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Original article

Identification of Heat Stress-Associated the HSPA1A (HSP70) Gene in Holstein and Turkish Grey Cattle

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Abstract

Genetic research focuses on breeds that adapt to harsh climatic conditions against the adverse effects of global warming in the livestock industry. Turkish Grey cattle are highly resistant to adverse climatic and natural conditions and against parasitic diseases. The HSPA1A gene encodes the HAPA1A (HSP70) protein, which protects cells against many stress factors. This study investigated polymorphisms in the HSPA1A gene by DNA sequencing in Holstein (n=70) and Turkish Grey Cattle (n=70). The 23 and 21 SNPs were detected in Turkish Grey and Holstein cattle, respectively. The six SNPs were identified in the 3´-UTR region and 18 SNPs in the exonic region (15 synonymous SNPs, 3 non-synonymous SNPs) of the gene. The three non-synonymous SNPs (nsSNP), rs382492082, rs385826597 and rs384294013 lead to Met5Ile, Met5Thr and Gly626Ala substitution, respectively. The effects of nsSNPs on protein structure and function were evaluated using ConSurf, HOPE project, SHIFT and DUET tools. The ConSurf and SHIFT analyses suggest that the amino acid substitutions are likely well-tolerated and have low evolutionary conservation, implying that these changes might not significantly impact the protein's function. In contrast, the HOPE project and DUET analyses indicate potential structural and functional disruptions caused by these mutations. Additionally, haplotype analysis indicates distinct genetic structures between Turkish Grey and Holstein cattle, suggesting diverse evolutionary pressures and historical recombination events. The SNPs identified in this study may guide genetic marker-assisted breeding to improve thermotolerance in domestic and exotic cattle.

Keywords: HSP70, HSPA1A, Thermotolerance, nsSNP, Cattle.

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INTRODUCTION

Global climate change and its associated challenges significantly complicate the lives of farm animals, especially those with economic importance. Climate change dramatically impacts animal welfare, fertility, health, and production (Paula-Lopes, Lima, Satrapa, & Barros, 2013; Summer, Lora, Formaggioni, & Gottardo, 2019). Selecting thermotolerant cattle is a crucial strategy to mitigate the effects of global warming. Intraspecific genetic variations lead to differences in thermotolerance capacity, enabling the genetic or genomic selection of cattle resistant to heat stress (Ansari-Mahyari, Ojali, Forutan, Riasi, & Brito, 2019).

Turkish Grey Cattle, which have a common origin with some local grey cattle breeds in Europe, descend from the wild ancestor *Bos taurus primigenius*, reported to have gone extinct in the 16th century (Felius, Koolmees, Theunissen, Consortium, & Lenstra, 2011; Kök, 2017; Pariset et al., 2010). This breed is favored in rural areas due to cold and heat tolerance and its resistance to parasites (Kök, 2017). Historically, poor farmers in Turkey's Thrace region widely used Turkish Grey Cattle for beef production (Soysal & Kök, 2006). However, their population has declined significantly. Currently, one herd is under ex-situ protection and twelve herds are under in-situ protection by the Ministry of Agriculture and Forestry (Kök, 2017).

Despite advanced technologies like cooling pads and mist spraying to control farm animals' environments against rising temperatures, managing heat stress in animals remains costly for enterprises. Therefore, modern, practical, and sustainable approaches are needed to address the challenges of heat stress caused by climate change. One such approach is to develop animal breeds with high thermotolerance by investigating the genetic mechanisms of heat-stress-resistant animals. Research on cattle breeds has identified many genes that may be associated with heat stress response (Bhat et al., 2016; Hassan et al., 2019; Kerekoppa et al., 2015; Onasanya et al., 2021). Instead of randomly searching for genes across the entire genome, a more effective strategy involves focusing on genes suspected to play a role in the trait of interest (Tesema, Taye, & Ayichew, 2019). The HSPA1A protein is particularly noteworthy in this context, as it is involved in various regulatory pathways related to the response to cell stress. It has significant cytoprotective effects, helping protect cells from heat shock by preventing cellular proteins from denaturing. Additionally, HSPA1A plays a crucial role in modulating the immune system and thermotolerance by ensuring proper protein folding and regulating apoptosis (Hassan et al., 2019; Kumar & Lapierre, 2021).

Heat stress in cattle can be determined by measuring the expression of genes or proteins that increase under stress conditions, such as heat shock proteins (HSPs). Among these, the HSPA1A protein is one of the most extensively studied. The expression of HSPA1A varies significantly among different cattle breeds and is higher in cattle adapted to hot and humid environments (Elayadeth-Meethal, Tiambo, Naseef, Kuruniyan, & Maloney, 2023). HSP70 has also been associated with thermotolerance using

whole genome sequencing methods (Wang et al., 2024). Studies have shown that HSPA1A gene expression is a better marker for heat stress than HSP60 and HSP90 (Guzmán et al., 2023; Taborda-Charris et al., 2023). Consequently, the HSPA1A protein is considered the most important indicator of heat stress (Kim, Ghassemi Nejad, Roh, & Lee, 2020).

