



## Association of polymorphic variants of KAP 1.3 gene with wool traits in Rambouillet sheep

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### ABSTRACT

The objective of the present study was to investigate the association of polymorphic variants of keratin-associated protein (KAP) 1.3 gene with wool traits in Rambouillet sheep. Genomic DNA was isolated from blood samples of 100 Rambouillet sheep. A 598 bp KAP 1.3 gene segment was amplified by PCR using *ovine* specific primers. One SNP was confirmed by sequencing, which was C264T nucleotide transition mutation. Restriction Fragment Length Polymorphism (RFLP) using *Bsr* I restriction enzyme revealed two alleles A and B with 0.645 and 0.355 allele frequencies, respectively. Genotypic frequencies were 0.41 for AA, 0.47 for AB and 0.12 for BB genotypes. The  $\chi^2$ -test value for KAP 1.3 gene was non-significant revealing that the population under study was in Hardy Weinberg Equilibrium (HWE). Significant differences for CWY and highly significant differences for FD, SL, WC and GFW were observed between the least squares means of sex. Males showed higher level of production for CWY (56.91±0.26%) and GFW (2.53±0.09 kg); whereas, females were found to be superior in FD (21.74±0.11  $\mu$ ), SL (6.07±0.15 cm) and WC (62.80±0.29). The least squares means of various genotypes for GFW differed significantly. Highest GFW production was recorded for BB genotype (2.47±0.13 kg) followed by AB genotype (2.05±0.07 kg) and least by AA genotypes (1.62±0.08 kg). These results suggested that polymorphisms in the KAP 1.3 gene might be a potential molecular marker for genetic selection for GFW in Rambouillet sheep.

**Key words:** Keratin associated protein 1.3, PCR-RFLP, Polymorphisms, Sheep, Wool traits

The major proteins that make up the wool fibre are keratin intermediate-filament proteins (KRTs) and keratin-associated protein (KAPs) (Gong *et al.* 2012). In general, the KAP gene is composed of single exon of less than 1000 bp. The matrix of KAPs are divided into three groups based on their amino acid compositions, viz. 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 (KAP6.n, KAP7.n, KAP8.n) (Marshall *et al.* 1991, Plowman 2003, Rogers *et al.* 2006).

The high sulphur KAP 1.3 gene is located on chromosome 11 (McLaren *et al.* 1997). Many studies (Rogers *et al.* 1993, McLaren *et al.* 1997, Beh *et al.* 2001, Liu *et al.* 2007, Xu *et al.* 2008, Chen *et al.* 2011, Kumar *et al.* 2016) have reported polymorphism in members of the KAP gene families. Furthermore, there have been reports

associating variation in the KAP loci with variation in fibre diameter (Beh *et al.* 2001, Liu *et al.* 2007, Xu *et al.* 2008, Chen *et al.* 2011), staple length (Rogers *et al.* 1994, Itenge-Mweza *et al.* 2007, Chen *et al.* 2011) and wool yield (Xu *et al.* 2008). The genetic variation at KAP 1.3 gene might play an important role in determining various wool traits in sheep (Xu *et al.* 2008, Wang *et al.* 2009, Chen *et al.* 2011).

As most important wool traits for the apparel industry include fibre diameter, staple strength and staple length. Identification of genes and their association with wool traits will be helpful for selecting the desired animals at the early age in sheep breeding programmes. Based on the above facts, this study was carried out to identify polymorphisms in the KAP 1.3 gene and to evaluate the association of polymorphic variants with wool traits in the resource population of Rambouillet sheep.

### MATERIALS AND METHODS

**Resource populations and DNA isolation:** The present study comprised of 100 blood samples, ram (32) and ewe (68) of Rambouillet sheep maintained at Sheep Breeding and Research Farm, Reasi (A hill area with variable climatic conditions, ranging from sub-tropical to the semi temperate), Jammu, along with their wool records, viz. clean wool yield (CWY), fibre diameter (FD), crimps per inch

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(CPI), staple length (SL), wool count (WC) and greasy fleece weight (GFW).

