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Tetra-primer ARMS-PCR assay for genotyping SNPs in ovine *GDF8* gene associated with mutton traits in sheep

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DNA polymorphism is useful for identifying variation in known genes of potential economic importance. Animals with the desired trait can be identified and selected to increase productivity and profitability. The identification of the role of growth differentiation factor 8 (*GDF8*) gene in muscle growth and development has led to extensive investigation of this gene in several species. Mutations in *GDF-8* gene were identified in bovine (Kambadur *et al.* 1997), ovine (Clop *et al.* 2006), caprine (Li *et al.* 2006), and canine (Mosher *et al.* 2007) that either inactivate the encoded protein or suppress its quantity causing enhanced muscling. In sheep, single nucleotide polymorphisms (SNPs) identified in the *GDF8* gene were reported to be associated with muscle depth (Hadjipavlou *et al.* 2008, Clop *et al.* 2006), increased forequarter weight, mutton tenderness (Kijas *et al.* 2007) and loin yield/proportion (Hickford *et al*. 2010). The g+6723G>A (renamed as g.6223A>G, Hickford *et al.* 2009.) mutation reported by Clop *et al.* (2006) was found to be virtually fixed in Australian Texel sheep for the A allele and located in the 32 untranslated region (3'UTR) of *GDF8* gene. There is also evidence for additional but as yet uncharacterized mutations possibly located in the promoter region, intron 1, intron 2, or 3'UTR of *GDF8* in various sheep populations (Kijas *et al.* 2007, Hickford *et al.* 2010, Ansary *et al.*2011, Arora *et al*. 2013). This SNP was established as a molecular marker for selection of sheep with improved muscle growth and carcass yields (Hadjipavlou *et al.* 2008, Johnson *et al.* 2009, Kijas *et al.* 2007).

In the present study, the SNPs g.-41C>A and g.6223A>G identified in the 5'UTR and 3'UTR of *GDF8* gene were selected for genotyping, as they were reported to be associated with meat quality characteristics and hyper-muscularity in Australian (Kijas *et al.* 2007) and Chinese (Gan *et al*. 2008) sheep. Although the cost of DNA sequencing has reduced considerably, it is still too expensive to detect SNPs in large number of samples. The tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) is a simple, rapid and economical method for genotyping SNPs. The technique first described by Ye *et al.* (2001), involves two pairs of primers; one specific for each of the nucleotide of the SNP requiring only PCR amplification and subsequent electrophoresis for the determination of genotypes. Therefore, the aim of the study was to design a simple and reliable T-ARMS-PCR assay for genotyping the *GDF8* c.-41C>A and g.6223A>G SNPs and validate their efficiency; and to investigate the effect of the variation on body weight and paunch girth of Indian sheep.

Blood samples of Sangamneri (96), Solapuri (94), Kolhapuri (95), Madgyal (98) and Lonand (57) sheep were collected from their distribution area in Maharashtra. Genomic DNA was extracted using standard phenol/chloroform extraction protocol followed by ethanol precipitation (Sambrook *et al.* 1989). Data were collected for body weight and paunch girth for all animals. Primers were designed using the online web service Primer1 (Collins *et al*. 2012). The details of the primers are given in Table 1.The T-ARMS-PCR was carried out in a final reaction volume of 10 µl, containing 40–80 ng of template DNA, concentration of primers as given in Table 1 and 1× Dream *Taq* master mix (Fermentas). A touch down cycling program was used for the amplification. The PCR amplified products were resolved on 2% agarose gel, in 1×TAE buffer with ethidium bromide.

Genotypes were determined by direct counting of banding pattern observed in the gel. The allelic frequencies were determined by counting the number of either allele and dividing by the total number of alleles. Genotypic frequencies of different patterns were estimated and allele frequencies were calculated from genotypic frequencies. Genetic diversity within and between the sheep populations was estimated in terms of allele frequency, genotype frequency and gene diversity using GenAlex 6 Software (Peakall and Smouse 2006). The association of genotype with body weight and paunch girth was analyzed using the least squares linear fixed model, where gender, age, sex and genotypes were taken as fixed effects. Least square

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Table 1. Summary of primers used for T-ARMS-PCR

