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High-resolution melting analysis of *Phospholipase C gamma 1 (PLCG1)* gene in Vechur and Kasargod cattle of Kerala

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Abstract

Phospholipase C gamma 1 (PLCG1) gene is located on chromosome 13 of the cattle genome and consists of 32 exons. It plays a key role in calcium signalling pathways that contribute to disease resistance in cattle. This study was conducted to identify the polymorphisms in the *PLCG1* gene in the native cattle of Kerala, namely the Vechur and Kasargod which are known for their disease resistance and adaptability to various agro-climatic conditions of the state. A 139bp fragment located in exon 31 of the *PLCG1* gene on chromosome 13 was selected for analysis. High-Resolution Melting (HRM) analysis revealed homozygous CC, AA and heterozygote CA genotypes in both cattle groups. The CA genotype exhibited the highest frequency in both Vechur (0.66) and Kasargod (0.60) cattle. Chi-square analysis indicated that the Vechur cattle population was not in Hardy Weinberg Equilibrium (HWE) ($p \leq 0.01$), whereas, the Kasargod cattle population was in HWE ($p \geq 0.05$).

Keywords: *PLCG1,* Vechur cattle, Kasargod cattle, HRM

The Vechur and Kasargod cattle are two genetic groups of dwarf cattle, native to Kerala, India. They are recognised for their disease resistance, adaptability to the region's hot and humid conditions, low maintenance cost and their significant role in maintaining the biological diversity. Vechur cattle are relatively small, with cows averaging about 133.6±3.7 kg in weight and 89.0±0.7 cm in height, while bulls average around 173.5±6.8 kg in weight and 99.8±1.4 cm in height (Iype, 1996). Red, black, white and its shades were commonly seen in these animals. Chocolate and grey colours were also seen. Hump is prominent for males (Iype, 2013). The Kasargod cattle were characterised by their small size and a variety of colours, including black, brown, white and chocolate, with some animals having spots. Adult males typically weigh an average of 182.5±22.7 kg, while females average 146.4±3.3 kg (Iype *et al*., 2016).

Phospholipase C gamma 1 (PLCG1) gene is located on chromosome 13 of the cattle genome and consists of 32 exons. The gene is involved in calcium signalling pathways that contribute to disease resistance in cattle. Enhanced

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expression of the *PLCG1* has been associated with regulation of intracellular calcium ion concentrations, which plays a role in the increased resistance of cattle to ticks, particularly in the skin following an artificial challenge with *Rhipicephalus (Boophilus) microplus* (Bagnall *et al*., 2009).

The *PLCG1* gene was reported to be associated with signalling pathways such as calcium signalling (Shen *et al*., 2013) and PI3K, AKT, HIF1-α and RAF/ MEK/ ERK cascade (Mandal *et al*., 2021) in human beings. Patel (2019) reported that the mature T-cell lymphomas in human beings were associated with frequent mutations in *PLCG1* gene by a comprehensive analysis of whole exome and targeted gene sequencing manner.

Khan *et al*. (2021) identified ten non-synonymous Single Nucleotide Polymorphisms (SNPs) in *PLCG1* that affected protein stability, significant change in the protein structure and post-translational modifications. Additionally, they examined 5' and 3' UTR regions, where they identified miRNA target sites and DNA binding sites by a computational in silico approach to predict high risk coding and non-coding SNPs of *PLCG1*.

The *PLCG1* has been identified as a candidate gene for immunity in Mengshan cattle and its involvement in calcium signalling pathway was also analysed using Whole Genome Sequencing (Cheng *et al.*, 2024).

Previous studies have explored the role of the *PLCG1* gene in disease resistance in various species, including humans and Mengshan cattle. However, there is limited research on *PLCG1* gene polymorphisms in native Indian cattle, such as Vechur and Kasargod. Most studies focus on specific pathways such as calcium signalling, but the genetic variations within *PLCG1* that influence disease resistance in these groups of animals remain underexplored. This research aims to identify *PLCG1* gene polymorphisms in Vechur and Kasargod cattle, which help us to understand the genetic variations present at the locus, design breeding strategies for enhancing the desired traits and prioritise the conservation goals in native cattle.