The intronless HSPA1A gene is on chromosome 23 (Hassan et al., 2019). This gene encodes the 641 amino acid-long HSPA1A protein (UniProt Consortium, 2018). The HSPA1A protein helps cells reorganize their vital functions and maintain normal operations under stress. Polymorphisms in the HSPA1A gene have been associated with thermotolerance, disease susceptibility, and production performance in cattle (Abbas et al., 2020; Badri, Alsiddig, Lian, Cai, & Wang, 2021; Hu et al., 2019). The structure of the HSPA1A protein consists of three main domains: the N-terminal ATPase domain, the substrate-binding domain, and the C-terminal regulatory domain. The N-terminal ATPase domain regulates the functional cycle of the protein by hydrolyzing ATP, while the substrate-binding domain plays a role in recognizing and binding misfolded proteins. The C-terminal regulatory domain regulates interactions with co-chaperone proteins and other protein-protein interactions (UniProt Consortium, 2018).

The purpose of this study is to determine the characterization of the HSPA1A gene in Turkish Grey and Holstein cattle. Turkish Grey cattle are known for their thermotolerance, allowing them to adapt to changing environmental conditions and resist parasites (Atalay & Kök, 2023; Kök, Atalay, Eken, & Savasci, 2017). Holstein cattle, on the other hand, are highly susceptible to heat stress (Cartwright, McKechnie, Schmied, Livernois, & Mallard, 2021). It is known that selection for increased milk yield has led to a decreased heat stress resistance (Liu et al., 2017; Santana, Bignardi, Pereira, Stefani, & El Faro, 2017). Therefore, this study aims to compare the HSPA1A gene variations between Turkish Grey and Holstein cattle. Identifying variations in genes associated with thermotolerance may help elucidate the molecular mechanisms of resistance to heat stress.

MATERIALS and METHODS

Molecular Genetics Analysis

gDNAs were isolated from tissue samples according to the protocol guidelines of the hybrid genomic DNA isolation kit (MG-GDNA-01, Turkiye). The three primer pairs to amplify the entire 2474 nucleotide-long HSPA1A gene were designed using the NCBI primer3 program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The PCR mix was 25 µL total volume containing 12.5 µL PCR master mix (Nucleo Gene, NG053), 1 µL (5 pmol) of each primer, 5 µL template DNA, and 5.5 µL nuclease-free water. PCR temperature and cycling conditions; First denaturation at 95 °C for 3 min, binding at 95 °C for 30 s for 40 cycles, then at 60/56/65 °C for 40 s for 40 cycles, 72 °C for 60 s for 40 cycles, and finally at 72 °C for 5 min in the synthesis process has been

used. With the designed primers, the HSPA1A gene of each bovine was amplified in PCR as 3 fragments (1001, 936, and 884 bp) (My Genie 96 Thermal Block, Bioneer Corporation, Republic of Korea). The PCR products were run on a 2% concentration agarose gel containing 6 µl of EtBr at 120 V and 80 mA current. The fragments of PCR products containing the target gene region were visualized under UV light (DNR BioImaging Systems Minibis Pro. Jerusalem, Israel). After it was determined that the product with suitable properties was obtained, the PCR products were purified by using the MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) purification kit to obtain the single band samples. The ABI 3730XL Sanger sequencing instrument (Applied Biosystems, Foster City, CA) and Cycle Sequencing Kit (BigDye Terminator v3.1) were used for DNA sequencing reactions. DNA sequences were analyzed using BioEdit v.2.7.5 (Hall, 1999).

Table 1. Detailed information about the primer pairs

Primer pairs	Primer binding region	Binding temperature	PCR product (bp)
F: GCCTGTTGTCGAAGTCCTCCC	27521871-27521891	60	1001
R: GAGGCGAAACCCCTGGAATA	27522871-27522852		
F: GGTGATGGTGATCTTGTTGGC	27521068-27521088	56	936
R: CGCAACGTGCTCATCTTTGAT	27522003-27521983		
F: ATAGCCATGGCGCCTAACAT	27520210-27520229	65	884
R: CAAGGCCAACAAGATCACC	27521093-27521074		

In silico analysis

The effects of nsSNPs on protein structure and function were evaluated using ConSurf (Ashkenazy, Erez, Martz, Pupko, & Ben-Tal, 2010), HOPE project (Venselaar, Te Beek, Kuipers, Hekkelman, & Vriend, 2010), SHIFT (McLaren et al., 2016) and DUET tools (Pires, Ascher, & Blundell, 2014). ConSurf is an online tool for identifying functionally essential protein regions by analyzing the evolutionary conservation of amino acid positions. It first collects homologous sequences of the target protein from various databases. These sequences are then aligned to the target protein sequence. The evolutionary conservation score (1-9) for each amino acid position in the target protein is calculated using these homologous sequences. In ConSurf results, a conservation score of 1 indicates the lowest level of conservation, meaning that the amino acid at this position is highly variable between different species, indicating that it is less critical for the protein's function or structure. Conversely, a score of 9 represents the highest level of conservation, indicating that the amino acid is highly conserved and likely essential for the proper function of the protein (Ashkenazy et al., 2010).

