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Polymorphism of Mitogen-activated protein kinase 12 *(MAPK12)* gene in Vechur and Kasargod cattle of Kerala

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Abstract

The mitogen-activated protein kinase 12 *(MAPK12)* gene, located on chromosome 5 of the cattle genome and consists of 12 exons. This gene plays a crucial role in sphingolipid signalling and Wnt signalling pathways, both of which are associated with thermo-tolerance in cattle. This study was conducted to investigate genetic polymorphisms in the MAPK12 gene in Vechur and Kasargod, the native, dwarf cattle of Kerala. They are known for their disease resistance, resilience to biotic and abiotic stresses and their ability to thrive under limited feed resources in varied agro-climatic conditions of the state. A missense mutation (G>A) was identified in exon 8 of the *MAPK12* gene at 119621852bp position by genotyping using High-Resolution Melting (HRM) analysis. The genotyping revealed AA and GA genotypes as distinct clusters in plots. The frequency of the AA genotype was the highest in both Vechur (0.91) and Kasargod cattle (0.79). Chi-square analysis indicated that both populations were in Hardy-Weinberg equilibrium (HWE) ($p \geq 0.05$). Structural annotation of the variation in *MAPK12* protein was examined by various computational tools, which indicate that the SNP lead to structural conformational changes in the protein structure when compared to the wild-type *MAPK12* protein.

Keywords: *MAPK12,* Vechur cattle, Kasargod cattle, HRM analysis, polymorphism

Kerala, a state in India, is home to a rich diversity of livestock genetic resources. Among these, the Vechur and Kasargod cattle stand out as unique, dwarf breeds native to the region. These cattle are renowned for their resilience to biotic and abiotic stresses, their ability to thrive under limited feed resources, and their adaptability to the varied agro-climatic conditions of the state (Iype, 1996; Iype *al*., 2016). The Mitogen-activated protein kinase 12 *(MAPK12)* gene, located on chromosome 5 of the cattle genome, consists of 12 exons and four transcripts. There are six different types of MAPKs. They were categorised into three subfamilies, such as ERK, JNK and p38. The *MAPK12* was included in the p38 subfamily, which is activated by factors related to different stress conditions (Teramoto and Gutkind, 2013). The gene has been significantly enriched in the prolactin signalling pathway, which plays a role in thermo-tolerance in livestock.

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Prolactin is a hormone that regulates its own release and expression (Hinuma *et al*., 1998), and elevated plasma prolactin levels have been linked to altered metabolic states in heat-stressed animals (Sunilkumar *et al*., 2011; Gupta *et al*., 2013). The *MAPK12* gene is also involved in regulating the body's water balance through aldosterone secretion, with animals adapted to hot, arid environments displaying lower water intake and turnover (Mirkena *et al*., 2010).

In addition, *MAPK12* is implicated in sphingolipid signalling and Wnt signalling pathways, both of which have been associated with thermo-tolerance in cattle, as observed in three East African Indicine breeds through Whole Genome Sequencing (Taye *et al*., 2018). Studying polymorphisms in this gene will offer valuable insights into genetic variations at this locus, facilitating the development of targeted breeding strategies to enhance desirable traits and guide the prioritisation of conservation efforts for native cattle. The studies on the polymorphism of the *MAPK12* in indigenous cattle of Kerala have not been published earlier. These kinds of studies are required for further exploration to prove the uniqueness of Vechur and Kasargod cattle of Kerala. This study was designed to investigate a 98bp long region in exon 8 of *MAPK12* to identify polymorphisms in Vechur and Kasargod cattle, contributing to a deeper understanding of their genetic makeup.

Materials and methods

Polymerase Chain Reaction

Vechur cattle (n=192) and Kasargod cattle (n=102), maintained under the farm and field level units of Vechur Conservation Project at the Centre for Advanced Studies in Animal Genetics and Breeding, Mannuthy, Thrissur, and at the Cattle Farm, Badiyadka, Kasargod, Kerala, were selected for the study. DNA was isolated from whole blood samples using the phenol-chloroform extraction method (Sambrook and Russell, 2001), and quality was assessed by agarose gel electrophoresis, with concentration and purity measured using a NanoDrop™ 2000C spectrophotometer (Thermo Scientific, USA).