Blood sample (5 ml) was collected from jugular vein aseptically in sterile EDTA coated vacutainer. Genomic DNA was isolated by HiPurATM 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.

#### PCR amplification and RFLP analysis of KAP 1.3 gene:

A pair of primers for amplification of KAP 1.3 gene was used on the basis of published sequences (Forward: 5' GGGTGGACAAGCAGACCAAACCTC 3' and Reverse: 5' TAGTTTGTGGACTGTACTACTGGC 3') as reported by Chen *et al.* (2011) to amplify a 598 bp PCR product. Polymerase chain reaction was carried out in a final reaction volume of 25 µl. PCR reaction mixture used for amplification of DNA was 2× PCR master mix 12.5 µl, forward primer (10 pmol/µl) 1.0 µl, reverse primer (10 pmol/µl) 1.0 µl, genomic DNA 3.0 µl and DNAase free water 7.5 µl. Amplification was performed in eppendorf thermal cycler, which was programmed specifically with respect to the KAP 1.3 gene as: Initial denaturation step at 94°C for 5 min followed by 34 cycles of cyclic denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, extension at 72°C for 40 sec and final extension at 72°C for 10 min. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6× loading dye buffer from each tube on 2% agarose gel containing ethidium bromide (1% solution @ 5 µl/100 ml) alongwith 100 bp DNA ladder at a constant voltage of 80 V for 45 min in 0.5× TBE buffer. The amplified product was visualized as a single compact band under UV transilluminator and documented by gel photography.

In order to detect the PCR-RFLP, the PCR product was digested with restriction enzyme *Bsr* I. The restriction digestion was carried out in 30 µl volume, i.e. 10 µl PCR product was taken into a microcentrifuge tube and 20 µl of master mix consisting of *Bsr* I: (1 µl) (10 Units/µl), buffer (2 µl) and autoclaved distilled water (17 µl) was added and incubated at 65°C for 6 h in the water bath. After restriction digestion, the restriction product mixtures were electrophoresed on 2.5% agarose gel containing 1% ethidium bromide @ 5 µl/100 ml at constant voltage of 80 V for 60 min using 0.5× TBE buffer till the dye front reached the last third of the gel. The DNA ladder, PCR-RFLP bands were visualized under UV transilluminator and photographed with gel documentation system. The band size was judged by comparing with molecular size marker and recorded. Genotyping of KAP 1.3 gene locus was carried out according to the band pattern of respective genotypes. Different patterns of amplified fragment of KAP 1.3 gene were sequenced in both directions by Xcelris Labs. Ltd. Nucleotide sequence alignments, translations and comparisons were carried out using the MEGALIGN software module and phylogenetic tree was developed with the MEGA 5.0 (Tamura *et al.* 2011).

**Statistical analysis:** Based on the results of genotyping for KAP 1.3 gene, genotypic frequencies, allelic frequencies, Hardy-Weinberg equilibrium (HWE), population genetic indexes such as gene homozygosity (Ho), gene heterozygosity (He), effective allele numbers (ne), Shannon's Information index (I) and fixation index (Fis) were as executed in POPGENE 32 version 1.32 software (Yeh *et al.* 1999, <http://www.ualberta.ca/~fyeh/fyeh>). The polymorphism information content (PIC) was calculated (Botstein *et al.* 1980). The data on CWY (%), FD (µ), CPI, SL (cm), WC, GFW (kg) were subjected to least squares analysis 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,  $Y_{ijk}$ ,  $k^{\text{th}}$  observation under  $j^{\text{th}}$  sex and  $i^{\text{th}}$  genotype;  $\mu$ , overall population mean;  $G_i$ , effect of  $i^{\text{th}}$  genotype;  $S_j$ , effect of  $j^{\text{th}}$  sex;  $e_{ijk}$ , random error associated with each observation and assumed to be normally and independently distributed with mean zero and variance  $(0, \sigma_e^2)$ .