Based on the physicochemical similarity between the natural and mutant amino acids, SIFT predicts whether an amino acid substitution will affect protein function. Scores close to zero are more

likely to be deleterious. Substitutions with a score < 0.05 are classified as 'deleterious', while others are classified as 'tolerable'.

The HOPE project is an online tool designed to analyze the effects of amino acid substitutions on protein structure and function. HOPE integrates information from various sources, including sequence conservation, structural data, and known protein-protein interactions. It uses a combination of homology modeling and literature-based information to predict the impact of a mutation. The tool evaluates factors such as changes in size, charge, and hydrophobicity of the mutated residue and its location within the protein (Venselaar et al., 2010).

The DUET program integrates the results from two distinct methods: mCSM and SDM, which analyze the structural and evolutionary context of the mutation. mCSM (mutation Cutoff Scanning Matrix) uses graph-based signatures to assess the effects of mutations on protein stability. At the same time SDM (Site Directed Mutator) utilizes statistical potentials derived from the analysis of evolutionary sequences. By combining these approaches, DUET provides a more robust and comprehensive prediction. The results are presented as ΔΔG values, where a negative value indicates a destabilizing effect and a positive value indicates a stabilizing effect (Pires et al., 2014).

Statistical analysis

The analysis conducted using Haploview v4.2 (Barrett, Fry, Maller, & Daly, 2005) followed the criteria established by Gabriel et al. (2002). The criteria for defining haplotype blocks included the following: a minimum of 80% confidence in the upper and lower confidence bounds of D', indicating strong linkage disequilibrium, the absence of historical recombination events between SNPs within a block, and at least 70% of the informative comparisons must show significant linkage disequilibrium (D' > 0.7).

RESULTS

DNA sequencing of the HSPA1A gene in 70 Holstein and 70 Turkish Grey cattle revealed 18 SNPs in the exon and 6 SNPs in the 3' UTR region. No genetic variation was observed in the 5' UTR of the gene. Detailed information about the identified SNPs is provided in Table 2. In total, 23 SNPs were detected in Turkish Grey cattle and 21 in Holstein. All identified SNPs, except rs480841468, were polymorphic in Turkish Grey cattle. In this breed, all individuals had the GG genotype at rs480841468, whereas this SNP was polymorphic in Holstein cattle. Additionally, rs479123543, rs383027605, and rs110559443 were monomorphic in Holstein but polymorphic in Turkish Grey cattle. Minor allele frequencies in the HSPA1A gene of Turkish Grey cattle and Holstein are represented in Table 2.

The three-dimensional protein model (AF-Q27975-F1) (Varadi et al., 2024) of cattle HSPA1A was obtained from UniProt (UniProt Consortium, 2018) and used as input for ConSurf analysis. The ConSurf score for both locations (M5 and G626) of amino acid substitution was 1. The low ConSurf

score (1) indicates that these positions are not highly conserved and may not be critical for the essential functions of the protein.

The SHIFT tool in the Ensemble Variant Effect Predictor tool (McLaren, Gil et al. 2016) was used to predict the functional effects of amino acid substitutions. The SIFT scores for the amino acid substitutions M5I, M5T, and G626A are 0.16, 0.28, and 1.00, respectively. The M5I (0.16) and M5T (0.28) scores are above the 0.05 threshold, suggesting that these mutations are likely tolerated and do not significantly impact the protein's function. On the other hand, the score for G626A is 1, which is the highest possible score, indicating that this substitution is well-tolerated and highly unlikely to affect the protein's function.

