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Polymorphism of Keratin - Associated Protein (*KAP*) 6.1 gene and its association with wool traits of Sandyno and Nilagiri breeds of sheep

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ABSTRACT

Polymorphic variants of keratin-associated protein (*KAP*) 6.1 gene with wool traits of Sandyno and Nilagiri breeds of sheep were investigated in this study. Genomic DNA was isolated from blood samples of 125 Sandyno, Nilagiri and Dorset x Nilagiri breeds of sheep along with 76 numbers of wool samples. A 528 bp segment was amplified by PCR using ovine specific primers for *KAP* 6.1 gene. SSCP analysis of *KAP* 6.1 gene in Dorset x Nilagiri crossbred sheep resulted in two genotypes for the *A/B* with no polymorphism. *KAP* 6.1 gene locus revealed allele frequencies of *A, B, C, D* and *E* in Sandyno sheep were 0.67, 0.23, 0.04, 0.01 and 0.04 and in Nilagiri were 0.75, 0.06, 0.11, 0.04 and 0.04 respectively. *KAP* 6.1 gene revealed departure from Hardy-Weinberg equilibrium. *KAP* 6.1 genes were found to have high degree of homozygosity (0.6667) in Nilagiri sheep. The effective number of alleles (N_e) for *KAP* 6.1 gene was 1.1690 and 1.7006 respectively in Sandyno and Nilagiri breeds of sheep. The PIC values for *KAP* 6.1 gene was 0.1341 and 0.3909 in Sandyno and Nilagiri breeds of sheep respectively. F_{IS} values for *KAP* 6.1 gene was positive (0.1909) in Nilagiri breed and it was negative (– 0.0110) in Sandyno breed. Least squares analysis of variance showed significant (P<0.05) effect between genotypes and between sexes for wool traits like GFW (kg), CWY (%) and FD (µ). Significant (P<0.05) effect of breed (Sandyno and Nilagiri) was observed for all the wool traits (GFW, CWY, FD, SL and Medullation %). Significant (p<0.05) higher CWY production was recorded for *AC* genotype (62.00 ± 1.73 %) while low value was recorded for *BB* genotype (56.25 ± 1.74 %). FD was found to be high in genotype *AC* (24.29 ± 1.17 µ) than in *AA* genotype (23.24 ± 0.49 µ). The homozygote *AA* was found to have more Medullation (%) and SL. Genotypes *BB* had higher GFW and lower CWY. The heterozygous genotype *AC* yielded more FD and CWY with lower most Medullation (%) and SL values. From the study, it may be concluded that *KAP* 6.1 gene might be a potential molecular marker for genetic selection of wool traits in Sandyno and Nilagiri breeds of sheep.

Key words: Keratin Associated Protein (*KAP*) 6.1, PCR-SSCP, Polymorphism, Sheep, Wool traits.

INTRODUCTION

The Indian Wool Industry is the 7th largest in the world and it accounts for about 1.8 per cent of total world production of wool with annual production in the range of 45-48 million kg. Out of the total production of raw wool about 10 % was apparel grade, 70 % carpet grade and 20 % coarse grade. Wool yield per sheep in India is about 800-1000 g/year. The annual growth of wool production is marginal and wool production has remained static for last 10 years.

Keratin Associated Protein was one of the major genes that influence the economically important traits in wool sheep hence gene mapping studies of keratin proteins have identified some chromosomal regions associated with variation in wool quality and production traits. The keratin intermediate-filament proteins (*KRT*s) and keratin-associated protein (*KAP*s) are the major proteins that make up about 90 per cent of the wool fibre (McLaren *et al.*, 1997). The *KRT*s form the skeletal structure of the wool fibre and are embedded in a matrix of *KAP*s. 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 *KAP*s are divided into 3 groups based on their amino acid compositions: the high-sulphur proteins (16–30% cysteine content) *KAP*1.n, *KAP*2.n, *KAP*3.n, ultra-high-sulphur proteins (30% cysteine content), *KAP*4.n, *KAP*5.n, *KAP*10.n and high- glycine-tyrosine proteins i.e., *KAP*6.n, *KAP*7.n, *KAP*8.n (Plowman, 2003; Rogers *et al.*, 2006, Barba *et al.*, 2009). Among all the classes of Keratin Associated Protein gene, *KAP* 6.1 genes are found to be polymorphic having impact on wool characteristics and was reported by various researchers.

