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Identification of single nucleotide polymorphism in the selected quantitative trait loci associated with resistance/susceptibility to tick infestation in Vechur cattle

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Abstract
The present study aimed to identify the polymorphisms in four QTLs that were reported to have an association with resistance / susceptibility to tick infestation in Vechur cattle of Kerala. The population under study comprised of 45 Vechur cattle from the farm. The QTLs and SNPs were selected from the Animal QTLdb. The SNPs 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 selected animals and genomic DNA was isolated and used for genotyping and polymorphism study by PCR-RFLP and confirmed by sequencing. Genotyping was done by direct counting and the allelic and genotypic frequencies were estimated by POPGENE1.32. The results revealed a polymorphic pattern for the SNPs rs41577070, rs41661020, and SNPrs43708490 in the population under study. The SNP rs29009970 was dimorphic. However, the Chi-square test revealed that Vechur were in HWE for SNPs rs 41577070, rs41661020, and rs43708490, whereas, the SNPs rs29009970 showed a significant departure from the equilibrium.

Keywords: Cattle, ticks, QTL, SNP, PCR-RFLP, HWE, Vechur

Introduction
The livestock sector has a central role in the socio-economic progress of India. The progress in this sector is attributed to the developments in various related fields like breeding, management, feeding, and related infrastructure etc. However, frequent outbreaks of infectious diseases and parasitism are the most important challenges critically affecting the growth of the Indian livestock sector. Control of internal and external parasites is important for maximum availability of nutrients to the animal system. Among the external parasites, ticks and tick-borne diseases poses a major threat to the growth, productivity, and economy of the livestock sector in India (Ghosh *et al.*, 2006) [4].
According to FAO (1984) [3], nearly 80 percent of the cattle population in the world is affected by TTBDs, especially in tropical and subtropical countries. In Kerala, TTBDs are a major concern for the livestock sector. There are several reports (Ravindran *et al*., 2002; Ravindran *et al*., 2007; Nair *et al*., 2011a; Nair *et al*., 2011b; Nair *et al*., 2013) [10, 11, 6, 7, 8] on the high prevalence of haemoprotozoan diseases in cattle in Kerala. The control of ticks in cattle became very challenging with the emergence of acaricide resistant ticks. In the above circumstances, it was thought that tick resistant breeds could be used as an alternative to the conventional tick control methods, or at least, would complement the conventional control methods. However, the identification and selection of tick resistant animals based on genetic parameters is difficult, time consuming and expensive. Moreover, the selection of tick resistant phenotypes on the basis of a repetitive tick count is subjected to uncontrolled environmental effects. Therefore, more objective procedures based on genomics, candidate genes and markers associated with tick resistance are desirable for identifying tick resistant phenotypes.
The present study was aimed to identify the polymorphisms in QTLs that were reported to have an association with resistance or susceptibility to tick infestation in Vechur cattle.

Materials and Methods
The design of this experiment was approved by the Institutional Animal Ethics Committee

(IAEC) of the college. Blood samples were collected from 45 Vechur cattle maintained in the Vechur Conservation Centre for Advanced Studies in Animal Genetics and Breeding (CASAGB), Mannuthy, Kerala. Genomic DNA was extracted from whole blood using the Wizard® Genomic DNA Purification Kit. The quality, concentration and purity of DNA were assessed and DNA working solution was prepared to a final concentration of 25 ng/µL.

The QTL #101148, #135798, #135800, and #135801 was downloaded from the Animal QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/index) and SNP rs41661020, rs43708490, rs41577070, rs29009970 located in the QTL was used for the polymorphism study by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay. Custom designed oligonucleotide primers used in this study are listed in (Table No.1). Sequences flanking the SNPs of the QTLs were retrieved from the databases like Animal QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/index), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and Ensemble (https://asia.ensembl.org/ index.html) used for designing the primers. Various bioinformatics tools like Editseq (DNASTAR), Oligo Analyzer from Integrated DNA Technologies (https://eu.idtdna.com/calc/analyzer), NEB cutter V2.0, (http://www.labtools.us/nebcutter), Primer 3 Input (version4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) and Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/) were also used.