Table 2. Detailed information about the identified SNPs

SNP ID	Alleles	Amino acid substitution	Amino acid position	Minor allel frequency of TGC	Minor allel frequency of THC
rs382492082	C/T	M/I (M)	5	C: 0.364	C: 0.479
rs385826597	A/G	M/T (M)	5	A: 0.357	G: 0.436
rs135145204	C/T	Y (S)	42	T: 0.071	T: 0.350
rs110903970	C/G/T	G (S)	52	T: 0.193 G: 0.321	T: 0.243 C: 0.371
rs480841468	G/A	R (S)	93	A: 0.000	A: 0.021
rs109475441	T/C	K (S)	108	T: 0.229	T: 0.286
rs479123543	G/A	N (S)	151	A: 0.079	A: 0.000
rs110374561	C/G	G (S)	191	G: 0.171	G: 0.043
rs110470368	C/T	L (S)	314	T: 0.293	T: 0.279
rs383027605	G/A	D (S)	352	A: 0.029	A:0.000
rs382780148	T/C	G (S)	402	C: 0.114	C: 0.029
rs110559443	G/A	A (S)	412	A: 0.100	A: 0.000
rs41257359	C/T	S (S)	544	T: 0.021	T: 0.043
rs209779684	G/A	S (S)	579	A: 0.257	A: 0.450
rs110397047	C/A	A (S)	587	A: 0.286	A: 0.450
rs384294013	C/G	G/A (M)	626	C: 0.150	C: 0.079
rs444090099	A/G	S (S)	631	G: 0.007	G: 0.029
rs41257358	A/G	D (S)	641	G: 0.286	G: 0.443
rs110850310	A/G	-	-	G: 0.307	G: 0.207
rs210622792	A/C	-	-	A: 0.329	A: 0.257
rs209163750	T/C	-	-	C: 0.386	C: 0.214

rs210291331	C/T	-	-	C: 0.264	C: 0.436
rs208710980	G/A	-	-	G: 0.307	G: 0.357
rs133110858	T/C	-	-	C: 0.321	C: 0.207

(M); missense, (S); synonymous mutation, TGC; Turkish Grey cattle, THC; Turkish Holstein cattle

According to the HOPE project results, the M5I occurs in a domain crucial for molecule binding and in contact with another essential binding domain. This change might disrupt the interaction between these domains, potentially affecting the protein's function. The size difference between the wild-type methionine and the smaller mutant isoleucine could create a space in the protein core, further impacting its stability and activity. The mutation of M5T occurs in a domain crucial for molecule binding and in contact with another important binding domain. This change might disrupt the interaction between these domains, potentially affecting the protein's function. The mutant threonine is smaller and less hydrophobic than the wild-type methionine, which could create a space and result in the loss of hydrophobic interactions in the protein core. This alteration may disturb signal transfer from the binding domain to the activity domain, impacting the overall stability and activity of the protein. The G626A substitution is a highly flexible residue for a larger, more hydrophobic one. Glycine's flexibility is crucial for the protein's function, and its mutation can disrupt this role. The increase in size from glycine to alanine might cause steric clashes, leading to bumps in the protein structure. Furthermore, glycine's unique ability to adopt unusual torsion angles is lost with this mutation, potentially forcing the local backbone into an incorrect conformation and disturbing the local structure. This alteration may significantly impact the protein's overall stability and function.

Table 3. ConSurf, SHIFT, HOPE and DUET analysis results.

Amino acid substitutions	**ConSurf Score**	**SHIFT score**	**mCSM ΔΔG (kcal/mol)**	**SDM ΔΔG (kcal/mol)**	**DUET ΔΔG (kcal/mol)**	**Change Results of mutant residue compared to native residue according to the HOPE server**	
						Hydrophobicity change	**Size change**
Met5Ile	1	0.16	-1.80	+0.73	-1.23	-	Smaller
Met5Thr	1	0.28	-1.49	-0.60	-1.14	The less hydrophobic	Smaller
Gly626Ala	1	1.00	-0.36	+0.01	-0.04	The more hydrophobic	Greater

For the M5I mutation, both the mCSM ΔΔG with a value of -1.8 kcal/mol and the DUET ΔΔG with a value of -1.233 kcal/mol also suggest a destabilizing effect, while the SDM ΔΔG indicates a stabilizing effect with a value of 0.73 kcal/mol. On the other hand, for the M5T mutation, all three predictions (mCSM, SDM, and DUET) show destabilizing effects with values of -1.488 kcal/mol, -0.6 kcal/mol, and -1.141 kcal/mol, respectively. For the G626A mutation, the mCSM ΔΔG of -0.362

kcal/mol indicated a destabilizing effect, and in addition, the DUET ΔΔG of -0.039 kcal/mol a very slightly destabilizing effect. However, the SDM ΔΔG with a 0.01 kcal/mol value indicated a slightly stabilizing impact.