The Nilagiri sheep is a dual utility (fine wool and meat), native to the Nilagiri hills of Tamil Nadu whose population was reported as 8000 by Ganesakale and Rathnasabapathy (1973). At present, this breed is endangered

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with 587 numbers existing, of which about 50 percent is maintained at Sheep Breeding Research Station, Sandynallah. The breed has been used along with Merino, in the development of another synthetic wool breed named Sandyno, which has better wool quality. So, Sandyno and Nilagiri breeds of sheep should be improved for fine wool production through Marker Assisted Selection. Considering above facts, the study was undertaken to investigate polymorphism of *KAP* 6.1 gene and assess the effect of polymorphism on wool traits of Sandyno and Nilagiri breeds of sheep.

MATERIALS AND METHODS

A total of 125 blood samples (51 numbers of Sandyno, 54 numbers of Nilagiri and 20 numbers of Dorset x Nilagiri crossbred sheep) and 76 numbers of wool samples (35 numbers of Sandyno and 41 numbers of Nilagiri) were collected along with production record from the Sheep Breeding Research Station (SBRS), Sandynallah, the Nilgiris. Different wool traits i.e., Greasy Fleece Weight (Kg), Clean Wool Yield (%), Fibre Diameter (µ), Staple Length (cm) and Medullation (%) were also recorded for individual sheep. Genomic DNA was isolated from whole blood using a modified method of Montgomery and Sise (1990) with slight modifications by using saturated Phenol: Chloroform: Isoamyl alcohol mixture. Purity and concentration of genomic DNA was determined by using spectrophotometry. Quality of genomic DNA was assessed in a one per cent agarose gel using horizontal gel electrophoresis technique. Good quality DNA samples with clear bands were selected for further study.

The *KAP* 6.1 gene were amplified by using F: (5′-CCAATGGCATGAAGGTGT-3′) and R: (5′-AAAAAGGGA AGGGTTGGTG-3′) as described by McLaren *et al.,* (1997). PCR reactions were carried out in a final reaction volume of 20 µl. Nineteen µl of reaction mixture comprising 0.5 µl (5 picomoles) of each forward and reverse primers, 7.5 µl of 2 x PCR master mixes (1.5 mM $MgCl_2$, Taq DNA polymerase, 100 µM dNTPs) and 10.5 µl of nuclease free water was aliquoted in each PCR tube and one µl template DNA was added to each tube to make the final volume. Master Mix was prepared with one additional sample to cover pipetting error. PCR tubes containing mixture were tapped gently and placed in a thermal cycler and subjected to PCR amplification. The thermal protocol used for amplifying the *KAP 6.1* gene of sheep consisted of an initial denaturation step at 94ºC for 4 min, followed by 35 cycles of denaturation (94ºC, 30 sec), annealing (62ºC, 45 sec) and DNA extension (72ºC, 30 sec) and a final extension step at 72ºC for 10 min. To confirm the targeted PCR amplification, five ml of each of PCR amplicons were electrophoresed through 2 per cent (w/v) agarose gel containing 0.5 mg/ml ethidium bromide in 1x TAE buffer. The horizontal gel electrophoresis was carried out at constant voltage of 5 V/cm for 40 min. After amplification, the PCR products were visualized on UV transilluminator to record the size of PCR fragments. The sizes and quantities of PCR products were verified by comparison with 100 bp DNA ladder.

To explore genetic polymorphism in *KAP* 6.1 gene, amplified PCR products were subjected for SSCP through 8% Polyacrylamide gel electrophoresis (acrylamide: bisacrylamide (29:1) 13.3 ml; 5 x TBE 10 ml; Ammonium persulfate (10 %) 250 µl; TEMED 100 µl; Triple distilled water 26.35 ml and total volume of 50 ml). Prepare the gel solution by combining all reagents and allow the gel to polymerize for at least 45 minutes. The reservoir was filled with 1 x TBE. Pre run was given for 30-40 minutes at constant power (80 W) to remove any polar impurity in the vertical gel electrophoresis system. After the pre-run was completed, denatured products were loaded in gel carefully. The electrophoresis was performed at 4°C for 24 hours at 120 V for 20×20 cm plate size. After the run was completed, gel was removed from the glass plates and silver staining was carried out according to Bassam *et al.,* (1991) with certain modifications to visualize the banding patterns.