Materials and methods

Selection of experimental animals and sample collection

Vechur cattle (n=192) and Kasargod (n=102) which were maintained under farm and field level units of Vechur Conservation Project, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur and Cattle Farm, Badiyadka, Kasargod, Kerala were selected for the study. Whole blood samples (10 mL) were collected in EDTA vials by jugular puncture and the genomic DNA was isolated using the phenol-chloroform extraction method (Sambrook and Russell, 2001). Quality of the isolated genomic DNA was checked by agarose gel electrophoresis and the concentration and purity was estimated by spectrophotometer (NanoDropTM 2000C). The DNA samples were diluted with TE buffer to a final concentration of 50ng/µL.

Polymerase chain reaction (PCR)

The National Centre for Biotechnology Information- Primer Basic Local Alignment Search Tool (Primer BLAST) and Primer 3 (V.0.4.0) open source software/ web application was used to design primers to amplify exon 31 region of *Bos taurus PLCG1* for HRM analysis. The primers of *PLCG1* ranging in the region of 69822215 - 69822353bp of chromosome 13 were used. The quality and specificity of the obtained primers were assessed using Primer BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and Sequence Manipulation Suite (SMS) software. Finally, the selected primers were custom-synthesised by Sigma Aldrich. The sequence information for the primers is given in Table 1. The optimisation of annealing temperatures for the gene fragment was performed through conventional gradient PCR, utilising the Bio-Rad T100™ thermal cycler. The temperature range tested was between 60°C and 65°C. The PCR products were subjected to gel electrophoresis using two per cent agarose to identify any additional (spurious) bands along with the GeneRuler 50 bp DNA (Thermo Scientific).

HRM and genotyping

The HRM reactions were carried out in CFX-Opus 96 Real-Time PCR System using 96-well plates or 8- well strips, which were sealed by adhesive seals or caps. Each DNA sample was amplified in triplicate as technical replicates. For each run, the control set included

Table 2. Reaction mixture used for HRM analysis

Sl. No.	Components	Volume (µL)
1	Sso Fast Evagreen HRM Master Mix	5.0
2	Forward primer (10 pmol/µL)	0.3
3	Reverse primer (10 pmol/µL)	0.3
4	Template DNA (50 ng/µL)	0.5
5	Nuclease free water	3.9
	Total	**10.0**

Table 1. Sequence and properties of primers designed

Oligo name	Primer sequence (5'-3')	Primer length (bp)	Tm (°C)	PCR product size (bp)
PLCG1.F	GAGGGCTCCTTTGAAGCTC	19	63	139
PLCG1.R	CTCTCAGGACCACTCCTCCA	20	63.0	

a non-template control (NTC) and a negative control (Nuclease-free water). The HRM was performed after PCR. The protocol involved an initial denaturation step at 95°C for 30s, followed by rapid cooling to 60°C for 1 min. Subsequently, the final denaturation was carried out by incrementally increasing the temperature from 65°C to 95°C at a rate of 0.2°C for 0.5s per step. Fluorescence data acquisition was performed at each step until reaching 95°C.

The resulting melting curve, analysed by precision melt analysis software (catalog number:1845025), reflects the DNA's specific melting behaviour. Peaks in this curve correspond to different DNA sequences or genotypes. The HRM software then identifies variations like SNPs or mutations, enabling genotyping by comparing sample melting curves to known standard peaks (wild-type or mutant controls). The optimised PCR reaction mix and thermal cyclic parameters are depicted in Table 2 and Table 3.