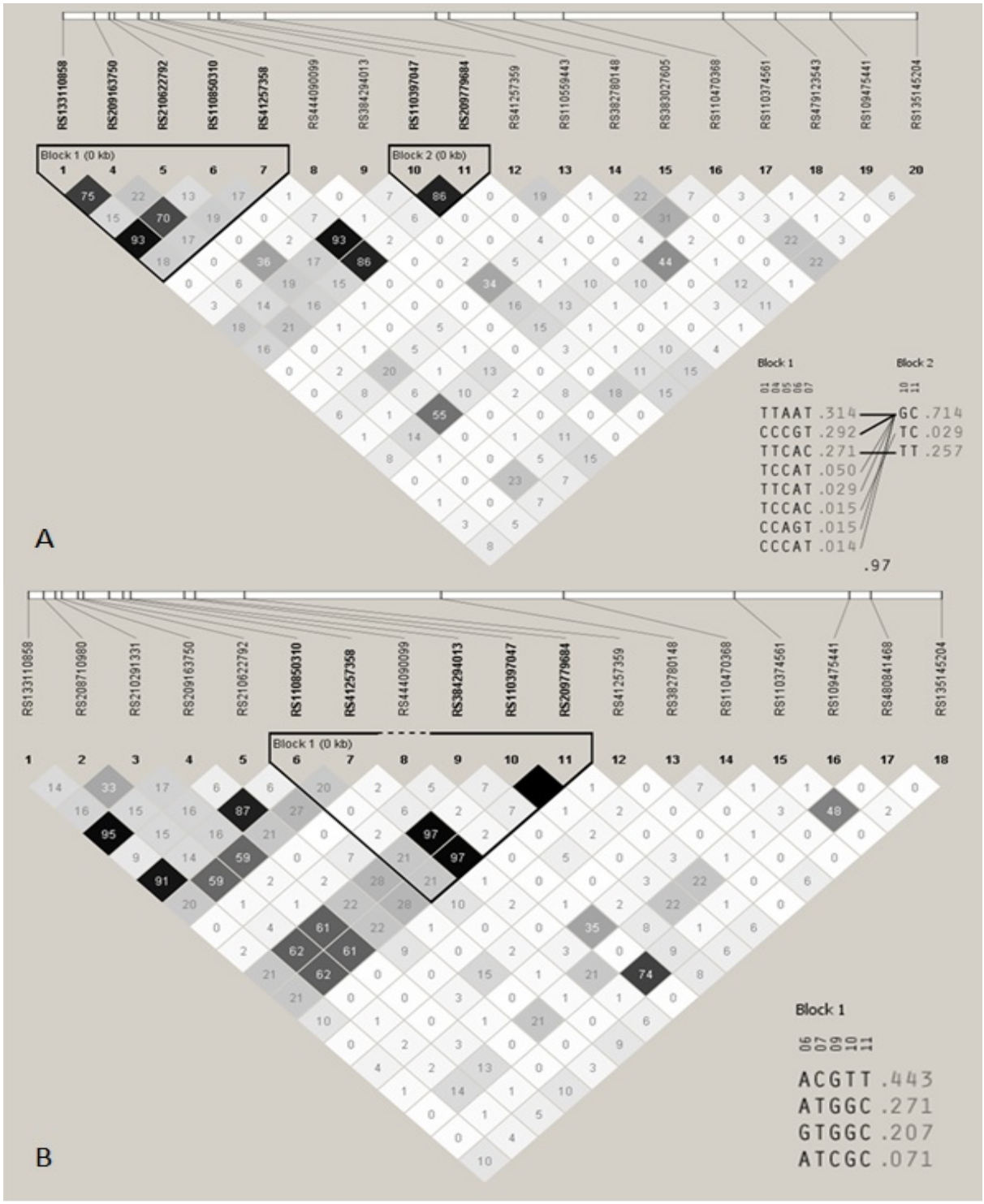

Figure 1. The Haploview v4.2. analysis result A. Turkish Grey cattle B. Holstein cattle

Based on the Haploview analysis results of SNPs in two populations, Turkish Grey cattle exhibit higher haplotype diversity in Block 1 (Figure 1A), with the major haplotypes being TTAA.T (31.4%), CCCGT (29.2%), and TTCAC (27.1%). In Block 2 (Figure 1A), Turkish Grey cattle have a dominant haplotype GC (71.4%). Holstein cattle shows a different pattern in Block 1 (Figure 1B), where the significant haplotypes are ACGTT (44.3%), ATGGC (27.1%), GTGGC (20.7%), and ATCGC (7.1%). Turkish Grey cattle demonstrate high linkage disequilibrium between blocks with an LD value of .97 (Figure 1A).

DISCUSSION

Changes in environmental temperatures lead to a decrease in meat and milk yield and reproduction in cattle (El-Tarabany & El-Bayoumi, 2015; Stamperna et al., 2021; Summer et al., 2019). Therefore, selecting cattle with high thermotolerance capacity is an important strategy to mitigate the adverse effects of heat stress. The HSP superfamily is a crucial target for elucidating the molecular mechanisms of resistance to heat stress. The HSPA1A protein is one of the most studied biomarkers among HSPs. Seasonal transitions and temperature changes significantly increase the expression of HSPA1A (Velayudhan et al., 2022).

Numerous SNPs have been identified in the HSPA1A gene in previous studies. Polymorphisms in the 5' UTR region were observed across various cattle breeds, including Gangatiri cattle (Dayal et al., 2024), Bali cattle (Suhendro et al., 2024), Holstein cattle (Abbas et al., 2020; Badri et al., 2021), Inner-Mongolia Sanhe cattle (Hu et al., 2019), and Western Sudan indigenous cattle (Ismaeel, Moussa, Eltahir, & Shakam, 2024). Some of these SNPs have been reported to be associated with heat stress tolerance (Abbas et al., 2020; Badri et al., 2021; Hu et al., 2019). However, in this study, no variation was detected in Holstein and Turkish Grey cattle's 5' UTR region of the HSPA1A gene.