The primers were designed in the exon 8 region of *B. taurus MAPK12* (NCBI NC_037332.1) sequence using the Primer 3 (www.ncbi.nlm.nih.gov). The specificity of the primers was confirmed through a BLAST search to ensure homology (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The forward primer (5' CGGTGTAGGATACGGTCGTT 3') and reverse primer (5' GCTTCTGTCTCAGTGGACATCT 3') targeted a region of the *B. taurus* chromosome 5, specifically between nucleotides 119621819bp and 119621916bp. A gradient PCR using a Bio-Rad T100™ thermal cycler with annealing temperatures ranging from 60°C to 65°C was done. An annealing temperature of 62.5°C was selected as optimum for PCR amplification. The concentration of master mix, primers, template DNA and nuclease-free water used in the reaction mixture was 5µL, 0.3µL (10pmol/µL), 0.5µL (50ng/µL) and 3.9 µL, respectively. Following amplification, the PCR products were analysed by gel electrophoresis on two per cent agarose gel. The presence of any spurious bands was monitored using the Gene Ruler 50bp DNA ladder (Thermo Scientific, USA) as a marker.

High Resolution Melting (HRM) analysis

The HRM reactions were performed on the CFX-Opus 96 Real-Time PCR System using 96-well plates or 8-well strips, sealed with adhesive seals or caps. DNA samples were amplified in triplicate, and each run included a non-template control (NTC) and a negative control (nuclease-free water). The reaction mixture consisted of 1µL of genomic DNA (50 ng/µL), 5µL of SsoFast EvaGreen HRM Master Mix (1X), 0.3 µL of each primer (10 pmol/µL), and 3.4µL of nuclease-free water. The HRM was conducted post-PCR with the following protocol: initial denaturation at 95°C for 30s, followed by rapid cooling to 60°C for 1 min. Final denaturation was achieved by gradually increasing the temperature from 65°C to 95°C at 0.2°C increments for 0.5s per step, with fluorescence data acquired at each step until 95°C. The optimised thermal cyclic parameters are depicted in Table 1.

Table 1. PCR amplification protocol for HRM analysis

Stage	Steps	Temperature (°C)	Duration
I	Initial denaturation	95.0	2 min
II	Denaturation	95.0	10 s
III Repeated for 40 cycles	Annealing/Extension	65.2	30 s
IV Melt curve analysis	95.0		30 s
	60.0		1 min
	65.0-95.0 (0.2°C increments)		0.5s/step
Steps 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			

Genotyping and sequencing

The resulting melting curve, analysed by precision melt analysis software (catalogue number: 1845025), reflects the DNA's specific melting behaviour. Peaks in this curve correspond to different DNA sequences or genotypes. The HRM enables genotyping by comparing sample melting curves to known standard peaks (wild-type or mutant controls). These genotypes were shown as distinct clusters. The melting profiles of the samples were compared to the reference genotype, represented as a horizontal line in the difference graph.

The PCR products from different genotypic clusters were selected and outsourced for sequencing using Sanger sequencing (Genspecs Pvt. Ltd., Ernakulam, Kerala) to confirm the different genotypes. These verified samples were then used as reference controls for HRM analysis of the remaining samples. The obtained sequences were aligned using EMBOSS Merger software and compared with sequences of the selected fragment in GenBank using BLAST.

Population parameters of MAPK12

Genotype and allele frequencies were calculated for the studied population, and Chi-square analysis was performed to assess HWE in both Vechur and Kasargod cattle populations.

Prediction of protein structure

The structure of the *MAPK12* protein was examined by SWISS-PROT and I-TASSER bioinformatics tools.