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 by PIC calculator. The data on CWY (%), FD (µ), SL (cm), GFW (kg), Medullation (%) were subjected to least squares analysis was done by using Statistical Package for Social Sciences (SPSS) programme. The following model was used for this purpose.

$$Y_{ijkl} = \mu + B_i + S_j + G_k + e_{ijkl}$$

Where,

Y_{ijkl} - Measurement of a trait on l^{th} sheep belonging to i^{th} breed and j^{th} sex and k^{th} genotype

µ - Overall population mean

B_i - fixed effect of i^{th} breed on Y_{ijk} where i_{1-2} (i_1-Nilagiri and i_2-Sandyno)

S_j - fixed effect of j^{th} sex on Y_{ijk} where j_{1-2} (J_1-Male and J_2-Female)

G_k - fixed effect of k^{th} genotype on Y_{ijk} where j_{1-4} (k_1 - *AA*, k_2 - *AB*, k_3 - *AC*, k_4 - *BB*)

e_{ijk} - random error

RESULTS AND DISCUSSION

The quantity and quality of DNA was assessed by Biophotometer and the mean yields of DNA isolated from Sandyno and Nilagiri breeds of sheep were 319.98 ± 53.33 and 483.38 ± 80.56 µg/ml respectively. The ratio of optical density at 260/280 of DNA for the above genetic groups ranged from 0.95 to 1.95 and from 1.28 to 1.99 respectively.

The extracted DNA samples were subjected to agarose gel electrophoresis to check the presence of DNA. The genomic DNA samples having good quality without smearing were used for further analysis. The PCR amplification of *KAP 6.1* gene yielded product at 528 bp (Fig 1) similar to the observation of McLaren *et al.*, (1997). In contrast, Liu *et al.*, (2014) reported that amplification of *KAP* 6 gene produced product of 498 bp size.

The SSCP pattern of 528 bp fragment of *KAP 6.1* gene produced (Fig 2) seven genotypes (*viz. AA, AB, AC, BB, BC, BE, DE)* in Sandyno sheep and six genotypes (viz. *AA, AB, AC, AD, CC, EE)* in Nilagiri sheep. The genotype frequencies of *AA, AB, AC, BB, BC, BE* and *DE* were in the order of 0.48, 0.32, 0.07, 0.02, 0.02, 0.07 and 0.02 respectively in Sandyno breed*; AA, AB, AC, AD, CC* and *EE* genotype frequencies were in the order of 0.59, 0.12, 0.14, 0.08, 0.04 and 0.04 respectively in Nilagiri breed (Table 1). The *A, B, C, D* and *E* allele frequencies in Sandyno sheep were 0.67, 0.23, 0.04, 0.01 and 0.04 respectively and in Nilagiri sheep were 0.75, 0.06, 0.11, 0.04 and 0.04 respectively. Presence of five alleles at *KAP 6.1* gene in this study was comparable with the report of Gong *et al.*, (2011) in Merino, Romney, Coopworth, and Cross-bred sheep. Whereas, Parsons *et al.*, (1993) observed alleles A1 and A2 with highest in A2 (0.78) allele in Medium Peppin flock by Restriction Fragment Length Polymorphism.

The Genotype *AA* was more prevalent in both Sandyno (0.48) and Nilagiri (0.59) breeds. The χ^2 values indicate that Sandyno and Nilagiri breeds differ significantly in their allele and genotype frequency having one genotype *(DE)* with frequency of 0.02 in Sandyno sheep. In Sandyno breed of sheep, the genotypes *BC, BE* and *DE* are of very less frequency (0.02) than the others (Table 1).

However, the reports of Feng *et al.*, (2012) revealed only three polymorphic variants (*AA, AB* and *BB*) in Chinese Merino sheep with highest frequency (0.457) of *BB* genotype and Zhou *et al.*, (2015) found three sequence variants in Merino cross lambs with highest frequency (0.405) for *AB* genotype. Deviation from the present study at *KAP 6.1* gene may be due to size of the population studied, breed differences and selective breeding practices.