The exon of the HSPA1A gene is polymorphic in Gangatiri cattle (Dayal et al., 2024), Red Sindhi cattle (Saeed et al., 2021), Tharparkar cattle (Bhat et al., 2016), and Zebu Breeds of Nigeria (Onasanya et al., 2021). In the current study, it was also found to be polymorphic in Holstein and Turkish Grey cattle. Three of the 18 SNPs identified in the exon region result in amino acid substitutions. The effects of these substitutions (M5I, M5Y, and G626A) on protein structure and function were evaluated in silico. ConSurf analysis revealed that the positions (5 and 626) of the amino acid substitutions have low evolutionary conservation (Table 3). Similarly, SHIFT analysis predicted these mutations would likely be tolerable (Table 3). However, analyses from the HOPE project and DUET suggest that these mutations might cause changes in the protein's structure and function (Table 3). The discrepancies between the results from different in silico tools can be attributed to the varying algorithms and models they use to predict the effects of mutations. ConSurf focuses on evolutionary conservation, while SHIFT assesses the likelihood of mutations being tolerable based on sequence homology and structural context. HOPE and DUET, on the other hand, provide insights into structural changes and protein stability.

The HOPE project analysis predicted that amino acid substitutions could have potentially harmful effects. The M5I substitution introduces a minor residue that could create a space in the protein core, potentially disrupting interactions between critical binding domains and affecting protein stability. Similarly, the mutation of M5T at the same position reduces residue size and hydrophobicity, leading to loss of hydrophobic interactions and possibly disturbing signal transfer from the binding domain to the activity domain. Lastly, the mutation of G626A changes a highly flexible and slight residue to a larger, more hydrophobic one, which could result in steric clashes and force the local backbone into an incorrect conformation. This change may abolish glycine's unique functional role, further destabilizing the protein structure.

For the M5I substitution, the DUET program algorithms mCSM and DUET predict a stability-degrading effect, while SDM predicts a stabilizing effect. This discrepancy highlights the variability in predictions from different algorithms and models that may differentially interpret the effect of mutation on protein stability. In contrast, the M5T mutation shows consistent results in all three prediction methods, all indicating a destabilizing effect on protein stability. This uniformity suggests that the M5T mutation has more apparent deleterious effect on protein stability. The differences in results between the two mutations emphasize the importance of considering multiple prediction methods and the specific context of the mutation when assessing its effects on protein stability. According to the different prediction methods the G626A mutation shows minimal effect on protein stability. mCSM and DUET results indicate a slight stability-disrupting effect, while the SDM result indicates an almost neutral, slightly stabilizing effect. But overall, mutation has a negligible effect on protein stability. These findings suggests that the G626A mutation does not significantly alter the structural integrity or function of the protein.

Based on the Haploview analysis results of SNPs identified by sequencing the same gene in two populations, there are distinct genetic structural differences between Turkish Grey and Holstein cattle. Turkish Grey cattle show higher haplotype diversity in Block 1 (TTAA.T 31.4%, CCCGT 29.2%, TTCAC 27.1%). In comparison, Block 2 has a very dominant haplotype (GC 71.4%) with high linkage disequilibrium (.97). In contrast, Holstein cattle have a dominant haplotype in Block 1 (ACGTT 44.3%) with other haplotypes at lower frequencies (27.1%, 20.7%, 7.1%). These differences suggest that different evolutionary pressures or historical recombination events are at play between the populations (Xu et al., 2015). Turkish Grey cattle exhibit more balanced haplotype frequencies and strong linkage disequilibrium, whereas Holstein cattle are characterized by a single dominant haplotype (ACGTT 44.3%) in Block 1. Reasons for Haplotype Diversity and LD in genetic mapping in Turkish Grey Cattle could be the following. The absence of a formal breeding program and potential population substructure or historical bottlenecks in Turkish Grey cattle may have contributed to the observed high LD. These

genetic regions under natural selection, possibly linked to local environmental adaptation, also maintained these strong LD patterns.

In previous studies, as in the present study, the 3' UTR region of the HSPA1A gene is polymorphic across various cattle breeds (Dayal et al., 2024; Suhendro et al., 2024). SNPs in the 3' UTR region can influence posttranscriptional regulation of gene expression by altering miRNA binding sites. Previous studies have also shown that miRNAs play a role in thermoregulation by targeting HSPs (Deb and Sengar 2020, Deb and Sengar 2021, Mishra 2022). Specifically, the 4693-T mutation has been reported to disrupt miRNA binding, leading to reduced transcriptional repression and increased expression of the HSF1 gene (Li, Zhang et al. 2015). Additionally, bta-mir-2898 has been found to correlate negatively with the expression of HSPs (Deb and Sengar 2020).