## RESULTS AND DISCUSSION

The present study revealed amplified PCR product on 2% agarose gel as a single compact band of 598 bp size in Rambouillet sheep under UV transilluminator and documented by gel documentation system. The PCR product of the 598 bp size was also reported for KAP 1.3 gene by Rogers *et al.* (1993) in six sheep breeds, Rogers *et al.* (1994) in Romney sheep, Itenge-Mweza *et al.* (2007) in Merino sheep, Xu *et al.* (2008) in 5 breeds of sheep of Xinjiang, Chen *et al.* (2011) in Chinese Merino sheep and Kumar *et al.* (2016) in Malpura, Avikalin, Chokla, Sonadi, Nali, Nellore, Garole, Magra, Deccani, Patanwadi and Kendrapara.

Restriction digestion of KAP 1.3 gene with *Bsr* I restriction enzyme revealed restriction fragments of 350 bp, 307 bp, 225 bp, 43 bp and 23 bp sizes. The RFLP pattern first was of 350 bp, 225 bp and 23 bp size symbolized as AA type, pattern second was of 350 bp, 307 bp, 225 bp, 43 bp and 23 bp size symbolized as AB type and pattern third was of 307 bp, 225 bp, 43 bp and 23 bp size symbolized as



Fig. 1. PCR-RFLP patterns of the KAP 1.3 gene in Rambouillet sheep at 2.5% agarose gel electrophoresis. Lanes 7, 10, 13 and 15, homozygous AA genotype; lanes 1, 2, 4, 6, 8, 9, 11 and 12, heterozygous AB genotype; lanes 3 and 14, homozygous BB genotype; lanes 5 and 16, 100 bp DNA ladder marker.

Table 1. Estimated sequence percent identity and divergence obtained from KAP 1.3 gene sequence data of different species