SSCP analysis of *KAP 6.1* gene in Dorset x Nilagiri crossbred sheep resulted in two genotypes for the *A/B* with no polymorphism (Fig 3). Absence of polymorphism in these two loci in Dorset x Nilagiri crossbred sheep intended for meat production further strengthens the evidence that these genes are possible candidate genes for wool traits being studied and can be utilized as markers for selection. No studies on polymorphism of *KAP* gene in Dorset breed of sheep could be traced in the literature for comparison.

KAP 6.1 gene had significant ($P < 0.01$) difference in genotype and allelic frequencies for both Sandyno and Nilagiri breeds. The departure from Hardy-Weinberg equilibrium in majority of the loci could be attributed to presence of null alleles, non-random mating, operation of selection over the years and shrinkage in population size (Pramod *et al.*, 2009).

KAP 6.1 gene was found to have high degree of homozygosity (0.6667) in Nilagiri sheep. The effective number of alleles (N_e) for *KAP* 6.1 gene was 1.1690; 1.7006 respectively in Sandyno and Nilagiri breeds of sheep (Table 2). The PIC values for *KAP* 6.1 gene was 0.1341; 0.3909 in Sandyno and Nilagiri breeds of sheep respectively. F_{IS} values for *KAP* 6.1 gene were positive (0.1909) in Nilagiri breed and it was negative (– 0.0110) in Sandyno breed (Table 2). The positive value indicates heterozygote deficiency in the Nilagiri population compared with the Hardy-Weinberg equilibrium. The negative values of F_{IS} indicates that the Sandyno population moved towards out breeding i.e., mating

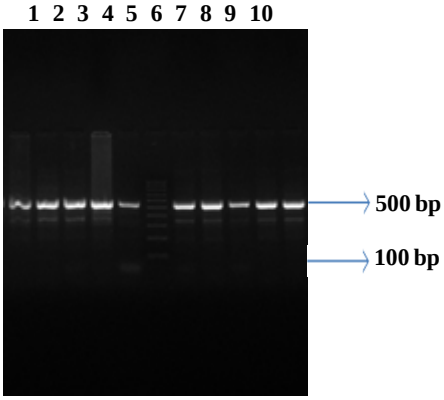

Fig 1: PCR amplified product of *KAP* 6.1 gene of Sandyno and Nilagiri sheep (2 % agarose gel electrop500 bp horesis) {Lane:1 to 10 samples; Lane M:Marker(100 bp)}.

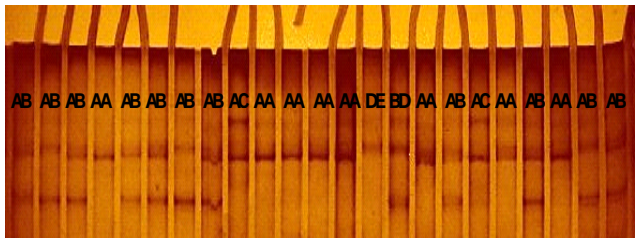

Fig 2: SSCP patterns of PCR products of *KAP* 6.1 genes in Sandyno and Nilagiri breeds of sheep (8 % PAGE electrophoresis).

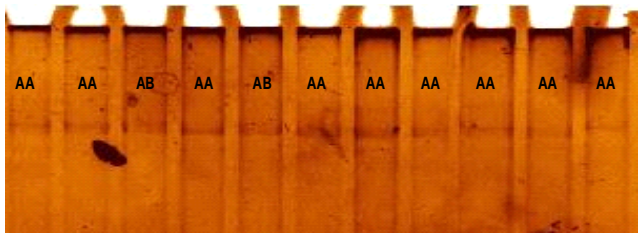

Fig 3: SSCP patterns of PCR products of *KAP* 6.1 gene in Dorset x Nilagiri crossbred sheep (8 % PAGE electrophoresis).

Table 1: Genotype and allele frequencies of *KAP* 6.1 gene in Sandyno and Nilagiri breeds of sheep.

