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Analysis of Single Nucleotide Polymorphisms in the QTLS Associated with Tick Resistance/Susceptibility in the Native Vechur and Cross red Cattle of Kerala State

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A B S T R A C T

Tick infestation is a major reason for economic loss in livestock production in the tropical countries. However, animals show variation in resistance and susceptibility to tick infestation. *Bos indicus* breeds were reported to be more resistant to tick infestation than *Bos taurus* cattle. The present study aimed to identify polymorphisms in four quantitative trait loci (QTLs) that were stated to be associated with resistance/susceptibility to tick infestation, in a population comprising native Vechur breed (n=45) and crossbred cattle (n=74) of the Kerala Veterinary and Animal Sciences University farms. The QTLs were selected from the Animal QTL Database (https://www.animalgenome.org/cgi-bin/QTLdb/index). The single nucleotide polymorphisms (SNP) and the QTLs (in parenthesis) used in this study were: rs41661020 (#101148), rs43708490 (#135798), rs41577070 (#135800) and rs29009970 (#135801). Blood samples were collected from the animals, isolated the DNA and used for genotyping and polymorphism study by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism and confirmed by sequencing. The results revealed polymorphic pattern for the SNPs rs41577070, rs41664020, and SNP rs43708490 in both Vechur and cross-bred populations. The SNP rs29009970 was monomorphic in the crossbred cattle, whereas, dimorphic in Vechur. Genotyping was done by direct counting and the allelic and genotypic frequencies were estimated by POPGENE 1.32. Chi-square test revealed that crossbreds and Vechur were in Hardy-Weinberg equilibrium for SNPs rs41577070, rs41664020, and rs43708490, whereas, the SNP rs29009970 showed a significant departure from the equilibrium.

Introduction

More than 80% of the cattle population in the world are affected by ticks and tick-borne infections (Food and Agriculture Organisation, 1984) which cause huge economic loss in cattle rearing in tropical and sub-tropical countries including India (Ghosh *et al.,* 2007). The world economy suffers an estimated loss of US$20 to US$30 billion per annum due to ticks and tick-borne infections (TTBDs) (Lew-Tabor and Valle, 2016). The

estimated loss due to TTBDs for the Indian economy is US$ 498.7 million per annum (Minjauw and McLeod, 2003).

Even though, acaricides form the mainstay of tick control methods, the development of acaricide resistant ticks (Li *et al.,* 2005), high price of acaricides and the likelihood of presence of its residues in the animal products made the use of chemical acaricides less attractive (Castro-Janer *et al.,* 2010).Hence, the researchers introduced the idea of using resistant breeds as an alternate strategy for control of tick burden in cattle.In general, *Bos indicus* breeds show significantly higher resistance to tick infestation than *Bos taurus* cattle (Kelly, 1932) and resistant breeds would transmit tick resistance traits to their descendants. However, the selection of resistant animals based on genetic parameters on the basis of repetitive tick counts is difficult and prone to uncontrolled environmental effects (Burrow, 2001; Henshall *et al.,* 2003). Therefore, procedures based on genomics are more objective and hence desirable for identifying tick resistant phenotypes. Hence, attempts have been directed to identify candidate genes and markers associated with resistance against ticks in cattle. Some of the early attempts based on blood protein polymorphisms (Francis and Ashton, 1967) revealed the association of the serum amylase locus with the tick number. Several studies have identified QTLs and SNPs that are associated with host resistance against tick infestation (Gasparin *et al.,* 2007 and Regitano *et al.,* 2008). Later, Machado et al (2010) mapped QTLs on *Bos taurus* (*B. taurus*) autosomes which were associated with season- specific resistance against *Rhipicephalus microplus* (*R. microplus*) in cattle. Mapholi *et al.,* (2016) also reported several genomic regions having QTLs associated with different tick count traits. Many single nucleotide polymorphism (SNP) in the candidate genes such as epidermal growth factor (EGF) latrophilin and seven transmembrane domain containing-1 (ELTD1) (Porto Neto *et al.,* 2010a), Integrin alpha-11 (ITGA11) (Porto Neto *et al.,* 2010b), Interferon gamma (IFNG) (Maryam *et al.,* 2012) and receptor-interacting serine-threonine kinase 2 (RIPK2) (Porto Neto *et al.,* 2012) were identified having association with resistance against ticks.