Sequencing of PCR products

Differences in melting curve shape that correlated to different genotypes were revealed by HRM curve shapes and the precision melt data. The different genotypes were shown as distinct clusters. Heterozygotes could be easily identified by a change in melting curve usually visible as a parabolic curve. Homozygous samples had sharper melting transitions, whereas heterozygotes showed broader transitions. The PCR products from different genotypic clusters were selected and outsourced for sequencing in order to confirm different genotypes. It was performed by automated sequencer using Sanger's

Table 3. PCR amplification protocol for HRM analysis

Stage	Steps	Temperature (°C)	Duration
I	Initial denaturation	95.0	2min
II	Denaturation	95.0	10s
III Repeated for 40 cycles	Annealing/Extension	65.2	30s
IV Melt curve analysis	95.0		30s
	60.0		1min
	65.0-95.0 (0.2°C increments)		0.5s/step
Steps from 2 to 3 were repeated for 40 cycles Melt curve analysis was done after PCR The data acquisition was performed during the final denaturation step			

Table 4. Genotype and allele frequencies of *PLCG1* based on HRM analysis in Vechur and Kasargod cattle

Parameters	Vechur			Kasargod		
	AA	CA	CC	AA	CA	CC
Observed numbers	45	125	18	23	51	11
Expected numbers	61.47	92.06	34.47	27.67	41.65	15.67
Observed frequency	0.24	0.66	0.09	0.27	0.60	0.13
Gene frequency(C)	0.57			0.57		
Gene frequency (A)	0.43			0.43		
Chi- square (*p*- value)	****24.07**			**4.28**		

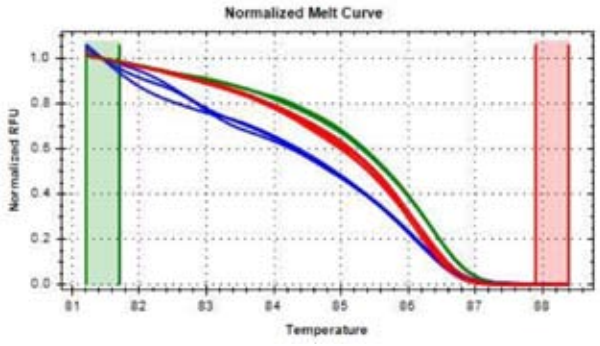

Fig. 1. Normalised melt curve of HRM analysis, showing heterozygous C/A (red), homozygous AA (green) and homozygous CC (blue) genotypes of *PLCG1*

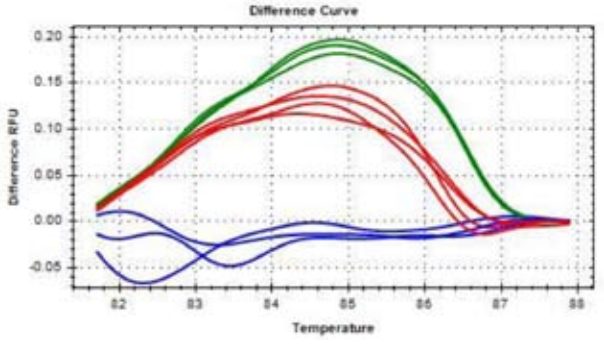

Fig. 2. Difference curve of HRM analysis in *PLCG1*, keeping homozygous CC genotype (blue) as reference

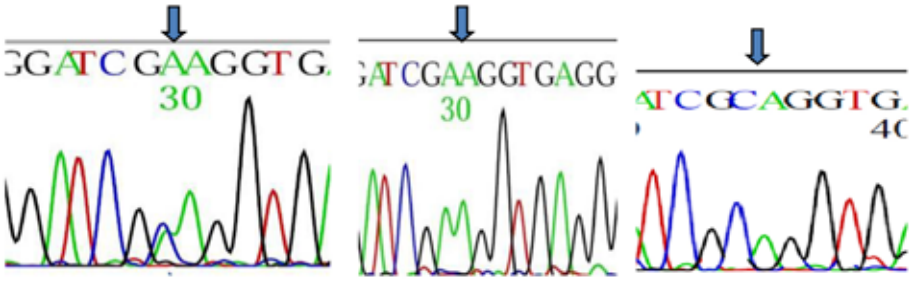

Fig. 3. Sequence map showing heterozygous C/A (i), homozygous AA (ii) and homozygous CC (iii) genotypes of *PLCG1*

dideoxy chain termination method by Genspecs, Pvt. Ltd., Ernakulam, Kerala. The obtained sequences were aligned using EMBOSS merger software and were compared with sequences of selected fragment in Gen Bank utilising BLAST.

