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Polymorphism of keratin-associated protein (KAP) 3.2 gene and its association with wool traits in Rambouillet sheep

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ABSTRACT

In the present study, association of polymorphic variants of keratin-associated protein (KAP) 3.2 gene with wool traits in Rambouillet sheep was investigated. Genomic DNA was isolated from blood samples of 100 Rambouillet sheep. A 393 bp segment was amplified by PCR using *ovine* specific primers for KAP 3.2 gene. The genotyping was done by using PCR-SSCP. KAP 3.2 gene locus revealed 3 genotypes, viz. AA, AB and BB with genotype frequencies 0.46 (46), 0.40 (40) and 0.14 (14), respectively, with allele frequencies for allele A (0.66) and allele B (0.34). The result showed that the population was in Hardy-Weinberg equilibrium. Population genetic indexes such as expected heterozygosity (He) 0.45, effective number of alleles (ne) 1.81, Shannon indices (I) 0.64, Fixation index of individual with sub-population (F_{IS}) 0.11, Standard error (SE) 0.03 and polymorphic information content (PIC) 35% were found. The least squares means of various genotypes for greasy fleece weight (GFW) differed significantly. The animals with AB genotype (2.08±0.08 kg) had higher GFW followed by AA genotype (1.75±0.08 kg), and BB genotypes (1.71±0.15 kg) was found to be associated. From the study, it may be concluded that KAP 3.2 gene might be a potential molecular marker for genetic selection in Rambouillet sheep.

Key words: Keratin associated protein 3.2, PCR-SSCP, Polymorphisms, Sheep, Wool traits

Out of 42 breeds of sheep in India 6 breeds are found in Jammu and Kashmir (J&K). According to the 19th Indian livestock census, sheep population in India is 63.77 million. Wool production stands at the modest level of 44.7 million kg (DAHDAF 2012). The total wool production estimated for the Jammu and Kashmir State in 2011–12 was 7.53 million kg, http://www.indiaenvironmentportal. org.in/files/file/J&K%20 Economic Survey% 202013–2014.pdf). As the efficiency of wool processing is dependent on the consistency of wool fibre, the most important wool traits for the apparel industry include fibre diameter (FD), staple strength, colour (lack of pigmented fibre) and staple length (SL). The use of genetic markers along with classical breeding methods in sheep breeding programmes would greatly accelerate the efficiency of wool.

The keratin intermediate-filament proteins (KRTs) and keratin-associated protein (KAPs) are the major proteins that make up about 90% of the wool fiber (McLaren *et al.* 1997). The KRTs form the skeletal structure of the wool fibre and are embedded in a matrix of KAPs. These proteins are connected through disulphide cross-linkages, which are important for the stability and the mechanical properties of wool (Feughelman 1996, Schweizer *et al.* 2006). The KAP genes are small, between 0.6 and 1.5 kb in size and are intron less (Powell 1996). The matrix KAPs are divided into 3 groups based on their amino acid compositions: the high-sulphur proteins (16–30% cysteine content) KAP1.n, KAP2.n, KAP3.n, ultra-high-sulphur proteins (30% cysteine content), KAP4.n, KAP5.n, KAP10.n and high-glycine-tyrosine proteins i.e., KAP6.n, KAP7.n, KAP8.n (Plowman 2003, Rogers *et al.* 2006, Barba *et al.* 2009).

The high sulphur KAP 3.2 gene is located in chromosome 11 (McLaren *et al.* 1997). Many studies have reported that the genetic variation at KAP 3.2 gene might play an important role in determining various wool traits in sheep (Wang *et al.* 2010[a, b], Wang *et al.* 2011). Furthermore, there are reports associating variation in the KAP loci with variation in fiber diameter (FD) (Chen *et al.* 2011), staple strength and length (Itenge-Mweza *et al.* 2009, Chen *et al.* 2011) and wool length and wool yield (Xu *et al.* 2008, Wang *et al.* 2011).

The Rambouillet sheep is considered as one of the best choices, being very well adopted under the temperate and sub-temperate agro-climatic conditions and hilly geographical topography prevailing in the states of J&K. It is the largest fine wool breed adaptable to wide variety of

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arid range conditions, has a well developed flocking instinct and is long lived. Fleece is long, dense and uniform over the whole body. Rambouillet ewes possess many desirable traits which have resulted in their inclusion in crossbreeding programs to improve wool production. Considering above facts, the study was undertaken to investigate polymorphism of KAP 3.2 gene and assess the effect of this polymorphism on wool traits in Rambouillet sheep.