Hence, the present study aimed to genotype a population of Vechur and crossbred cattle for SNPs in the QTLs associated with resistance /susceptibility to tick infestation in cattle.

Materials and Methods

Genotyping and polymorphism study was carried out by PCR-RFLP assay and confirmed by Sanger sequencing. The design of the experiment was approved by the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Pookode (IAEC/COVAS/PKD/13/2019 dated 17-01-19).Native Vechur (n = 45) and crossbred cattle (n = 74) of the Kerala Veterinary and Animal Sciences University farms formed the population under study. Blood samples were collected and genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega Cat. No. A1125) as per the kit's protocol. The quality, concentration and purity of DNA were evaluated and the DNA working solution was prepared with a final concentration of 25ng/µL. Four QTLs that were reported to have an association with resistance against ticks in cattle were identified from the Animal QTLdb (https://www. animalgenome.org/cgi-bin/QTLdb/index) and one SNP from each QTL was used for genotyping and polymorphism study by PCR-RFLP. The SNPs and the QTLs (in parenthesis) used in the study were: rs41661020 (#101148), rs43708490 (#135798), rs41577070 (#135800) and rs29009970 (#135801). The

sequences flanking the SNPs were downloaded from dbSNP (https://www.ncbi. nlm. nih.gov /snp/) and Ensemble (https://asia.ensembl.org/ index.html) databases and primers were designed (Table1).

The PCR conditions were optimized for each primer pairs which were then used in the final PCR assays. Each PCR reaction mix of final volume of 25µL prepared in PCR tubes comprised of 12.5µL Emerald Amp GT PCR master mix (Takara Cat. No. RR310A), 1µL forward primer (10pmol/µL), 1µL reverse primer (10pmol/µL), 8.5µL nuclease-free water and 2µL DNA working solution (25nm/µL). The amplification conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 seconds; annealing at optimized temperatures (Table 1) for 30 seconds and extension at 72°C for 30 seconds. The final extension was done at 72°C for 5 min. The amplification was verified by subjecting 2µL PCR product to 2 per cent (w/v) submarine agarose gel electrophoresis with 100bp DNA ladder (HiMedia, MBT049-50LN) as a marker.

The restriction enzymes (REs)MboII (#R0148S), AluI (#R0137S) and HpyCH4IV (#R0619S) of New England Bio labs were used for the digestion of the PCR products as per the manufacturer's protocol. A digestion reaction mix prepared in PCR tubes contained 2.5µL of 10 x RE buffer, one unit of restriction enzyme and approximately 22.3µL of the PCR product. The digestion reaction mix was incubated overnight (14 to 16 h) at the optimum temperature prescribed by the manufacturer. Next, enzymes were deactivated at the required temperature in a water bath. The digestion products were subjected to 2.5 per cent (w/v) submarine horizontal agarose (low EEO) gel electrophoresis at 125 V for 3 h with 50bp marker (HiMedia Cat. No: MBT084-50LN). The fragmentation pattern for each SNP was documented with gel documentation system. Details of the cattle SNPs, REs and expected fragment sizes are listed in Table 2.

For confirming the genotypes and polymorphisms, the PCR products were sent to M/S AgriGenom Lab Private Limited, Cochin, India for sequencing by Sanger dideoxy chain termination method. The sequence data were analyzed by FinchTV 1.4.0 (https://digitalworldbiology.com/FinchTV).
The number of individuals belonging to different genotypes were recorded by direct counting (Falconer and Mackay, 1996). The allele and genotype frequencies were estimated by POPGENE 1.32 (https://sites.ualberta.ca/~fyeh/index.html).
The Chi-square test was used to test whether the population was in Hardy-Weinberg equilibrium (HWE).

Results and Discussion

The genotypes and allele frequencies of the SNPs in the population under study are given in the tables 3 to 6. The PCR-RFLP results were confirmed by sequencing (Fig. 1 to 4).

In brief, the PCR-RFLP revealed a polymorphic pattern for the SNP rs41577070 in the cattle population under study, whereas, only two genotypes were seen for the SNPs rs41661020 and rs43708490. However, for the SNP rs29009970, the PCR-RFLP revealed only GC genotype in the crossbred population and GG and GC genotypes in the Vechur population.