Breed / Group	Total number of animals (n)	Observed Genotypic frequency										Expected Genotype frequency									
		AA	*AB*	*AC*	*BB*	*BC*	*BE*	*DE*	*AA*	*AB*	*BB*	*AC*	*BC*	*CC*	*AD*	*BD*	*CD*	*DD*	*AE*	*BE*	*CE*	*DE*	*EE*
Sandyno	44	0.48 (21)	0.32 (14)	0.07 (3)	0.02 (1)	0.02 (1)	0.07 (3)	0.02 (1)	0.45 (19.66)	0.31 (13.56)	0.05 (2.18)	0.06 (2.71)	0.02 (0.91)	0.00 (0.06)	0.01 (0.67)	0.00 (0.22)	0.00 (0.04)	0.00 (0.00)	0.06 (2.71)	0.02 (0.91)	0.00 (0.18)	0.00 (0.04)	0.00 (0.06)
		AA	***AB***	***AC***	***AD***	***CC***	***EE***		***AA***	***AB***	***BB***	***AC***	***BC***	***CC***	***AD***	***BD***	***CD***	***DD***	***AE***	***BE***	***CE***	***DE***	***EE***
Nilagiri	51	0.59 (30)	0.12 (6)	0.14 (7)	0.08 (4)	0.04 (2)	0.04 (2)		0.57 (28.97)	0.09 (4.57)	0.00 (0.14)	0.16 (8.38)	0.01 (0.65)	0.01 (0.54)	0.06 (3.04)	0.00 (0.23)	0.00 (0.43)	0.00 (0.05)	0.06 (3.04)	0.00 (0.23)	0.00 (0.43)	0.00 (0.15)	0.00 (0.05)

Breed / Group	Total number of animals (n)	Allele frequency					χ^2 value	P value
		A	***B***	***C***	***D***	***E***		
Sandyno	44	0.67	0.23	0.04	0.01	0.04	29.27**	0.00
		A	***B***	***C***	***D***	***E***		
Nilagiri	51	0.75	0.06	0.11	0.04	0.04	73.70**	0.00

Figures in parentheses indicate the number of animals.
**Highly significant ($P < 0.01$), NS: Not significant.

of individuals who are less related than the average relationship of the population. The results showed that *KAP 6.1* gene was found to be reasonably informative in Sandyno breed of sheep. In contrast, Itenge-Mweza, (2012) reported *KAP* 6.1 gene as non-polymorphic and un-informative in Merino cross sheep.

The GFW of *BB* genotype (1.57 ± 0.18 Kg) was higher when compared to *AC* genotype (0.97 ± 0.10 Kg) (Table 3). But, Zhou *et al.* (2015) reported non-significant higher GFW in *BC* genotype (2.26 ± 0.13 Kg) while lowest in *AB* genotype (2.15 ± 0.09 Kg) in Merino cross lambs. Similar results were reported by Yang *et al.* (2012) having GFW values significantly higher in *BB* genotype than that of *AA* and *AB* genotypes in Chinese Merino sheep.

The CWY of *AC* genotype (62.00 ± 1.73 %) was higher when compared to *AB* genotype (60.29 ± 2.39 %) and *AA* genotype (58.80 ± 1.23 %) while low value was recorded for *BB* genotype (56.25 ± 1.74 %) with significant difference (Table 3). In contrary Zhou *et al.* (2015) observed higher yield in *AB* genotype (75.9 ± 1.35 %) and lower yield in *BC* genotype with significant difference (72.0 ± 1.85 %) in Merino cross lambs.

The FD value was observed utmost in *AC* genotype (24.29 ± 1.17 µ) followed by *BB* genotype (23.56 ± 0.98 µ) and *AB* genotype (23.50 ± 1.12 µ) while the lowest was seen in *AA* genotype (23.24 ± 0.49 µ) with significant difference (Table 3) which was similar to the report of Zhou *et al.* (2015) whose finding revealed higher FD values in *BC* genotype (20.3 ± 0.49 µ) with significant difference and lowest in *BB* genotype(18.4 ± 0.39 µ) in Merino cross lambs and conflicting results were reported by Yang *et al.* (2012) with no significant difference between the genotypes and wool FD in Chinese Merino sheep. The finding was similar to the report of Parson *et al.* (1994) who revealed association between *KAP* 6 gene and wool fibre diameter in Peppin Merino using restriction enzyme *BamH1*.

SL value was highest for *AA* genotype (6.23 ± 0.25 cm) and *AC* genotype (5.61 ± 0.18 cm) yields less (Table 3) when compared to other genotypes which was in contrary with reports of Zhou *et al.* (2015) who proposed higher SL values in *BC* genotype (8.58 ± 0.36 cm) and lower in *AB* genotype (8.13 ± 0.29 cm) in Merino cross lambs.