MATERIALS AND METHODS

Resource populations and DNA isolation: Blood samples were collected from 100 Rambouillet sheep i.e., male (32) and female (68), selected randomly from Sheep Breeding and Research Farm, Reasi, Jammu, (India). Different wool traits i.e., clean wool yield (CWY)%, FD (μ), crimps per inch (CPI), SL (cm), wool count (WC) and greasy fleece weight (GFW) in kg were also recorded for the individual sheep. Genomic DNA was isolated from the venous blood samples by HiPurA^{TM} SPP Blood DNA kit. Purity and concentration of genomic DNA was determined by using nano-drop spectrophotometer. Genomic DNA quality was assessed by using 0.8% horizontal submarine agarose electrophoresis. Gel was then examined on UV trans-illuminator for checking of quality. The genomic DNA samples having good quality (intact bands with no smearing) were used for further analysis.

PCR amplification: A pair of primers for amplification of KAP 3.2 gene was used on the basis of latest published sequence as reported by Wang *et al.* (2010[a,b]) primer sequences: forward (5' CCA AGA CTT CTC TCA TCA ACC 3'); reverse (5' GCA TTA AGA CTT GAG CAG CTC 3') primers. PCR was carried out in a final volume of 25μl. For KAP 3.2 gene reactions was carried out in PCR tubes containing volume of 5 μl (30ng/μl) of genomic DNA, 1μl (20pmol/μl) of forward and reverse primers, 12.5μl of 2X PCR master mix and 5.5μl of DNase free distilled water. Amplification was performed in eppendorf thermal cycler and was programmed for amplification of KAP 3.2 gene: Initial denaturation step at 94°C for 4 min followed by 35 cycles of cyclic denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min were carried out. To confirm the targeted PCR amplification, 5 μl of PCR product from each tube was mixed with 1 μl of 6X loading dye buffer each on 2% agarose gel containing ethidium bromide (1% solution @ 5μl/100ml) along with 100 bp DNA ladder at a constant voltage of 80 V for 45 min. in 0.5X TBE buffer.

Single-strand conformational polymorphism analysis of PCR amplicons: To explore genetic polymorphism in KAP 3.2 gene; amplified PCR products were subjected for SSCP through 10% polyacralamide gel electrophoresis (acrylamide: bisacrylamide (49:1) 10 ml; 5X TBE 6 ml; ammonium persulfate (20%) 150μl; TEMED 30 μl; autoclaved TDW 13.82 ml and total volume 30 ml). The gel was put in to electrophoresis tank with notched plate facing towards the buffer reservoir. The reservoir was filled with 1X TBE. The gel was given pre run at 250V for 30 min to remove any polar impurity, in the vertical gel electrophoresis system. PCR product (amplicon) was mixed with formamide dye; mixture was then loaded in gel with the help of long tip micropipette carefully. Electrophoresis was performed at 4°C for 8 h at 200V. DNA bands on the gel were visualized by the silver staining technique (Bassam *et al.* 1991).

Statistical analysis: The allele and genotype frequencies were calculated and Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies using a Chi-square (χ^2)-test along with population genetic indexes such as gene homozygosity (Ho), gene heterozygosity (He), effective allele numbers (Ne), fixation index (Fis) and Shannon's Information index (I) were executed in POPGENE 32 version 1.32 software (Yeh *et al*. 1999). The polymorphism information content (PIC) was calculated according to Botstein *et al.* (1980). The data on CWY (%), FD (μ), CPI, SL (cm), WC, GFW (kg) were subjected to least squares analysis was done by using Statistical Package for Animal Breeding (SPAB2) programme (Sethi 2006). The following model was used for this purpose:

$$Y_{ijk} = \mu + G_i + S_j + e_{ijk}$$

where, Y_{ijk}, k^{th} observation under j^{th} sex and i^{th} genotype; μ, overall population mean; G_i, fixed effect of i^{th} genotype on Yijk, where i_{1-3} (i_1 indicates genotype MM, i_2 indicates genotype MN and i_3 indicates genotype NN); S_j, fixed effect of j^{th} sex on Yijk, where j_{1-2} (j_1 indicates male and j_2 indicates female) and e_{ijk} = random error

RESULTS AND DISCUSSION

A total of 15–20 μg of DNA was obtained from the 300μl of the blood. DNA samples having OD values from 1.7 to 1.9 only were used for further analysis. Horizontal agarose gel electrophoresis was performed to check quality of the DNA using 0.8% agarose. The gel was photographed by gel documentation system. The genomic DNA samples having good quality without smearing were used for further analysis.

