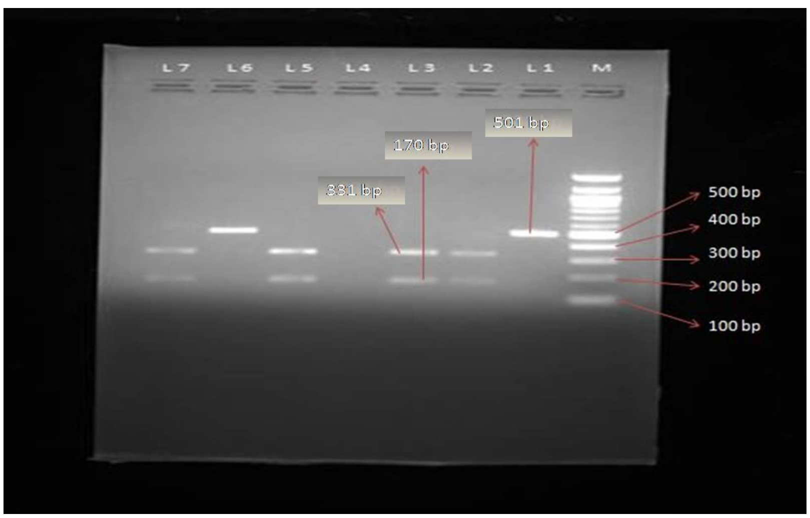

Fig. 3: PCR-RFLP of KAP 8.2 gene using Pvu II

Notably, there was a greater number of AB heretozygotes in comparison to AA homozygotes. The chi-square (χ^2) test revealed that the calculated value for KAP 8.2 gene was more than the tabulated value at 5% with1degrees of freedom (Table 5). Hence the population under study was not found to be in Hardy-Weinberg Equilibrium for KAP 8.2 gene. The finding was in consistent with those determined in Inner Mongolian Cashmere goats (Lui *et al.,* 2009)**.**

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Table 5: Distribution of genotype and allele frequencies of KAP 8.2 gene

Loci		Genotype			Allele Frequency	
		AA	**AB**	**BB**	**A**	**B**
Pvu II	Obs.	10	15	0		
	Exp.	12.1429	10.7143	2.1429	0.700	0.300
	Genotype frequency	0.400	0.600	0		
	χ^2	4.2352 *				

*χ^2= chi-square value, Obs.: Observed number, Exp.: Expected frequencies on the basis of Hardy-Weinberg law, *: Significant (P > 0.05)*

Sequencing was done using both forward and reverse primers for a few samples representing AA, AB genotype in KAP 8.2 gene by automated DNA sequencer (Applied Biosystem, USA). The obtained sequences were confirmed by BLAST analysis and submitted to NCBI (Fig. 4).

ACTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCTCCTCCTGTG
GTTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCAGCTCCTGCCAGC
TGGGCTCCTCTCTCTACAGCCAGGAGAGCTGCTGTGAGCCCATCAGGACCCAGACTGTGGTG
TCCAGTCCCTGCCAGACGTCCTGCTACCGCCCGAGGACCTCCACGTTCTTCAGTCCCTGCCAG
ACGACTTGCTCTGGATCTCTGGGCTTCGGTTCCAGTAACTTGCAATCTGCTGGTCAAGTCCCA
TCTCTGGGCTTTGGATCCGGTGGTTTCCAATCCGTGGGTCACAGCCCCAACATTTTCTCATCC
CTAAGTTGTAGATCCAGCTTTTACCGTCCCACCT

Fig. 4: Nucleotide sequences alignment of KAP 8.2 Gene

Based on scores obtained by BLAST, other sequences of KAP 8.2 gene in *Capra hircus* were downloaded from GeneBank database for comparative analysis. Multiple alignment of the sequence was performed. The sequences were aligned using ClustalW software as shown in Fig. 5. Pvu II restriction site was detected at position 170 of a total 501 bp by partial sequence.

Fibre diameter of NLHG was found to differ significantly (P < 0.05) according to the genotypes. Goats with AA genotypes had significantly thinner fibre diameter (0.214 ± 0.006 mm) as compared to that of AB genotypes (0.257 ± 0.007 mm). However, fibre yield and fibre length did not differ in respect of the two genotypes (Table 6).

Table 6: Mean ± S.E for fibre characteristics according to KAP 8.2 genotype

Genotype	Fibre diameter(mm)	Fibre yield(g)	Fibre length(cm)
AA	0.214 ± 0.006^a	193.2 ± 2.659	12.474 ± 0.889
AB	0.257 ± 0.007^b	198.13 ± 3.46	13.052 ± 0.917

Mean in a column with different superscripts differ significantly (P < 0.05).

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)


KAP8.2          ACTGCTGCTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCT
KAP8.2          ACTGCTGCTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCT
KAP8.2          ACTGCTGCTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCT
KAP8.2          ------ACTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCT
KAP8.2          ------ACTCTGGAAACTTCTCCTCCCGCTCCCTCCGGGACCACCTGCGCTACTCAGGCT
                       *****************************************************

KAP8.2          CCTCCTGTGGCTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCA
KAP8.2          CCTCCTGTGGCTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCA
KAP8.2          CCTCCTGTGGTTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCA
KAP8.2          CCTCCTGTGGTTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCA
KAP8.2          CCTCCTGTGGTTCCTCCTTCCCCAGCAACCTGGTCTACAGGACTGACCTCTACTCTCCCA
                ********** *************************************************

KAP8.2          GCTCCTGCCAGCTGGGCTCCTCTCTCTACAGCCAGGAGACCTGCTGTGAGCCCATCAGGA
KAP8.2          GCTCCTGCCAGCTGGGCTCCTCTCTCTACAGCCAGGAGACCTGCTGTGAGCCCATCAGGA
KAP8.2          GCTCCTGCCAGCTGGGCTCCTCTCTCTACAGCCAGGAGAGCTGCTGTGAGCCCATCAGGA
KAP8.2          GCTCCTGCCAGCTGGGCTCCTCTCTCTACAGCCAGGAGAGCTGCTGTGAGCCCATCAGGA
KAP8.2          GCTCCTGCCAGCTGGGCTCCTCTCTCTACAGCCAGGAGAGCTGCTGTGAGCCCATCAGGA
                *************************************** ********************

KAP8.2          CCCAGACTGTGGTGTCCAGTCCCTGCCAGACGTCCTGCTACCGCCCGAGGACCTCCACGT
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Fig. 5: Nucleotide alignment of partial sequences of KAP 8.2 gene using CLUSTAL W

Conclusion

The present study showed polymorphic banding pattern in most of the samples of NLHG of Nagaland with respect to KAP 8.2 gene. The absence of BB genotype in the population studied might be due to limited sample size. Chi-square (χ^2) test revealed that the population under study was not in Hardy-Weinberg Equilibrium for KAP 8.2 gene. Presence of polymorphism in KAP 8.2 opens interesting prospects for future selective breeding programme, especially based on marker-assisted selection.

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