The results of the Chi-square test are shown in the tables 7 to 10. By comparing the observed and expected frequencies, it was found that cattle population under study was in HWE in the case of SNP rs41577070,

rs41661020 and rs43708490. However, both the Vechur and crossbred populations were departed from HWE significantly ($p \leq 0.01$) for SNP rs29009970 (Table 9).

Table.1 Oligonucleotide primers with optimized annealing temperatures for the primer pairs used in the study

Cattle SNP ID	Primer Name	Sequence (5' to 3')	Ta (˚C)
rs41577070	TR-2N-F	GCTGAGCCTTGCAAGAACAT	60.8˚C
	TR-2N-R	AGGAGGGTCACAAGGAGTCA	
rs41661020	TR-3-F	GGACTGAGAAAATAGTAGCC	54.0˚C
	TR-3-R	TCTTTTCCTACAACCTCC	
rs29009970	TR-4-F	GTATGTGGTTTTAGGTAGG	54.0˚C
	TR-4-R	CAAACACACACAAAACGC	
rs43708490	TR-5-F	CCTCAGGTTCCTACTGATG	53.0˚C
	TR-5-R	AGGGTCATGTTTTCCATC	

Table.2 Details of cattle SNPs, allele, restriction enzymes used and expected fragmentation patterns

Cattle SNP ID	SNP allele	REs	Fragmentation pattern (approx. size in bp)		
			AA	AB	BB
rs41577070	A/G	MboII	118,174	118,174,292	292
rs41661020	T/C	AluI	238	96,142,238	96,142
rs29009970	C/G	HpyCH4IV	281	98,183,281	183,98
rs43708490	A/G	HpyCH4IV	276	98,178,276	98,178

Table.3 Genotype and allele frequencies of SNP rs41577070 in Vechur and crossbred cattle

Population	Genotype frequency			Allele frequency	
	AA	AG	GG	A	G
Crossbred (74)	0.32 (24)	0.53 (39)	0.15 (11)	0.59	**0.41**
Vechur (45)	0.33 (15)	0.49 (22)	0.18 (8)	0.58	**0.42**
Pooled population (119)	**0.33 (39)**	**0.51 (61)**	**0.16 (19)**	**0.58**	**0.42**

Figures in parenthesis are actual numbers

Table.4 Genotype and allele frequencies of SNP rs41661020 in Vechur and crossbred cattle

Population	Genotype frequency			Allele frequency	
	TT	TC	CC	T	C
Crossbred (74)	0.65 (48)	0.35 (26)	0.0	0.83	**0.17**
Vechur (45)	0.73 (33)	0.27 (12)	0.0	0.87	**0.13**
Pooled population (119)	**0.68 (81)**	**0.32 (38)**	**0.0**	**0.84**	**0.16**

Figures in parenthesis are actual numbers

Table.5 Genotype and allele frequencies of SNP rs29009970 in Vechur and crossbred cattle

Population	Genotype frequency			Allele frequency	
	GG	CG	CC	G	C
Crossbred (74)	0.0	1.0 (74)	0.0	0.5	**0.5**
Vechur (45)	0.07 (3)	0.93 (42)	0.0	0.53	**0.47**
Pooled population (119)	**0.03 (3)**	**0.97 (116)**	**0.0**	**0.51**	**0.49**

Figures in parenthesis are actual numbers

Table.6 Genotype and allele frequencies of SNP rs43708490 in Vechur and crossbred cattle

Population	Genotype frequency			Allele frequency	
	AA	AG	GG	A	G
Crossbred (74)	0.96 (71)	0.04 (3)	0.0	0.98	**0.02**
Vechur (45)	0.96 (43)	0.04 (2)	0.0	0.98	**0.02**
Pooled population (119)	**0.96 (114)**	**0.04 (5)**	**0.0**	**0.98**	**0.02**