Lower Medullation (%) of *AC* genotype (2.71 ± 1.24 %) was observed when compared to *AA* genotype (4.84 ± 1.63 %) with higher Medullation (%). The examination of literature did not reveal any association studies related to Medullation (%) and hence, cannot be compared. The selection should be practiced towards decreased Medullation (%) which is *AC* genotype also accounts for increased CWY. Deviation in the result obtained in the present study may be due to gene x environmental interaction, changes in the level of expression of gene.

Table 2: Heterozygosity statistics and genetic diversity at *KAP 6.1* gene in Sandyno and Nilagiri breeds of sheep.

Breed	Gene	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	*Ne*	PIC	F_{IS}
Sandyno	*KAP 6.1*	0.5000	0.5000	0.4997	0.4997	1.9785	0.4441	-0.0110
Nilagiri	*KAP 6.1*	0.6667	0.3333	0.5840	0.4160	1.7006	0.3909	0.1909

Ne = Effective number of alleles; PIC = Polymorphic information content; F_{IS} = Fixation index.

Table 3: Least square mean ± S.E for wool traits for *KAP* 6.1 gene in Sandyno and Nilagiri breeds of sheep.

Particulars	N	GFW(kg)	CWY (%)	FD (µ)	SL(cm)	Medullation (%)
Overall mean	76	1.34 ± 0.07	59.41 ± 0.87	23.53 ± 0.41	5.96 ± 0.14	3.94 ± 0.90
Sex						
Male	14	1.65 ± 0.16 ^{a}	54.71 ± 1.31^{a}	21.39 ± 0.72^{a}	5.95 ± 0.19	4.66 ± 3.28
Female	62	1.27 ± 0.08 ^{b}	60.50 ± 0.98 ^{b}	24.02 ± 0.45^{b}	5.96± 0.17	3.77 ± 0.84
Breed						
Nilagiri	41	0.94 ± 0.05 ^{a}	61.78 ± 1.17 ^{a}	24.89 ± 0.55^{a}	5.82 ± 0.23 ^{a}	6.27 ± 1.43 ^{a}
Sandyno	35	1.89 ± 0.08 ^{b}	56.14 ± 1.06 ^{b}	21.65 ± 0.40^{b}	6.14 ± 0.15 ^{b}	0.72 ± 0.47 ^{b}
Genotype						
AA	25	1.35 ± 0.10^{b}	58.80 ± 1.23^{ab}	23.24 ± 0.49^{b}	6.23 ± 0.25	4.84 ± 1.63
AB	15	1.52 ± 0.19^{b}	60.29 ± 2.39^{ab}	23.50 ± 1.12^{b}	5.65 ± 0.24	3.47 ± 1.52
AC	17	0.97 ± 0.10^{a}	62.00 ± 1.73^{b}	24.29 ± 1.17^{a}	5.61 ± 0.18	2.71 ± 1.24
BB	19	1.57 ± 0.18^{b}	56.25 ± 1.74^{a}	23.56 ± 0.98^{b}	5.90 ± 0.29	2.91 ± 1.85

Mean values possessing different superscripts differ significantly ($p<0.05$)

Males had higher Medullation (%) (4.66 ± 3.28 %) and GFW (1.65 ± 0.16 Kg); whereas, females were found to have higher FD (24.02 ± 0.45 µ), SL (5.96 ± 0.17 cm) and CWY (60.50 ± 0.98 %).

CONCLUSION

The SSCP polymorphism of *KAP 6.1* gene revealed seven genotypes in Sandyno sheep and six genotypes in Nilagiri sheep. The Genotype *AA* was more prevalent in both Sandyno (0.48) and Nilagiri (0.59) breeds. The *AC* genotype of *KAP 6.1* gene was associated with higher CWY production (62.00 ± 1.73 %) and low value was recorded for *BB* genotype (56.25 ± 1.74 %) and the homozygote *AA* was found to have association with higher Medullation (%) and SL and the genotype *BB* had a higher GFW and lower CWY. Fibre diameter had a high positive correlation with Medullation (%) and CWY and negatively correlated with GFW.

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