The genotype and allele frequencies in the studied population were calculated. Chi-square analysis was conducted to check the Hardy Weinberg Equilibrium (HWE) in both Vechur and Kasargod cattle populations.

Results and discussion

The polymorphism of the 139bp segment of the *PLCG1* gene located in exon 31 on chromosome 13 was assessed using the HRM protocol in both populations of Vechur and Kasargod cattle. The amplified product generated three distinct melt curves which corresponds to three different genotypes homozygous CC, AA and heterozygote CA in the study population (Fig.1 and Fig. 2). The amplicons displaying these varied melt curves were sequenced to confirm the genotypes as heterozygote CA, homozygous AA and CC as illustrated in Fig. 3.

The expression of *PLCG1* can cause an increased resistance of cattle to ticks by regulating the intracellular calcium ion concentrations, particularly in the skin following an artificial challenge with ticks (Bagnall *et al*., 2009).

The *PLCG1* gene was reported to be associated with immunity pathways such as calcium signalling (Shen *et al*., 2013) and PI3K, AKT, HIF1-α and RAF/ MEK/ ERK cascade (Mandal *et al*., 2021) in human beings. Patel (2019) reported that the mature T-cell lymphomas in human beings were associated with frequent mutations in *PLCG1* gene. The author also highlighted five hotspot mutations in *PLCG1* gene, which could enhance the gene activity by elevating inositol phosphate production, downstream NFκB and NFAT activity, augmenting the TCR signalling and demonstrating bonafide gain of function properties by a comprehensive analysis of whole exome and targeted gene sequencing manner.

Khan *et al*. (2021) identified ten non-synonymous Single Nucleotide Polymorphisms (SNPs) in *PLCG1* that affected protein stability, significant change in the protein structure and post-translational modifications. Additionally, they examined 5' and 3' UTR regions, where they identified miRNA target sites and DNA binding sites by a computational in silico approach to predict high risk coding and non-coding SNPs of *PLCG1*. The *PLCG1* has been identified as a candidate gene for immunity in Mengshan cattle and its involvement in calcium signalling pathway using Whole Genome Sequencing (Cheng *et al.*, 2024). The findings of these studies emphasise the significant role played by the gene in disease resistance in both animals and humans. The present study identified a polymorphism in 139bp long segment of the *PLCG1* gene located in exon 31, suggesting that the gene could play a similar role in the native cattle of Kerala. Future researches are needed to explore the use of this SNP as a molecular marker in larger population and to develop more targeted strategies for breeding disease-resistant cattle.

The genotype and allele frequencies of *PLCG1* observed in the studied population are given in Table 4. Chi-square analysis indicated that Vechur cattle population was not in HWE ($p \leq 0.01$), whereas, the Kasargod cattle population was in HWE ($p \geq 0.05$).

Genotyping study of *PLCG* using HRM analysis confirmed three genotypes: AA, CA and CC. The frequencies of the allele 'C' and genotype 'CA' were found to be the highest in both Vechur and Kasargod cattle populations. The Chi-square analysis reveals that Vechur cattle population deviate significantly from HWE, suggesting potential factors such as selection or inbreeding affecting this population. The deviation from HWE may be due to the presence of factors such as inbreeding, population substructure, or errors in genotyping (Chen *et al*., 2017). In contrast, the Kasargod cattle population appears to be in HWE indicating a stable allele frequency distribution. Identifying and validating these SNPs can help in breeding programmes aimed at improving disease resistance in these cattle populations. This work underscores the importance of genetic markers in livestock improvement strategies.

Conclusion

The HRM genotyping of *PLCG1* revealed AA, CA and CC genotypes. In both populations heterozygotic frequency was higher and the Vechur cattle population

was found to be deviated from HWE. The identified SNP can exert either synonymous or non-synonymous effects, depending on its position and impact. It can serve as reliable marker for marker-assisted selection, especially, based on the results of association studies conducted in larger populations.

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Conflict of interest

The authors declare that they have no conflict of interest.

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