Present study revealed the amplified PCR product on 2% agarose gel as a single compact band of 393 bp size in Rambouillet sheep under UV transilluminator and documented by gel documentation system (Fig. 1). The PCR product of the same bp size was also reported by Wang *et al.* (2010[a,b]) in Tibetan sheep.

The PCR product of 393 bp was subjected to

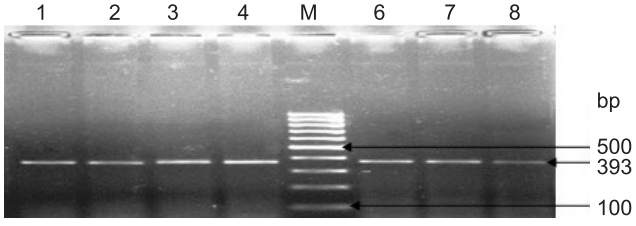

Fig. 1. PCR amplified product of KAP 3.2 gene of Rambouillet sheep at 2% agarose gel electrophoresis. Lane 1–4, 6–8: samples and lane M: 100 bp DNA ladder.

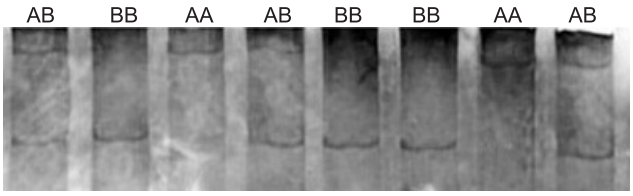

Fig. 2. The 10% PAGE electrophoresis patterns of 393 bp PCR products of KAP 3.2 gene in Rambouillet sheep.

polyacrylamide gel electrophoresis used for the detection of SSCP variants as 2 variants and 3 patterns of different SSCP variants were found in each case i.e., AA, AB and BB (Fig. 2). The SSCP variants with same patterns were also reported by Wang *et al.* (2010[a,b]), Wang *et al.* (2011) for KAP 3.2 gene in Tibetan sheep.

The genotypic frequency in Rambouillet sheep for KAP 3.2 gene was 0.46 for AA genotype; 0.40 for AB genotype and 0.14 for BB genotype whereas, the gene frequencies were 0.66 for A alleles and 0.34 for B alleles, respectively, in Rambouillet sheep. The χ^2-test values for KAP 3.2 gene were nonsignificant revealing that the population under study was in Hardy Weinberg equilibrium (HWE). The results obtained in the present study are not in agreement with those reported by Wang *et al.* (2010[a,b]) for KAP 3.2 gene. Deviation from the reported studies at KAP 3.2 genes may be due to breed differences, evolutionary pressure and selective breeding practices.

Population indices: Expected heterozygosity (He) value was 0.45. The results obtained in this study were not in agreement with those reported by Wang *et al.* (2010b) for Tibetan sheep (0.50) and Wang *et al.* (2011) Tibetan (0.50), Oula (0.47) and Qiaoke (0.29) sheep. Effective number of alleles (ne) was 1.81. The results obtained in this study were not in agreement with those reported by Wang *et al.* (2010b) for Tibetan sheep (2.00) and Wang *et al.* (2011) in Tibetan (2.00), Oula (1.87) and Qiaoke (1.40) sheep. Shannon indices (I), an information statistic index, was found to be 0.64. The index value was slightly lower than those reported by Wang *et al.* (2010b) for Tibetan sheep (0.69) and Wang *et al.* (2011) for Tibetan (0.69), and Oula (0.66) sheeps, However Wang *et al.* (2011) reported lower index value for Qiaoke (0.46) sheep. Fixation index of individual with sub-population (F_{IS}) was 0.11. The estimated polymorphic information content (PIC) values were with median polymorphism as 0.35. Thus, 35% of the offspring should be informative (Table 1). PIC compares the polymorphism levels across markers and is used to determine the usefulness of markers for specific studies. Our results were not in agreement with those reported by Wang *et al.* (2010b) for Tibetan sheep (0.38) and Wang *et al.* (2011) in Tibetan (0.38), Oula (0.36) and Qiaoke (0.24) sheep. Deviation from the reported studies at KAP 3.2 genes for (He), (ne), (I) and PIC values may be due to breed differences, evolutionary pressure, selective breeding practices and availability of lesser number of breedable adults.