Table 1: Oligonucleotide primers used in this study

Primer Name	Sequence (5' to 3')
TR-1-F	GCTGAGCCTTGCAAGAACAT
TR-1-R	AGGAGGGTCACAAGGAGTCA
TR-2-F	GGACTGAGAAAATAGTAGCC
TR-2-R	TCTTTTCCTACAACCTCC
TR-3-F	GTATGTGGTTTTAGGTAGG
TR-3-R	CAAACACACACAAAACGC
TR-4-F	CCTCAGGTTCCTACTGATG
TR-4-R	AGGGTCATGTTTTCCATC

First, the PCR reaction was carried out using custom designed primers in a final volume of 25µL reaction mixture in 0.2 µL thin PCR tubes. The PCR reaction mix contained: 2µL genomic DNA working solution, 12.5µL Emerald Amp GT PCR master mix (Takara Cat. No. RR310A), 1µL Forward and Reverse primer for each QTL and nuclease free water to make the volume 25µL. The amplification was carried out using the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 seconds; The annealing temperature were Optimized for each primer pairs for 30 sec (Table No. 2) and extension at 72 °C for 30sec and final extension at 72 °C for 5 min. The PCR products were resolved in 2 percent (w/v) submarine horizontal low EEO agarose gel electrophoresis with a 100bp DNA ladder (Himedia, MBT049-50LN) as marker for verification of specific amplification.

Table 2: Optimized annealing temperatures for primer pairs used in this study

Primer Name	Ta (˚C)
TR-1-F/ TR-1-R	60.8˚C
TR-2-F/ TR-2-R	54.0˚C
TR-3-F/ TR-3-R	54.0˚C
TR-4-F/ TR-4-R	53.0˚C

The restriction enzyme (RE) digestion of the PCR products was done by using enzyme (Table No. 3) as per the manufacturer's instruction. The digestion reaction mix contained 2.5µL of 10X RE buffer, one unit of restriction enzyme and approximately 22.3 µL of PCR product. The RE digestion reaction was carried out in 200µL thin PCR tubes by incubating overnight (14 to 16 hrs) at the optimum temperature and then the enzyme was deactivated at the required temperature in a water bath.

Table 3: Restriction enzymes, their manufacturers and the catalog numbers used in the study

Restriction enzymes	Manufacturers	Cat. No.
MboII	New England Bio labs	#R0148S
AluI	New England Bio labs	#R0137S
HpyCH4IV	New England Bio labs	#R0619S

Next, the digestion products were subjected to 2.5 percent (w/v) submarine horizontal low EEO agarose gel electrophoresis with 50bp marker (Himedia Cat. No: MBT084-50LN) and the fragmentation pattern was photographed by a gel documentation system. Details of the cattle QTLs, SNPs, REs and expected fragment sizes are listed in Table 4.

Table 4: Details of cattle QTLs, SNPs, allele, REs and expected fragmentation pattern

Cattle QTL ID	Cattle SNP ID	SNP allele	Primers	RE	Fragmentation pattern (approx. size in bp)		
					AA	AB	BB
135800	rs41577070	A/G	TR-1-F TR-1-R	MboII	118,174	118,174,292	292
101148	rs41664020	T/C	TR-2-F TR-2-R	AluI	238	96,142,238	96,142
135801	rs29009970	C/G	TR-3-F TR-3-R	HpyCH4IV	281	98,183,281	183,98
135798	rs43708490	A/G	TR-4-F TR-4-R	HpyCH4IV	276	98,178,276	98,178

The genotype was confirmed by sequencing of the PCR products by M/S AgriGenom Lab Private Limited, Cochin, India by Sanger dideoxy chain termination method. The sequence data were analyzed by Finch TV 1.4 (https://finchtv.software. informer.com/1.4).