Figures in parenthesis are actual numbers

Table.7 Testing of genotypes of SNP rs41577070 for HWE in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df = 1)
		AA	AG	GG	
Crossbred	Observed	24.0	39.0	11.0	**0.48^{ns}**
	Expected	25.45	36.10	12.45	
Vechur	Observed	15.0	22.0	8.0	**0.003^{ns}**
	Expected	14.90	22.20	7.89	
Pooled population	Observed	39.0	61.0	19.0	**0.31^{ns}**
	Expected	**40.47**	**58.06**	**20.47**	

ns - Non- Significant at 5% level
df – degrees of freedom

Table.8 Testing of genotypes of SNP rs41661020 for HWE in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=1)
		TT	TC	CC	
Crossbred	Observed	48.0	26.0	0.0	**3.21^{ns}**
	Expected	50.21	21.58	2.21	
Vechur	Observed	33.0	12.0	0.0	**0.97^{ns}**
	Expected	33.74	10.52	0.74	
Pooled Population	Observed	81.0	38.0	0.0	**4.17^{ns}**
	Expected	**83.97**	**32.06**	**2.97**	

ns- Non- Significant at 5% level
df – degrees of freedom

Table.9 Testing of genotypes of SNP rs29009970 for HWE in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=1)
		GG	GC	CC	
Crossbred	Observed	0.0	74.0	0.0	**73.00****
	Expected	18.37	37.25	18.37	
Vechur	Observed	3.0	42.0	0.0	**33.59****
	Expected	12.67	22.65	9.67	
Pooled Population	Observed	3.0	116.0	0.0	**106.63****
	Expected	**31.14**	**59.71**	**28.14**	

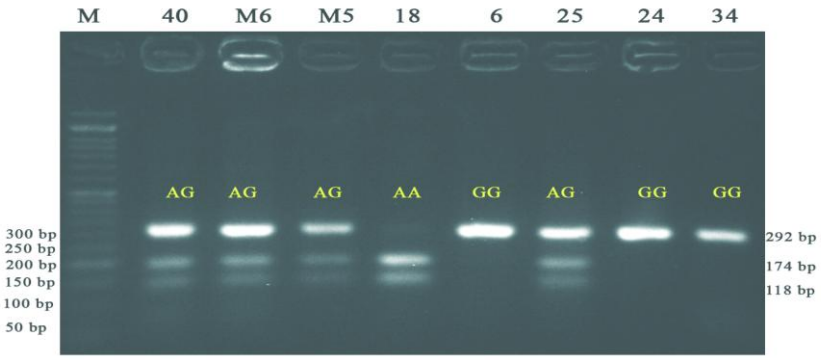
** - Significant at 1% level
df – degrees of freedom

Table.10 Testing of genotypes of SNP rs43708490 for HWE in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=1)
		AA	AG	GG	
Crossbred	Observed	71.0	3.0	0.0	**0.02^{ns}**
	Expected	71.02	2.96	0.0	
Vechur	Observed	43.0	2.0	0.0	**0.01^{ns}**
	Expected	43.01	1.98	0.01	
Pooled population	Observed	114.0	5.0	0.0	**0.04^{ns}**
	Expected	**114.04**	**4.92**	**0.04**	

Ns- Non-significant at 5% level
df – degrees of freedom

Fig.1 Study of genetic polymorphism at SNP rs41577070 of QTL #135800. Representative photographs of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are given on the top of the gel and fragment sizes are shown on the right side. M: Himedia 50bp DNA ladder marker, 2.5% agarose gel, 125 V for 3hrs (A). Chromatogram confirming the AG genotype (B), GG genotype (C) and AA genotype (D)



(A)