Conclusion

This study provides valuable insights into the genetic diversity of the HSPA1A gene in Holstein and Turkish Grey cattle, emphasizing the need for a multifaceted approach to assess SNP effects on protein function and stability. Our findings reveal that while no variation was detected in Holstein and Turkish Grey cattle's 5' UTR region of the HSPA1A gene, the exon region exhibits polymorphisms with potential implications for protein structure and function. Additionally, the haplotype analysis indicates distinct genetic structures between Turkish Grey and Holstein cattle, suggesting diverse evolutionary pressures and historical recombination events. Further research is warranted to explore the functional consequences of these polymorphisms in vivo and in vitro to develop strategies for enhancing thermotolerance in cattle through marker-assisted genetic selection.

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Conflicts of interest

The authors declare that they have no conflict of interest.

REFERENCES

Abbas, Z., Hu, L., Fang, H., Sammad, A., Kang, L., Brito, L. F., . . . Wang, Y. (2020). Association analysis of polymorphisms in the 5′ flanking region of the HSP70 gene with blood biochemical parameters of lactating Holstein cows under heat and cold stress. *Animals, 10*(11), 2016.

Ansari-Mahyari, S., Ojali, M. R., Forutan, M., Riasi, A., & Brito, L. F. (2019). Investigating the genetic architecture of conception and non-return rates in Holstein cattle under heat stress conditions. *Tropical Animal Health and Production, 51*, 1847-1853.

Ashkenazy, H., Erez, E., Martz, E., Pupko, T., & Ben-Tal, N. (2010). ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic acids research, 38*(suppl_2), W529-W533.

Atalay, S., & Kök, S. (2023). The Comparison of Polymorphisms in the Heat Shock Transcription Factor 1 Gene of Turkish Grey Cattle and Holstein Cattle. *Kafkas Universitesi Veteriner Fakültesi Dergisi, 29*(5), 429-435.

Badri, T., Alsiddig, M., Lian, L., Cai, Y., & Wang, G. (2021). Single nucleotide polymorphisms in HSP70–1 gene associated with cellular heat tolerance in Chinese Holstein cows. *Animal Gene, 20*, 200114.

Barrett, J. C., Fry, B., Maller, J., & Daly, M. J. (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics, 21*(2), 263-265.

Bhat, S., Kumar, P., Kashyap, N., Deshmukh, B., Dige, M. S., Bhushan, B., . . . Singh, G. (2016). Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. *Veterinary World, 9*(2), 113.

Cartwright, S. L., McKechnie, M., Schmied, J., Livernois, A. M., & Mallard, B. A. (2021). Effect of in-vitro heat stress challenge on the function of blood mononuclear cells from dairy cattle ranked as high, average and low immune responders. *BMC Veterinary Research, 17*, 1-11.

Dayal, S., Kumar, B., Kumari, R., Kumar, J., Ray, P. K., Chandran, P., & Dey, A. (2024). Molecular Characterization and Seasonal Variation in Expression of HSP70. 1 Gene in Gangatiri Cattle and Its Comparison with Buffalo. *Biochemical Genetics*, 1-15.

El-Tarabany, M. S., & El-Bayoumi, K. M. (2015). Reproductive performance of backcross Holstein× Brown Swiss and their Holstein contemporaries under subtropical environmental conditions. *Theriogenology, 83*(3), 444-448.

Elayadeth-Meethal, M., Tiambo, C. K., Naseef, P. P., Kuruniyan, M. S., & Maloney, S. K. (2023). The profile of HSPA1A gene expression and its association with heat tolerance in crossbred cattle and the tropically adapted dwarf Vechur and Kasaragod. *Journal of Thermal Biology, 111*, 103426.

Felius, M., Koolmees, P. A., Theunissen, B., Consortium, E. C. G. D., & Lenstra, J. A. (2011). On the breeds of cattle—historic and current classifications. *Diversity, 3*(4), 660-692.

Guzmán, L. F., Martínez-Velázquez, G., Villaseñor-González, F., Vega-Murillo, V. E., Palacios-Fránquez, J. A., Ríos-Utrera, Á., & Montaño-Bermúdez, M. (2023). Expression of heat shock protein genes in Simmental cattle exposed to heat stress. *Animal bioscience, 36*(5), 704.

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Paper presented at the Nucleic acids symposium series.

Hassan, F.-u., Nawaz, A., Rehman, M. S., Ali, M. A., Dilshad, S. M., & Yang, C. (2019). Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. *Animal Nutrition, 5*(4), 340-350.