Results and discussion

HRM analysis of MAPK12

The polymorphism of the *MAPK12* gene was assessed using the HRM protocol in both populations of Vechur and Kasargod cattle. The amplified product was a 98bp fragment, which produced two distinct melt curves that correspond to two different genotypes in the validation population (Fig.1 and Fig. 2). The amplicons displaying these varied melt curves were sequenced to confirm the genotypes as AA and heterozygote GA, as illustrated in Fig. 3.

The *MAPK* signalling cascade is essential for transmitting extracellular signals to the nucleus and consisted of three level kinase cascade which regulated various biological processes such as adipogenesis, apoptosis, migration of cells, muscle development and survival of the cells (Wang *et al*., 2020; Chen *et al*., 2022). The MAPKKK (MAPKK kinase) protein is the most upstream in the signalling pathway, which activates MAPKK (MAPK kinase) in response to extracellular signals, which in turn activate MAPKs and the activated MAPKs primarily phosphorylate target proteins at the TxY motif within the T-loop and affects serine and threonine residues (Li *et al*., 2023).

The *MAPK12* was significantly enriched in "prolactin signalling pathway", which was involved in thermo-tolerance mechanisms in livestock. Prolactin was a hormone that stimulates its own release and regulates its expression (Hinuma *et al*., 1998) and elevated plasma prolactin levels have been linked to altered metabolic states in heat-stressed animals (Sunilkumar *et al*., 2011; Gupta *et al*., 2013). The gene regulated the water balance of the body through the secretion of aldosterone, and the animals adapted to hot arid environments display lower overall water intake and turnover (Mirkena *et al*., 2010). It elicited cellular responses favouring survival or apoptosis (Sugimoto *et al*., 2012). The *MAPK12* is involved in sphingolipid signalling and Wnt signalling pathways were observed to be associated with thermo-tolerance in three East African Indicine cattle breeds by Whole Genome Sequencing (Taye *et al*., 2018). These findings underscore the critical role played by the gene in the thermo-tolerance mechanisms of livestock. So, in this study, the polymorphism of the *MAPK12* gene in Vechur and Kasargod cattle from Kerala was identified, which indicated that the gene could play a similar role in the native cattle of Kerala. Future research into the specific polymorphisms within the *MAPK12* gene and their functional implications could provide more targeted strategies for breeding heat-tolerant cattle and marker-assisted selection.

Sequencing of the amplicons of the *MAPK12* revealed the presence of two SNPs at position 671 (a G→A transition) within the coding regions. Further analysis indicated that the c.671G>A mutation is a non-synonymous mutation (SO:0001583), resulting in a missense variant with moderate effect. This mutation causes a codon change from TGT to TAT, leading to an amino acid substitution of p.Thr224Ile (from Threonine to Isoleucine). The SIFT score for this mutation is 0.43 in the canonical transcript (Ensembl database). This amino acid substitution alters the polarity of the residue from polar (Threonine) to non-polar (Isoleucine), which could potentially affect protein folding, stability and interactions with other molecules.

Population parameters of MAPK12

The genotypic frequency of 'AA' was notably higher in both Vechur (0.91) and Kasargod (0.79) cattle than the heterozygous 'GA' genotype (Table 2). The frequency of allele 'A' was observed to be high in Vechur cattle (0.95) compared to Kasargod cattle (0.89). Chi-square analysis indicated that both populations were in HWE, suggesting that the allele and genotype frequencies are stable over time. It indicated the absence of factors such as inbreeding in the population, population substructure, or errors in genotyping (Chen *et al*., 2017).