		Percent identity																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
Divergence	1	█	73.0	99.6	99.8	99.6	99.6	99.5	99.3	99.6	99.6	99.8	99.7	99.5	99.3	99.3	84.3	73.6	84.6	86.9	1	AY835589.1 <i>Ovis aries</i>
	2	33.7	█	74.5	72.9	73.1	72.9	73.1	72.3	72.9	72.7	73.2	72.5	72.7	72.5	72.8	78.0	74.1	74.2	71.6	2	BC1011150.1 <i>Homo sapiens</i>
	3	0.4	31.4	█	99.4	99.2	99.2	99.1	98.9	99.2	99.2	99.6	100.0	99.2	99.1	99.1	84.3	72.9	84.7	90.5	3	FJ936476.1 <i>Capra aegagrus</i>
	4	0.0	33.7	0.4	█	99.5	99.5	99.3	99.1	99.5	99.5	99.6	99.5	99.5	99.3	99.3	84.0	73.4	84.7	88.4	4	FJ936477.1 <i>Ovis ammon collium</i>
	5	0.4	33.6	0.8	0.4	█	99.3	99.5	98.9	99.3	99.3	99.5	99.3	99.3	99.1	99.5	83.8	73.2	84.5	88.2	5	FJ936479.1 <i>Ovis canadensis canadensis</i>
	6	0.4	33.9	0.8	0.4	0.7	█	99.5	99.1	99.8	99.3	99.5	99.3	99.6	99.5	99.5	84.5	73.4	85.0	88.4	6	FJ936499.1 <i>Ovis orientalis gmelini</i>
	7	0.5	33.6	1.0	0.5	0.5	0.5	█	98.9	99.5	99.1	99.3	99.1	99.5	99.3	99.6	84.0	73.2	84.7	88.0	7	FJ936533.1 <i>Ovis vignei</i>
	8	0.2	34.2	0.6	0.2	0.5	0.4	0.5	█	99.1	99.3	99.1	98.9	99.3	99.1	98.9	84.0	73.4	84.3	87.8	8	FJ936540.1 <i>Ovis orientalis</i> × <i>vignei</i>
	9	0.2	33.9	0.6	0.2	0.5	0.0	0.4	0.2	█	99.3	99.5	99.3	99.6	99.5	99.5	84.5	73.4	85.0	88.4	9	FJ936545.1 <i>Ovis orientalis</i> × <i>vignei</i>
	10	0.2	34.0	0.6	0.2	0.5	0.5	0.7	0.0	0.4	█	99.5	99.3	99.3	99.1	99.1	83.8	73.6	84.5	88.2	10	FJ936578.1 <i>Ovis ammon sevetzovi</i>
	11	0.2	33.4	0.4	0.2	0.5	0.5	0.7	0.4	0.4	0.4	█	99.5	99.5	99.3	99.3	84.3	73.6	84.8	87.7	11	HM460695.1 <i>Ovis aries</i> KAP 1.3 A allele
	12	0.3	34.5	0.0	0.4	0.7	0.7	0.9	0.5	0.5	0.5	0.5	█	99.3	99.2	99.2	84.3	72.9	84.1	87.1	12	JQ772533.1 <i>Capra hircus</i> breed Bakerwal
	13	0.5	34.3	0.8	0.4	0.7	0.4	0.5	0.2	0.2	0.5	0.5	0.7	█	99.8	99.2	84.5	73.2	84.6	86.5	13	KAP 1.3 AA genotype.seq
	14	0.7	34.6	1.0	0.5	0.9	0.5	0.7	0.4	0.4	0.7	0.7	0.9	0.2	█	99.3	84.3	73.2	84.4	86.4	14	KAP 1.3 BB genotype.seq
	15	0.7	34.0	1.0	0.5	0.5	0.5	0.4	0.5	0.4	0.7	0.7	0.9	0.8	0.7	█	84.0	73.2	84.6	86.2	15	X02925.1 Sheep B2C gene
	16	17.9	26.2	17.9	18.0	18.5	17.6	18.2	17.7	17.6	18.2	17.9	17.9	17.6	17.9	18.2	█	78.3	82.3	84.7	16	XM_001917477.1 PREDICTED <i>Equus caballus</i>
	17	32.8	32.0	34.0	32.9	33.5	33.1	33.5	32.4	33.1	32.5	32.8	34.0	33.5	33.5	33.5	25.6	█	76.1	74.2	17	XM_002724546.1 PREDICTED <i>Rattus norvegi</i>
	18	17.4	31.6	17.3	17.1	17.6	16.8	17.3	17.2	16.8	17.4	17.1	18.0	17.4	17.6	17.3	20.3	28.9	█	80.2	18	XM_003482987.1 PREDICTED <i>Sus scrofa</i>
	19	14.5	35.9	10.3	12.5	13.0	12.7	13.2	12.8	12.7	12.8	13.5	14.3	15.0	15.2	15.4	17.2	31.9	23.1	█	19	XM_870828.5 PREDICTED <i>Bos taurus</i>