The numbers of individuals belonging to different genotypes were recorded by direct counting (Falconer and Mackay, 1996) [2]. The allelic and genotype frequencies were estimated by POPGENE 1.32 (https://www.mybiosoftware.com/ popgene-1-32-population-genetic- analysis.html) for the

analysis of genetic variation among and within populations. The Chi-square test was used to test whether the population was in Hardy-Weinberg equilibrium (HWE).

Results and Discussion

The current study was formulated with the objective to identify the polymorphisms in the selected QTLs that are reported to have association with resistance/susceptibility to tick infestation in Vechur cattle. However, the polymorphism study was carried out by calculating the allele and genotype frequencies. The Chi-square test was used to test whether the population was in HWE.

Genotyping and polymorphism study

Blood samples were collected from the animals under study and genomic DNA was isolated. The concentration and purity of DNA were evaluated and those samples with a minimum optical density ratio of 1.6 (260/280) were used for further procedure. The quality of DNA was assessed by horizontal submarine 0.8 percent agarose gel (w/v) electrophoresis. Good quality DNA samples having a single, clear band without much shearing were obtained (Fig. 1A). The DNA working solution was prepared by diluting the DNA stock solution with nuclease free water to a final concentration of 25ng/µL (Fig. 1B).

The amplified products were screened in submarine 2 percent (w/v) agarose gel electrophoresis (Fig.2). One SNP each from the four QTLs were used for the polymorphism study by PCR-RFLP. The PCR-RFLP technique is most suitable for SNP genotyping especially in small scale laboratories (Yang *et al*., 2010) [12].

The number of individuals belonging to different genotypes was recorded by direct counting (Falconer and Mackay, 1996) [2] and the allelic and genotypic frequencies were estimated by POPGENE 1.32. The Chi-square test was used to test whether the population was in HWE. The HWE is a basic model of population genetics that forms the foundation for evolutionary genetics (Joshi, 2011) [5]. The Hardy-Weinberg principle and its statistical testing have several applications such as allelic variability, microsatellite genotyping, error detection and accuracy of haplotypes estimation. Significant deviations from HWE provide strong evidence that some of the assumptions behind the Hardy-Weinberg principle (like large random mating population with no selection, mutation or migration) are not satisfied and alternative hypotheses could be generated (Chen, 2010) [1].

Table 5: Details of cattle QTLs, SNPs, Genotype, allele frequency and Testing of genotypes for HWE in Vechur cattle (Total-45 cattle)

Cattle QTL ID	Cattle SNP ID	Genotype frequency			Allele frequency		Observed and Expected heterozygosity				χ2value (df=1)
135800	rs41577070	**AA**	**AG**	**GG**	**A**	**G**		**AA**	**AG**	**GG**	
		0.33 (15)	0.49 (22)	0.18 (8)	0.58	0.42	Observed	15.0	22.0	8.0	0.003ns
							Expected	14.90	22.20	7.89	
101148	rs41661020	**TT**	**TC**	**CC**	**T**	**C**		**TT**	**TC**	**CC**	
		0.73 (33)	0.27 (12)	0.0	0.87	0.13	Observed	33.0	12.0	0.0	0.97ns
							Expected	33.74	10.5	0.74	
135801	rs29009970	**GG**	**CG**	**CC**	**G**	**C**		**GG**	**CG**	**CC**	
		0.07 (3)	0.93 (42)	0.0	0.53	0.47	Observed	3.0	42.0	0.0	33.59**
							Expected	12.67	22.65	9.67	
135798	rs43708490	**AA**	**AG**	**GG**	**A**	**G**		**AA**	**AG**	**GG**	
		0.96 (43)	0.04 (2)	.....	0.98	0.02	Observed	43.0	2.0	0.0	0.01ns
							Expected	43.01	1.98	0.01	

Ns-non-significant at 5% level df – degrees of freedom
Figures in parenthesis are actual numbers.