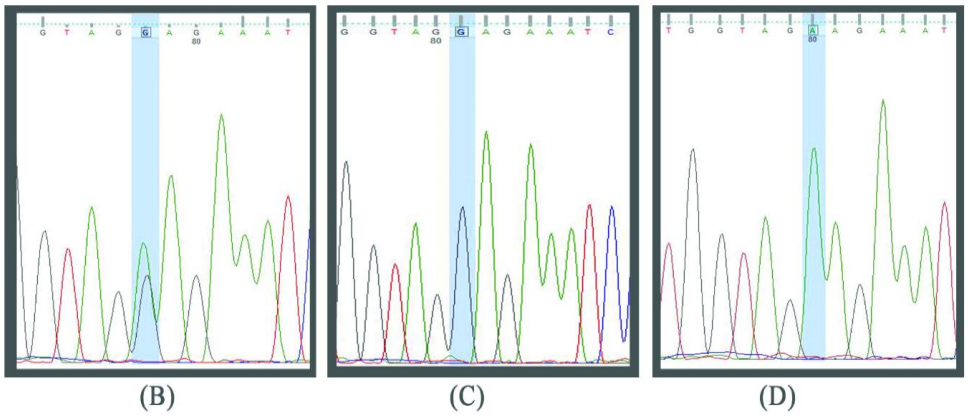
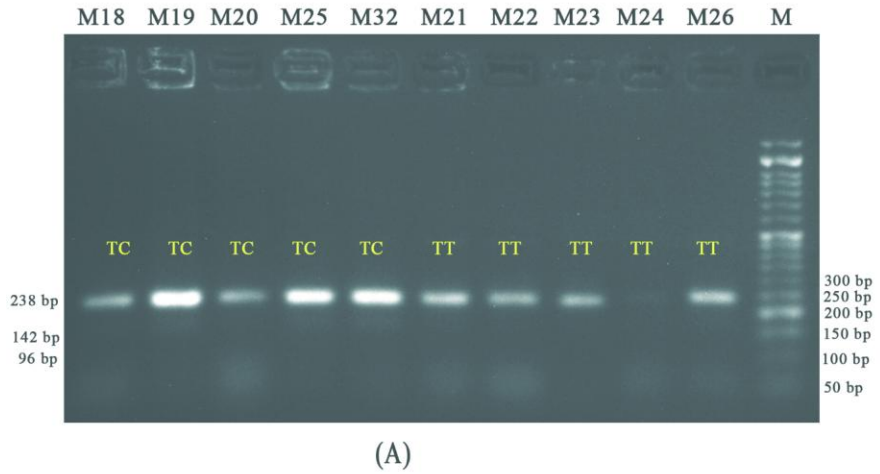


Fig.2 Study of genetic polymorphism at SNP rs41661020 of QTL 101148. Representative photographs of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are given on the top of the gel and fragment sizes are shown on the right side. M: Himedia 50bp DNA ladder marker, 2.5% agarose gel, 125 V for 3hrs (A). Chromatogram confirming the TC genotype (B) andTT genotype (C)



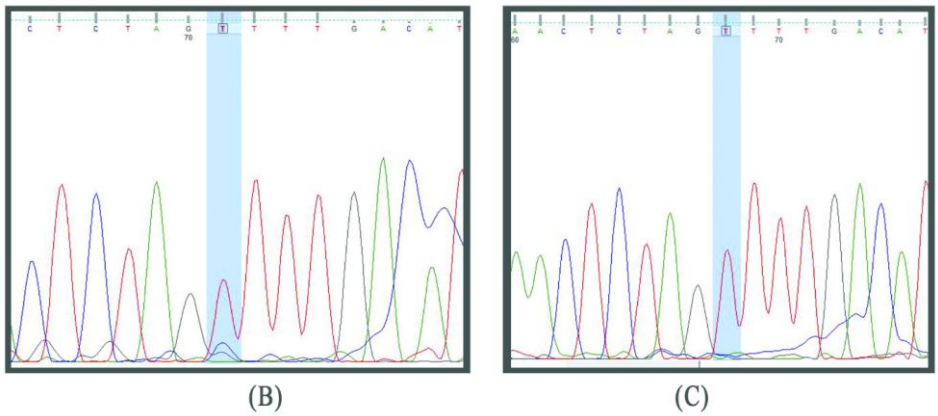


Fig.3 Study of genetic polymorphism at SNP rs29009970 of QTL 135801. Representative photographs of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are given on the top of the gel and fragment sizes are shown on the right side. M: Himedia 50bp DNA ladder marker, 2.5% agarose gel, 125 V for 3hrs (A). Chromatogram confirming the GC genotype (B) andGG genotype (C)