Table 1. Population genetic indexes of KAP 3.2 gene in Rambouillet sheep

Ho	He	na	ne	I	Fis	SE	PIC
0.55	0.45	2.00	1.81	0.64	0.11	0.03	0.35

Ho, Expected homozygosity; He, expected heterozygosity; na, observed number of alleles; ne, effective number of alleles; I, Shannon's information index; Fis, fixation index of individual with sub-population; SE, standard error, and PIC, polymorphic information content.

Association of KAP 3.2 gene with wool traits: Least squares analysis of variance showed nonsignificant effect of sex on CPI, whereas significant (P<0.05) effect on CWY and highly significant (P<0.01) effect on FD, SL, WC and GFW. The effect of genotypes for CWY, FD, CPI, SL and WC were found to be non-significant, whereas significant (P<0.05) effect on GFW in Rambouillet sheep. Males have higher level of production of CWY (56.84±0.29%) and GFW (2.37±0.11 kg); whereas, female were found to be superior in FD (21.72±0.10μ), SL (5.94±0.13 cm) and WC (62.72±0.27; Table 2).

The least squares means of heterozygous genotypes for GFW differed significantly (P<0.05) than both the homozygotes. High GFW production was recorded for AB genotype (2.08±0.08 kg) followed by AA genotype (1.75±0.08 kg) and BB genotypes (1.71±0.15 kg; Fig. 3). Our results were not in agreement with those of Wang

Table 2. Least squares means and standard error for wool traits for KAP 3.2 gene in Rambouillet sheep

Particulars	Clean wool yield (%)	Fibre diameter (μ)	Crimps per inch	Staple length (cm)	Wool count	Greasy fleece weight (kg)
Mean (100)	56.51±0.18	22.08±0.10	13.33±0.21	5.42±0.13	61.66±0.27	1.84±0.07
Sex	*	**	NS	**	**	**
Male (32)	56.84±0.29[b]	22.43±0.17[b]	13.18±0.34	4.90±0.21[a]	60.59±0.44[a]	2.37±0.11[b]
Female (68)	56.17±0.18[a]	21.72±0.10[a]	13.48±0.21	5.94±0.13[b]	62.72±0.27[b]	1.32±0.07[a]
Genotype	NS	NS	NS	NS	NS	*
AA (46)	56.35±0.22	21.98±0.13	13.08±0.26	5.28±0.16	61.84±0.33	1.75±0.08[a]
AB (40)	56.53±0.22	21.95±0.13	13.35±0.26	5.11±0.16	62.20±0.33	2.08±0.08[b]
BB (14)	56.65±0.41	22.30±0.24	13.56±0.48	5.87±0.30	60.94±0.61	1.71±0.15[a]

** (P<0.01), * (P<0.05), nonsignificant (NS) and values bearing same superscripts in a column under different subgroup did not differ significantly and figures in parentheses are number of observations.

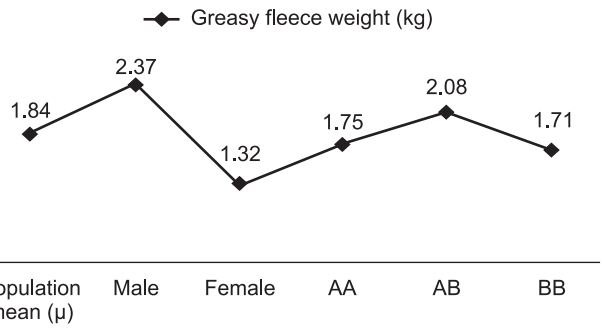

Fig. 3. Least squares means of sex and genotypes at KAP 3.2 gene for greasy fleece weight (kg) in Rambouillet sheep.

et al. (2010b). They reported that the wool length with genotype AA was significantly higher (P<0.05) than those with AB genotype and wool yield of individuals with genotype AA was significantly higher (P<0.05) than those with BB genotype of Tibetan sheep and similar finding were also reported by Wang *et al.* (2011) for Tibetan, Oula and Qiaoke breeds of sheep. Deviation in the results obtained in the present study may be due to breed differences, gene × environment interaction, level of expression of gene, breeding polices followed within the population and difference in the managemental practices.

KAP 3.2 gene was found polymorphic with AA, AB and BB genotypes as revealed by PCR-SSCP analysis. The polymorphisms in the KAP 3.2 gene might be a potential molecular marker for GFW in Rambouillet sheep.

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