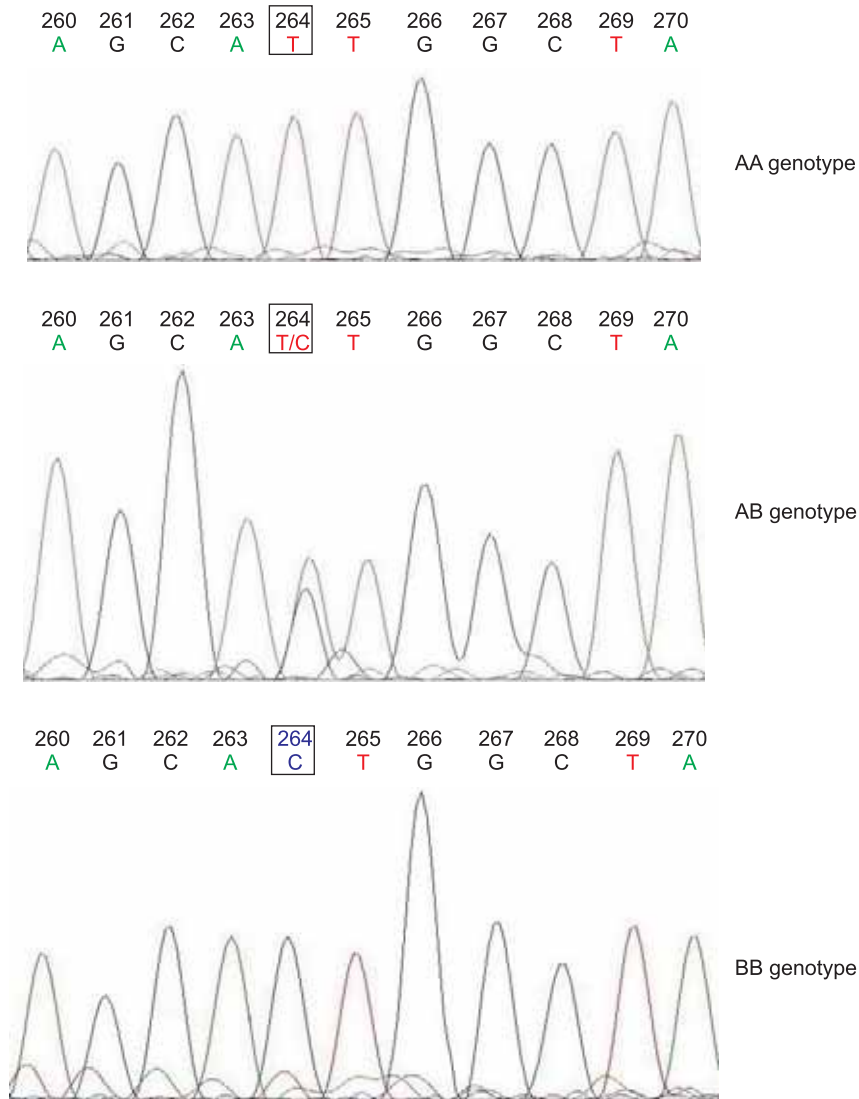
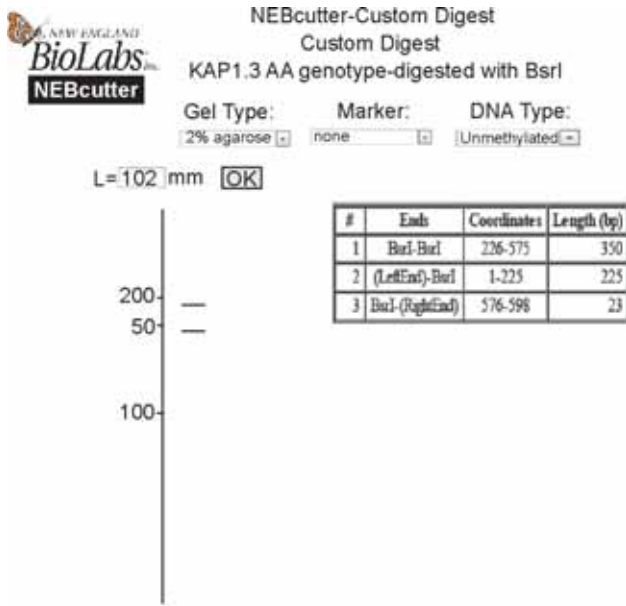
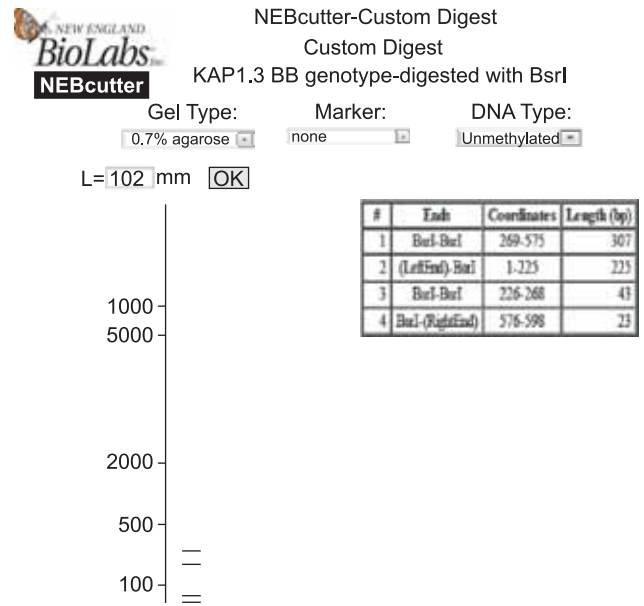


Fig. 2. Sequencing maps from different genotypes in Rambouillet sheep KAP 1.3 gene.