	Primer	Primer sequence	Product size (bp)	Concentration of primer used (pM)
SNP g.-41C>A				
	FIP	agcaaaagaaaagtaaaaggaggc	C=136	0.5
	RIP	aatacaatctttttccttagctcttattt	A=275	0.5
	FOP	ggaatacagtataaaagattcactggtg	357	0.3
	ROP	gagctgtttccaggcgaagc	357	0.3
SNP g.6223A>G				
	FIP	tgtcattgtattcaaatctcacca	A=213	0.2
	RIP	tttataagtattaaaataatggcac	G=165	0.2
	FOP	atatgcaaatggttagatggttat	330	0.2
	ROP	caatttatttgattaacaaaatcctga	330	0.2

means were compared using Tukey HSD method. Statistical analyses were performed using JMP Pro of SAS software, version 9.3 (Statistical Analysis System 2012).

The T-ARMS-PCR was optimized for genotyping SNPs g.-41C>A and g.6223A>G in the 5'UTR and 3'UTR of the *GDF8* gene, respectively, as they were reported to be associated with increased forequarter weight, mutton tenderness and muscularity in sheep (Kijas *et al.* 2007, Gan *et al.* 2008, Hadjipavlou *et al*. 2008). Strong association of double muscling and SNP g.-41>A was reported in Chinese sheep (Gan *et al.* 2008). This SNP was previously identified in the heterozygous condition in Indian sheep (Arora *et al.* 2013). The T-ARMS-PCR was first optimized for primer concentration, thermal cycling and other parameters to resolve the genotypes on agarose gel. The standardized protocol was then used to genotype a total of 440 samples of Sangamneri, Solapuri, Kolhapuri, Madgyal and Lonand sheep and the genotype of each sample was recorded. For SNP g.-41C>A, the length of the PCR product amplified by outer primers was 357 bp. The homozygous AA genotype was represented by 357 bp and 275 bp bands and CC genotype was represented by 357 bp and 136 bp bands. The heterozygous AC was represented by three band lengths of 357, 275 and 136 bp (Fig.1a). Representatives (in triplicate) of each genotype were confirmed by sequencing the amplified products using the outer primers FOP and ROP. The allele and genotype frequency for each sheep population at SNP g.-41C>A was estimated (Table 2). Frequency of the A allele varied from 0.293 (Solapuri) to 0.469 (Sangamneri). The frequency of C allele on the other hand ranged from 0.531 (Sangamneri) to 0.707 (Solapuri). The mean allele frequency of A allele (0.382) was lower than that of C allele (0.618). The most abundant genotype was the heterozygous AC (49%), followed by CC (37.4%) and AA (13.7%). The corresponding frequencies of the three genotypes fluctuated between 0.070 (Lonand) and 0.271 (Sangamneri) for AA; 0.246 (Lonand) and 0.511 (Solapuri) for CC; and between 0.394 (Solapuri) and 0.684 (Lonand) for AC genotype (Table 2). The gene diversity or expected heterozygosity values ranged from 0.414 (Solhapuri) to 0.498 (Sangamneri) with an average of 0.465.

For the g.6223A>G mutation, the outer primers gave an amplicon of 330 bp. The GG genotype was represented by two bands of 330 bp and 165 bp. The A allele was neither observed in the heterozygous nor in the homozygous state (Fig. 1b) in the ovine samples investigated. Since the G allele at g.6223A>G was fixed in the Indian sheep investigated, no further association study could be performed. Absence of the g.6223A allele was also reported for several breeds like Suffolk (Hadjipavlou *et al.* 2008), Black Suffolk, Coopworth, English Leicester etc (Kijas *et al*. 2007), which may be attributed to the small sample size of the studies. It may be noted that hypermuscularity is not observed in any of the Indian sheep breeds/populations investigated.