Table 2. Genotype and allele frequencies of *MAPK12* based on HRM analysis in Vechur and Kasargod cattle

Parameters	Vechur			Kasargod		
	AA	GA	GG	AA	GA	GG
Observed numbers	173	17	0	61	16	0
Expected numbers	173.38	16.24	0.38	61.83	14.34	0.83
Observed frequency	0.91	0.089	0	0.79	0.21	0
Gene frequency (A)	0.95			0.89		
Gene frequency (G)	0.04			0.10		
Chi-square (*p*- value)	**-0.34**			**-0.63**		

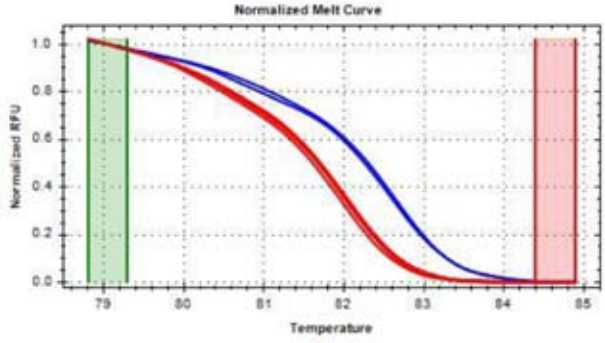


Fig. 1. Normalised melt curve of HRM analysis, showing homozygous AA (red) and heterozygous GA (blue) genotypes of *MAPK12*

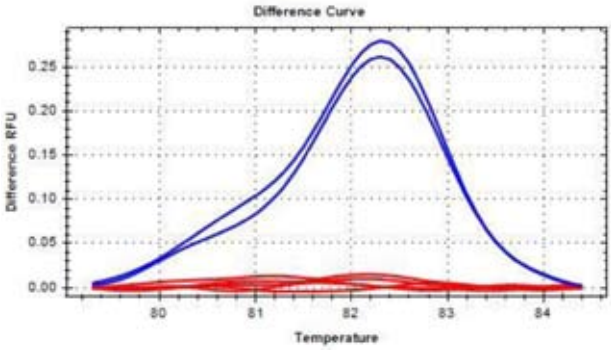
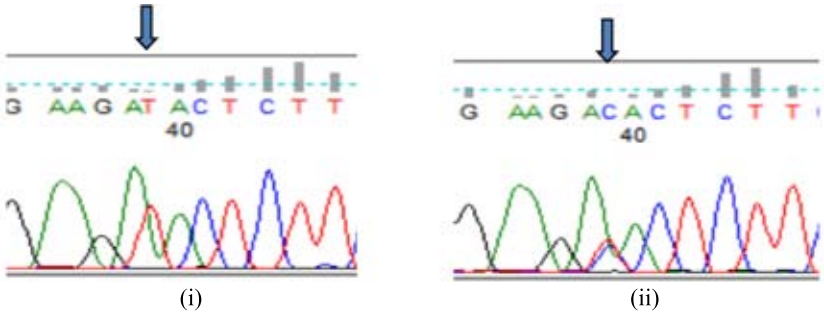
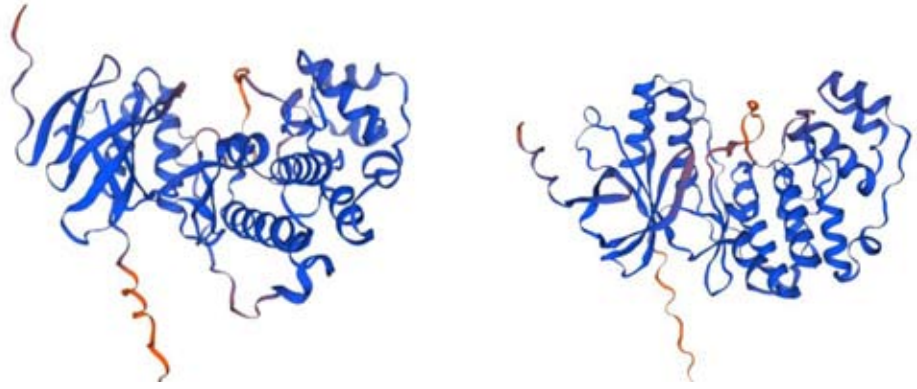


Fig. 2. Difference curve of HRM analysis in *MAPK12,* keeping homozygous AA genotype (red) as reference