The virtual gel was generated by interpolating experimental data.



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Fig. 3. Screensort showing the *Bsr* I digestion of sequence of AA genotype (<http://tools.neb.com/NEBcutter2/>).

Fig. 4. Screensort showing the *Bsr* I digestion of sequence of BB genotype (<http://tools.neb.com/NEBcutter2/>).

BB type. The 43 bp and 23 bp fragments were not visible on electrophoresis (Fig. 1). Three different RFLP patterns were obtained at two restriction sites corresponding to AA, AB and BB genotypes. Different RFLP patterns using *Bsr* I restriction enzyme were reported by Rogers *et al.* (1993) in six sheep breeds, Xu *et al.* (2008) in 5 breeds of sheep of Xinjiang, Chen *et al.* (2011) in Chinese Merino sheep and Kumar *et al.* (2016) in Malpura, Avikalin, Chokla, Sonadi, Nali, Nellore, Garole, Magra, Deccani, Patanwadi and Kendrapara. These differences may be due to breed difference, evolutionary pressure and selective breeding strategies. DNA sequencing allows the detection of changes in the nucleotide sequence and confirmed that allele A lacks a *Bsr* I site as a result of a C→T nucleotide substitution at

single base 264 position of PCR product as shown in Fig. 2 was reported by Rogers *et al.* (1993). Band patterns were further confirmed on the basis of sequence digested of AA and BB genotypes by using NEBcutter2 (<http://tools.neb.com/NEBcutter2/>) as shown in Figs 3 and 4, respectively for the presence of 43 bp and 23 bp fragments, as they were not visible on electrophoresis. Sequence percent identity and divergence obtained from gene sequence data of different species as shown in the Table 1 indicated higher divergence obtained for AA genotype with *Homo sapiens* followed by *Rattus norvegicus* whereas percent identity was higher with BB genotype of Rambouillet sheep and vice-versa. Phylogenetic analysis of KAP 1.3 gene following Neighbour-joining algorithm