The PCR-RFLP result revealed a polymorphic pattern for the SNP rs41577070 of QTL#135800 (Fig.3), SNP rs 41661020 of QTL#101148 (Fig.4), SNP rs 29009970 of QTL# 135801 (Fig.5), SNP rs 43708490 of QTL # 135798 (Fig.6). The Chi-square test revealed that Vechur were in HWE for SNPs rs 41577070, rs41661020, and rs43708490, was truly indicating inbreeding in the population whereas, the SNPs rs29009970 showed a significant departure from the equilibrium as the observed heterozygosity was significantly higher (at one percent level) than the expected heterozygosity (Table 5). Several factors cause deviation of polymorphic loci in a population 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) [2, 9]. In the Vechur cattle, 93 percent of the animals were CG genotypes. Therefore, it could be inferred that there might be some selective advantage for the CG heterozygotes.

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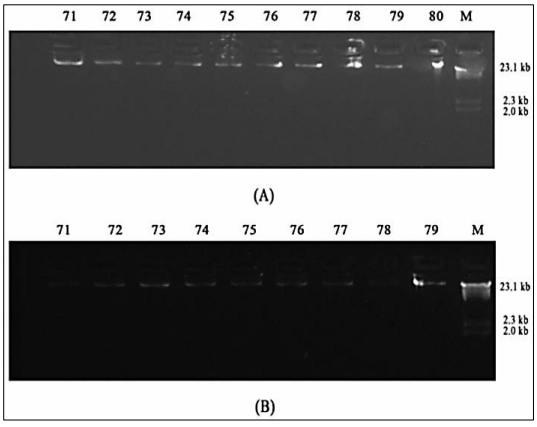

Fig 1: Representative photograph of genomic DNA for checking of its quality (A) and dilution of genomic DNA to make uniform concentration of DNA @ 25ng/µl for all the samples (B).Sizes of the bands from the marker given on the left side and sample numbers are given on the top of the gel. M: Geneilabs Lambda DNA/Hind III Digest Marker, 1% Agarose gel, 125 V for 2 hrs

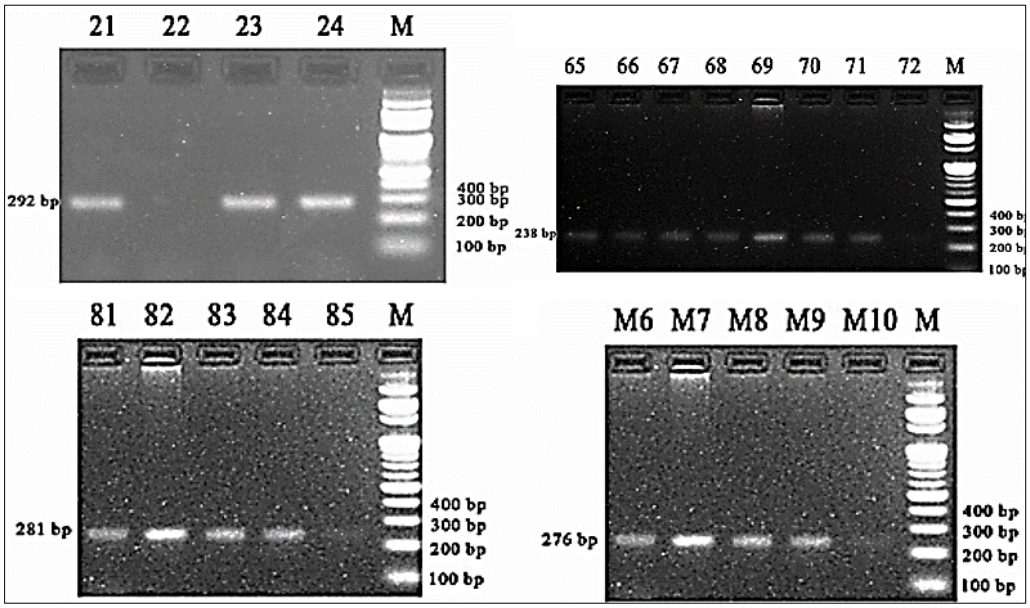

Fig 2: Representative photograph of amplification products during optimization of PCR for the primer pairs TR-1, TR-2, TR-3, TR-4 respectively. The Amplicon size ranges from 238 to 303 bp and are shown on the left side of gel. Sizes of the bands from the marker are given on the right side and sample numbers are noted on the top of each gel. M: Himedia 100 bp DNA ladder marker, 2% Agarose gel, 125V for 2.5 hrs