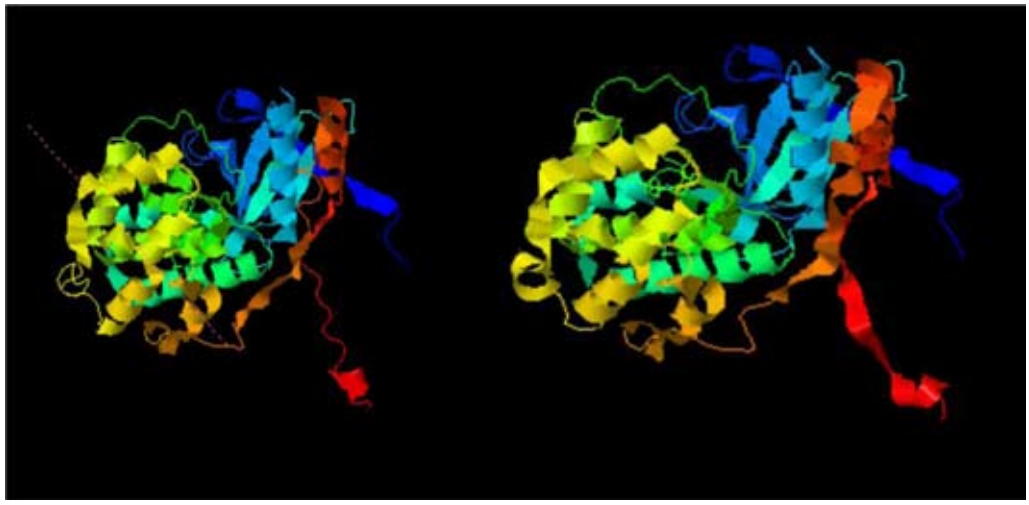
(i) (ii)

Fig. 3. Sequence map showing homozygous TT* (i) and heterozygous CT* (ii) genotypes of *MAPK12* (*reverse complement)



(i) (ii)

Fig. 4. SWISS PROT showing the predicted structure of wild (i) and mutant (ii) genotypes of *MAPK12* protein



(i) (ii)

Fig. 5. I-Tasser result showing the predicted structure of wild (i) and mutant (ii) genotypes of the *MAPK12* protein

Both populations being in HWE means that the observed allele and genotype frequencies are stable, implying no significant evolutionary forces are influencing them. The differences in allele and genotype frequencies could reflect distinct historical breeding practices or environmental adaptations between the two populations. Overall, Vechur shows a strong dominance of allele 'A', whereas Kasargod has more genetic variability with the presence of allele 'G'.

Structural implication of SNP on MAPK12 protein

The protein structure of *MAPK12* protein predicted by SWISS-PROT indicated a mild structural conformational change in the protein structure (Fig. 4).

The structural annotation of the *MAPK12* protein was conducted using the I-TASSER, which uses the SPICKER program to cluster all the decoys based on the pair-wise structure similarity. The C-score usually ranges from [-5, 2], with higher values indicating greater confidence in the model and lower values suggesting less confidence. In the study, structural conformational change was observed in the tertiary structure of the *MAPK12* protein with a C-score of 0.06 for wild-type protein and 0.21 for mutant Protein (Fig. 5). The increase in C-score> - 1.5 signifies a model with high confidence (Roy *et al.*, 2010).

Conclusion

The HRM genotyping of the *MAPK12* gene revealed AA and GA genotypes, with the AA genotype being more frequent in the Vechur (0.91) and Kasargod (0.79) populations. These populations showed a higher frequency of the altered A allele compared to *B. taurus* cattle, indicating they are genetically distinct at this locus. Furthermore, both populations were found to be in HWE, suggesting that the allele and genotype frequencies are stable over time. The structural impact of the variation was examined by computational tools indicated structural conformational change in the protein. Based on the study, it can be concluded that the identified SNP might be contributing to the expression of traits associated with thermo-tolerance in native cattle.

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Conflict of interest

The authors declare that they have no conflict of interest

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