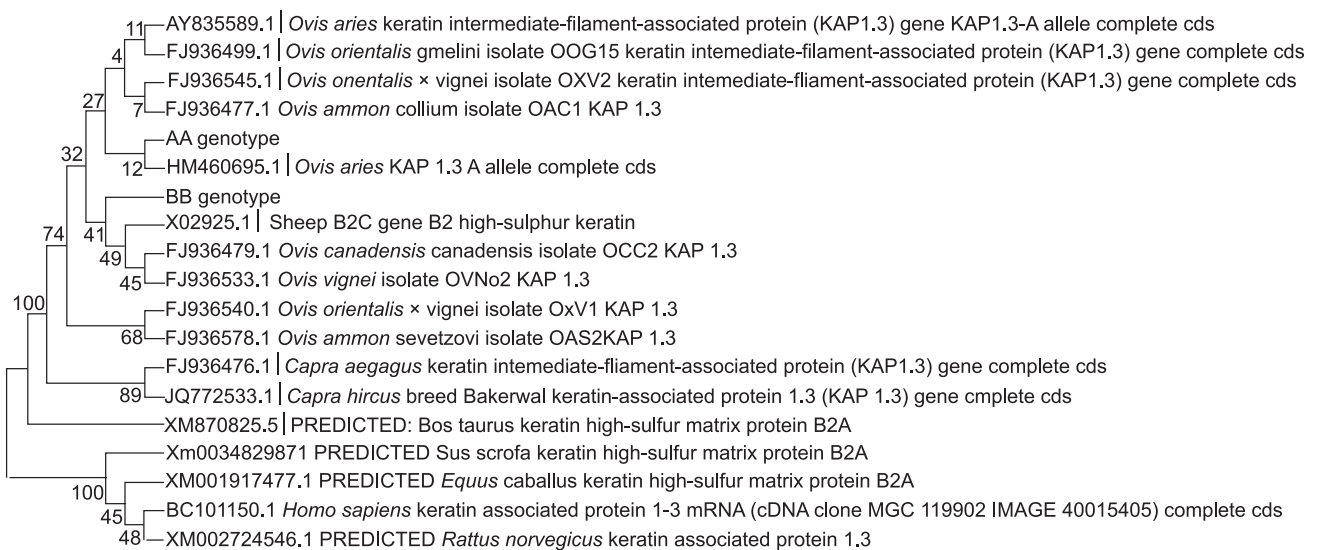


Fig. 5. Neighbour-joining tree obtained from KAP 1.3 gene sequence data of different species with boot strap values.

revealed that *Ovis* spp. and *Capra* spp. were found in same group belong to one cluster whereas, *Bos Taurus*, *Homo sapiens*, *Sus scrofa*, *Equus caballus* and *Rattus norvegicus* had distinct cluster on the basis of bootstrap values as presented in Fig. 5.

The genotypic frequencies in Rambouillet sheep for KAP 1.3 gene were 0.41 for AA genotype, 0.47 for AB genotype and 0.12 for BB genotype; whereas, the gene frequencies were 0.645 for A allele and 0.355 for B allele respectively. The  $\chi^2$ -test value for KAP 1.3 gene was nonsignificant revealing that the population under study was in Hardy Weinberg Equilibrium (HWE). Similar results were reported by Xu *et al.* (2008) in Merino (Mln), Tao Saite (Tst), Suffolk (Sfk) and the lake (Hu) sheep and Kumar *et al.* (2016) in Malpura, Avikalin, Chokla, Nali, Nellore, Garole, Magra and Deccani. However, results obtained in the present study were not in agreement with those reported by Xu *et al.* (2008) in Turk (Tks) sheep and Chen *et al.* (2011) in Chinese Merino sheep and Kumar *et al.* (2016) in Sonadi, Patanwadi and Kendrapara for KAP1.3 gene which may be due to breed differences, non-random mating and selective breeding practices (Falconer and Mackay 1996). Population genetic indexes such as Ho (0.54), He (0.46), ne (1.85), I (0.65),  $F_{IS}$  (-0.03), i.e. a negative  $F_{IS}$  value represents an excess of heterozygotes may occur if mates are less closely related than the average within subpopulations, SE (0.03) and PIC (0.35) indicated medium polymorphism (Table 2).

*Association of KAP 1.3 genotypes with wool traits:* Least squares analysis of variance showed non-significant effect of sex on CPI, whereas significant ( $P<0.05$ ) effect of sex

was found 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 non-significant, whereas highly significant ( $P<0.01$ ) effect of genotypes was obtained for GFW in Rambouillet sheep (Table 3). Males had higher level of production on CWY ( $56.91\pm0.26\%$ ) and GFW ( $2.53\pm0.09$  kg); whereas, female were superior in FD ( $21.74\pm0.11$   $\mu$ ), higher SL ( $6.07\pm0.15$  cm) and WC ( $62.80\pm0.29$ ). The least squares means of various genotypes for GFW differed highly significantly ( $P<0.01$ ). High GFW production was recorded for BB genotype ( $2.47\pm0.13$  kg) followed by AB genotype ( $2.05\pm0.07$  kg) and least by AA genotype ( $1.62\pm0.08$  kg). The results revealed in the present study were in accordance as reported by Xu *et al.* (2008) for SL. Wang *et al.* (2009) reported apparent relationship with the yield of wool ( $P<0.01$ ) and the wool length ( $0.01<P<0.05$ ) in sheep of Town stud farm in Jilin Province. Whereas, Xu *et al.* (2008) reported significant ( $P<0.05$ ) association of FD with AA genotype ( $18.13$   $\mu$ m) in five breeds of sheep, Chen *et al.* (2011) reported individuals carrying AA genotype had highly significant ( $P<0.01$ ) effect to yield lower FD ( $22.72$   $\mu$ m) in Chinese Merino sheep. Deviation in the results of the study may be due to breed differences, gene  $\times$  environment interaction, level of expression of gene, breeding policies followed within the population and difference in the managerial practices.