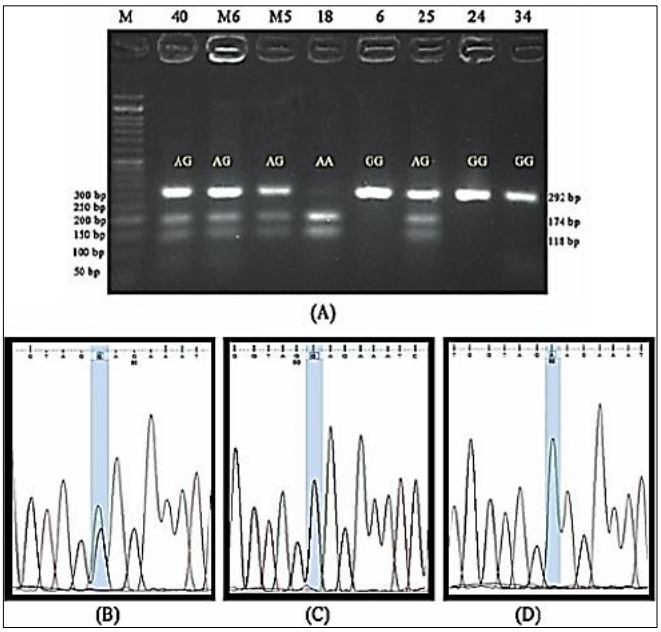

Fig 3: Study of genetic polymorphism at SNP rs41577070 of QTL#135800. Representative photograph of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are noted on the top of each gel and fragment sizes are shown on the right side. M: Himedia 50 bp DNA ladder marker,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

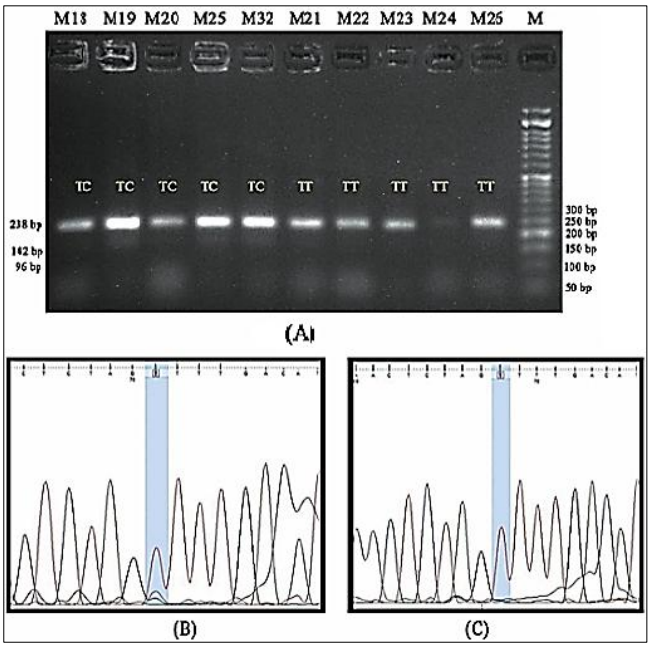

Fig 4: Study of genetic polymorphism at SNP rs 41661020 of QTL#101148. Representative photograph of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are noted on the top of each gel and fragment sizes are shown on the right side. M: Himedia 50 bp DNA ladder marker, 2.5% Agarose gel, 125V for 3 hrs (A).Chromatogram confirming the TC genotype (B), TT genotype (C)