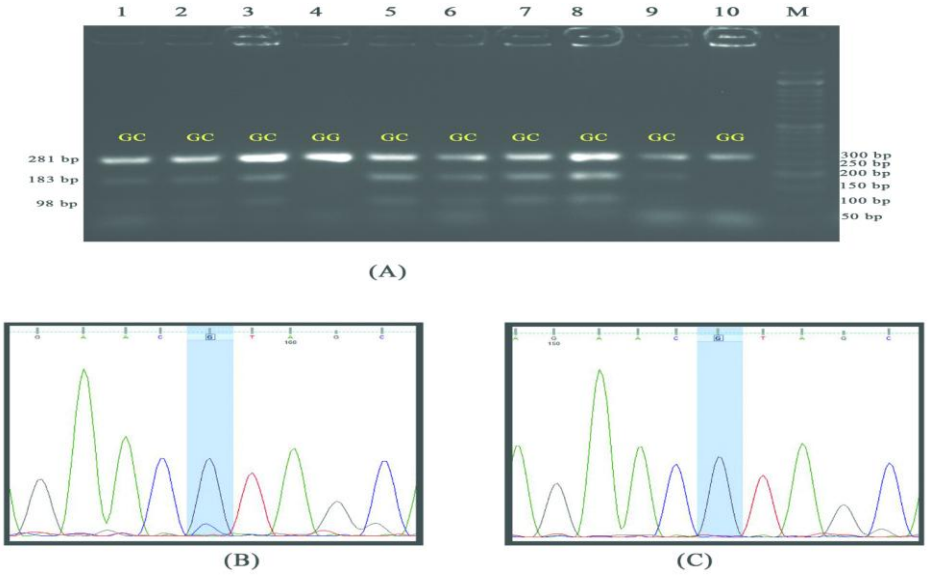
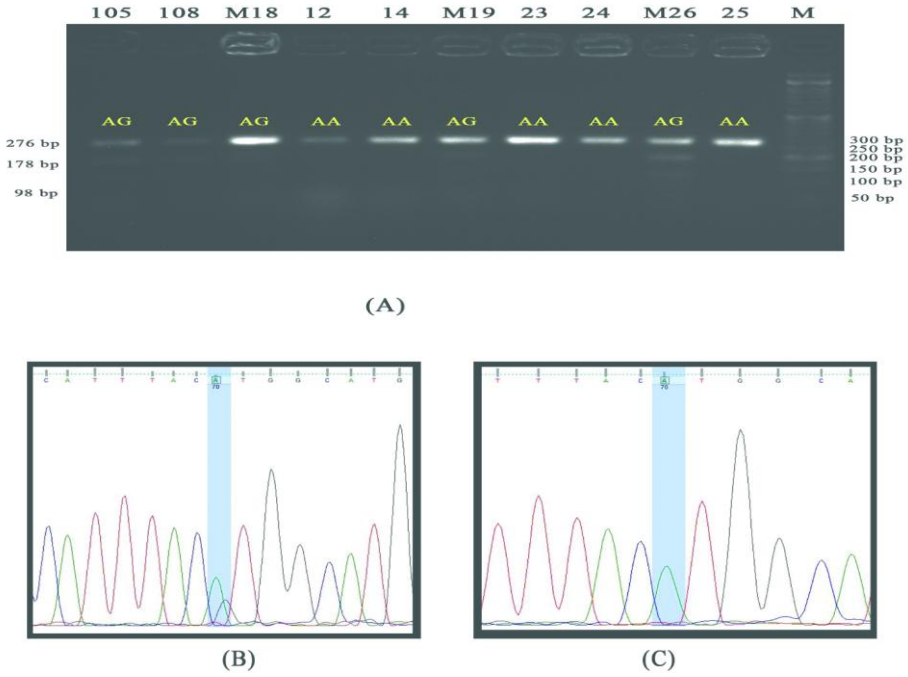


Fig.4 Study of genetic polymorphism at SNP rs43708490 of QTL 135798. Representative photographs of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are given on the top of the gel and fragment sizes are shown on the right side. M: Himedia 50bp DNA ladder marker, 2.5% agarose gel, 125 V for 3hrs (A). Chromatogram confirming the AG genotype (B) and AA genotype (C)



Several factors may be responsible for the deviation of polymorphic loci from the HWE such as small population size, presence of null alleles, Wahlund effect, genetic drift, non-random mating, mutation, migration and selective forces operating at certain loci (Falconer and Mackay, 1996; Phyu *et al.*, 2017). For SNP rs29009970, only CG heterozygote was present in the crossbreds. In the Vechur cattle, 93 per cent of the animals were CG genotypes. Therefore, it could be inferred that there might be some selective advantage for the CG heterozygotes in the cattle population under study.

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