KAP 1.3 gene was found polymorphic with AA, AB and BB genotypes using *Bsr* I restriction enzyme as revealed by PCR-RFLP analysis. The polymorphisms in the KAP 1.3 gene might be a potential molecular marker for superior GFW in Rambouillet sheep.

Table 2. Frequency of genotypes and alleles along with population genetic indexes for KAP 1.3 gene in Rambouillet sheep

Genotype	Gene/Allele		$\chi^2$ -test (HWE)	Population genetic indexes								
	AA	AB		BB	A	B	Ho	He	ne	I	$F_{IS}$	SE
0.41 (41)	0.47 (47)	0.12 (12)	0.645	0.355	0.05 <sup>NS</sup>	0.54	0.46	1.85	0.65	-0.03	0.03	0.35

HWE, Hardy-Weinberg equilibrium; NS, nonsignificant; Ho, gene homozygosity; He, gene heterozygosity; ne, effective allele numbers; I, shannon's information index;  $F_{IS}$ , fixation index; SE, standard error; PIC, polymorphism information content.

Table 3. Least squares means and standard error of sex and genotypes in KAP 1.3 gene on wool traits in Rambouillet sheep

Sex/Genotype	Wool traits					
	CWY (%)	FD ( $\mu$ )	CPI	SL (cm)	WC	GFW (kg)
$\mu$ (100)	56.56 $\pm$ 0.17	22.00 $\pm$ 0.10	13.26 $\pm$ 0.20	5.36 $\pm$ 0.13	62.00 $\pm$ 0.25	2.05 $\pm$ 0.06
Sex	*	**	NS	**	**	**
Male (32)	56.91 $\pm$ 0.26 <sup>b</sup>	22.25 $\pm$ 0.14 <sup>b</sup>	13.34 $\pm$ 0.30	4.65 $\pm$ 0.19 <sup>a</sup>	61.20 $\pm$ 0.38 <sup>a</sup>	2.53 $\pm$ 0.09 <sup>b</sup>
Female (68)	56.20 $\pm$ 0.20 <sup>a</sup>	21.74 $\pm$ 0.11 <sup>a</sup>	13.18 $\pm$ 0.23	6.07 $\pm$ 0.15 <sup>b</sup>	62.80 $\pm$ 0.29 <sup>b</sup>	1.56 $\pm$ 0.07 <sup>a</sup>
Genotype	NS	NS	NS	NS	NS	**
AA (41)	56.77 $\pm$ 0.23	21.81 $\pm$ 0.13	13.80 $\pm$ 0.27	5.21 $\pm$ 0.18	61.83 $\pm$ 0.34	1.62 $\pm$ 0.08 <sup>a</sup>
AB (47)	56.22 $\pm$ 0.21	22.15 $\pm$ 0.12	13.15 $\pm$ 0.24	5.24 $\pm$ 0.16	61.83 $\pm$ 0.31	2.05 $\pm$ 0.07 <sup>b</sup>
BB (12)	56.68 $\pm$ 0.41	22.03 $\pm$ 0.22	12.83 $\pm$ 0.47	5.63 $\pm$ 0.30	62.33 $\pm$ 0.59	2.47 $\pm$ 0.13 <sup>c</sup>

CWY, Clean wool yield (%); FD, fibre diameter ( $\mu$ ); CPI, crimps per inch; SL, staple length (cm); WC, wool count; GFW, greasy fleece weight (kg);  $\mu$ , population mean; \*\*Highly significant ( $P<0.01$ ); \*Significant ( $P<0.05$ ); NS, nonsignificant. Values bearing different superscripts in a column differ significantly and figures in parentheses are number of observations.

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