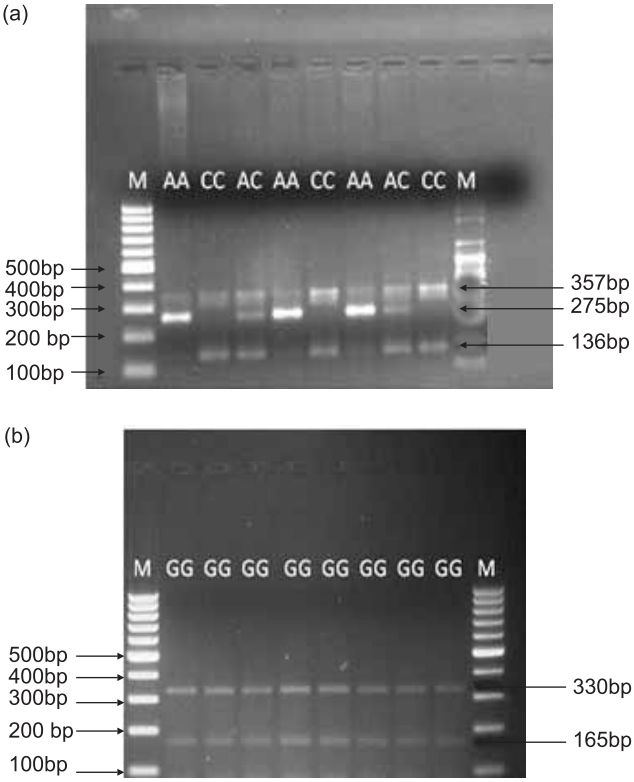

Fig.1 (a). Genotypes of SNP g.-41C>A and (b) genotypes of SNP g.6223A>G in 2% agarose gel, alongside 100 bp ladder**.**

The association analysis for genotypes of g.-41C>A SNP

Table 2. Genotype and allele frequencies at SNP g.-41C>A

Sheep	Sample no.	AA	AC	CC	A	C	Gene diversity
Sangamneri	96	0.271	0.396	0.333	0.469	0.531	0.498
Solhapuri	94	0.095	0.394	0.511	0.293	0.707	0.414
Kolhapuri	95	0.126	0.474	0.400	0.363	0.637	0.463
Madgyal	98	0.122	0.500	0.378	0.372	0.628	0.467
Lonand	57	0.070	0.684	0.246	0.412	0.588	0.485
Mean	–	0.137	0.490	0.374	0.382	0.618	0.465

Table 3. Least square means with standard error for body weight (BW) and paunch girth (PG)

Fixed effect	BW (kg)	PG (cm)
Gender	***	***
Female	35.5±0.35	76.6±0.26
Male	50.9±0.76	84.1±0.57
Age	***	***
12 -24 months	41.2±0.57	78.8±0.43
>2 years	45.3±0.48	81.9±0.36
Ecotype	***	***
Lonand	36.3±0.81	78.1±0.61
Solapuri	45.2±0.67	80.9±0.50
Madgyal	53.2±0.66	85.0±0.49
Kolhapuri	38.6±0.65	76.1±0.48
Sangamneri	42.9±0.64	81.5±0.48
Genotype SNP g.-41C>A	P=0.254	P=0.0493*
AA	42.4±0.77	79.5±0.57
AC	43.6±0.50	80.9±0.38
CC	43.7±0.51	80.8±0.38

*P<0.05, ***P<0.001.

was then performed to study the effect of genotype on body parameters in mutton-type sheep from Maharashtra, India. The phenotypic/biometric records of body weight and paunch girth were analyzed for all the 440 samples investigated. Fixed effects of gender, age and ecotype were highly significant for body weight as well as paunch girth (P<0.0001). The effect of genotype on paunch girth was observed to be significant (P<0.05), however, for body weight the effect was non-significant (P=0.254). Animals with the heterozygous AC and homozygous CC genotype had greater paunch girth (Table 3). The allele frequency distribution in Indian sheep was comparable to that observed in Poll Dorset, Pollwarth, Tibetan and White Suffolk sheep (Kijas *et al.* 2007). The Texel breed, which is meat type showed the least frequency for the A allele (0.028), whereas highest frequency of A allele was observed in English Leicester (0.875), which is a dual purpose longwool breed (Kijas *et al.* 2007). However, Gan *et al.* (2008) reported that the A allele was more common in the meat breeds than in the non meat breeds investigated by them. Our study, on the other hand revealed a higher frequency of the C allele in all the investigated sheep populations, which are mainly reared for mutton.

SUMMARY

Our study indicates the effects of genotypes on paunch girth. The results need to be verified on a larger number of samples. Nonetheless, our study emphasizes the efficacy of the T-ARMS PCR assay for increasing throughput. These assays will enable rapid and cost effective screening of large number of samples for association studies. The generated information can then be utilized in formulation of breeding policies for Indian sheep.

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