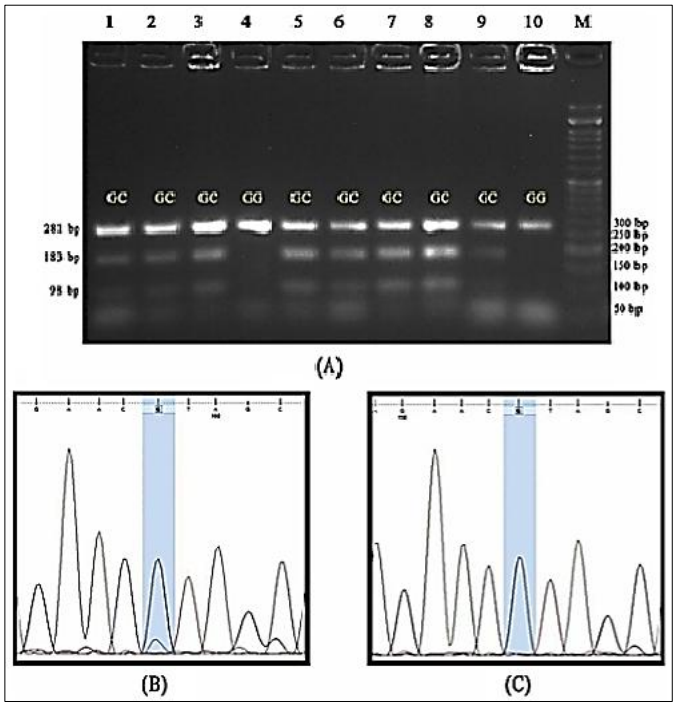

Fig 5: Study of genetic polymorphism at SNP rs 29009970 of QTL # 135801. Representative photograph of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are noted on the top of each gel and fragment sizes are shown on the right side. M: Himedia 50 bp DNA ladder marker, 2.5% Agarose gel, 125V for 3 hrs (A). Chromatogram confirming the GC genotype (B), GG genotype (C).

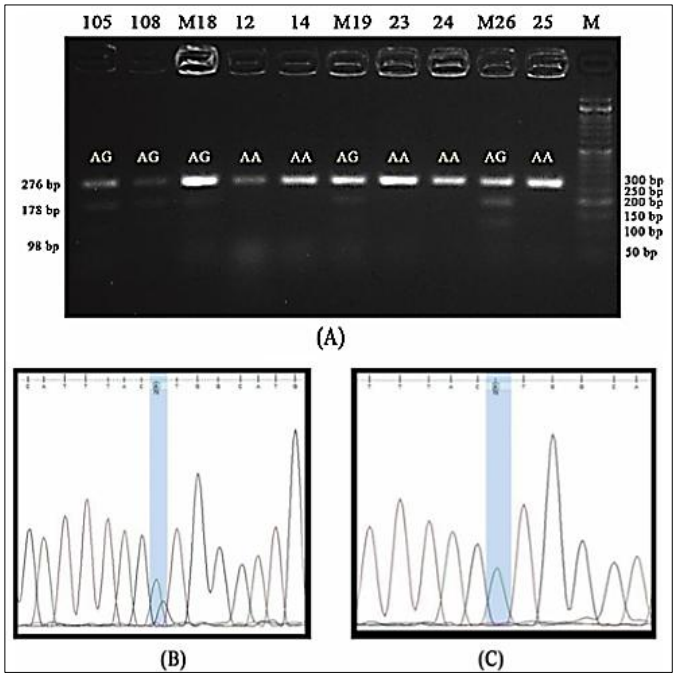

Fig 6: Study of genetic polymorphism at SNP rs 43708490 of QTL # 135798. Representative photograph of PCR-RFLP results. This locus was polymorphic in the population studied. Sample numbers are noted on the top of each gel and fragment sizes are shown on the right side. M: Himedia 50 bp DNA ladder marker, 2.5% Agarose gel, 125V for 3 hrs (A).Chromatogram confirming the AG genotype (B), AA genotype (C).

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