Hu, L., Ma, Y., Liu, L., Kang, L., Brito, L. F., Wang, D., . . . Xu, Q. (2019). Detection of functional polymorphisms in the hsp70 gene and association with cold stress response in Inner-Mongolia Sanhe cattle. *Cell Stress and Chaperones, 24*(2), 409-418.

Ismaeel, F., Moussa, M., Eltahir, H. A., & Shakam, H. M. (2024). Identification of single nucleotide polymorphisms (SNPs) in 5′ and 3′ untranslated regions (5′ UTR and 3′ UTR) of HSP70 gene in some Western Sudan indigenous cattle. *Journal of Advanced Veterinary Research, 14*(4), 742-748.

Kerekoppa, R. P., Rao, A., Basavaraju, M., Geetha, G. R., Krishnamurthy, L., RAO, T. V. N., . . . Mukund, K. (2015). Molecular characterization of the HSPA1A gene by single-strandconformation polymorphism and sequence analysis in Holstein-Friesiancrossbred and Deoni cattle raised in India. *Turkish Journal of Veterinary & Animal Sciences, 39*(2), 128-133.

Kim, W.-S., Ghassemi Nejad, J., Roh, S.-G., & Lee, H.-G. (2020). Heat-shock proteins gene expression in peripheral blood mononuclear cells as an indicator of heat stress in beef calves. *Animals, 10*(5), 895.

Kök, S. (2017). Comparison of genetic diversity between the ex-situ conservation herd and smallholders of Turkish grey cattle. *Pakistan Journal of Zoology, 49*(4).

Kök, S., Atalay, S., Eken, H. S., & Savasci, M. (2017). The genetic characterization of Turkish grey cattle with regard to UoG Cast, CAPN1 316 and CAPN1 4751 markers. *Pakistan Journal of Zoology, 49*(1).

Kumar, A. V., & Lapierre, L. R. (2021). Location, location, location: subcellular protein partitioning in proteostasis and aging. *Biophysical Reviews, 13*(6), 931-941.

Liu, Z., Ezernieks, V., Wang, J., Arachchillage, N. W., Garner, J., Wales, W., . . . Rochfort, S. (2017). Heat stress in dairy cattle alters lipid composition of milk. *Scientific reports, 7*(1), 961.

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R., Thormann, A., . . . Cunningham, F. (2016). The ensembl variant effect predictor. *Genome biology, 17*, 1-14.

Onasanya, G. O., Msalya, G. M., Thiruvenkadan, A. K., Sreekumar, C., Tirumurugaan, G. K., Fafiolu, A. O., . . . Okpeku, M. (2021). Heterozygous single-nucleotide polymorphism genotypes at heat shock protein 70 gene potentially influence thermo-tolerance among four zebu breeds of Nigeria. *Frontiers in Genetics, 12*, 642213.

Pariset, L., Mariotti, M., Nardone, A., Soysal, M. I., Ozkan, E., Williams, J., . . . Bodò, I. (2010). Relationships between Podolic cattle breeds assessed by single nucleotide polymorphisms (SNPs) genotyping. *Journal of Animal Breeding and Genetics, 127*(6), 481-488.

Paula-Lopes, F., Lima, R. S. d., Satrapa, R. A., & Barros, C. M. (2013). Physiology and endocrinology symposium: Influence of cattle genotype (Bos indicus vs. Bos taurus) on oocyte and preimplantation embryo resistance to increased temperature. *Journal of Animal Science, 91*(3), 1143-1153.

Pires, D. E., Ascher, D. B., & Blundell, T. L. (2014). DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. *Nucleic acids research, 42*(W1), W314-W319.

Saeed, A., Wajid, A., Abbas, K., Ayub, G., Din, A. M., Ain, Q., . . . Hussain, T. (2021). Novel polymorphisms in complete coding region of heat shock protein 70.1 gene in subtropically adapted red sindhi cattle breed.

Santana, M., Bignardi, A., Pereira, R., Stefani, G., & El Faro, L. (2017). Genetics of heat tolerance for milk yield and quality in Holsteins. *Animal, 11*(1), 4-14.

Soysal, M., & Kök, S. (2006). The last survivors of Grey cattle resisting extinction. A case study of characteristics and sustainability of traditional systems of native Grey cattle breeds. *Options Méditerranéenes A, 78*, 55-63.

Stamperna, K., Giannoulis, T., Dovolou, E., Kalemkeridou, M., Nanas, I., Dadouli, K., . . . Amiridis, G. S. (2021). Heat Shock Protein 70 improves in vitro embryo yield and quality from heat stressed bovine oocytes. *Animals, 11*(6), 1794.

Suhendro, I., Noor, R. R., Jakaria, J., Priyanto, R., Manalu, W., & Andersson, G. (2024). Association of heat-shock protein 70.1 gene with physiological and physical performance of Bali cattle. *Veterinary World, 17*(1), 17.

Summer, A., Lora, I., Formaggioni, P., & Gottardo, F. (2019). Impact of heat stress on milk and meat production. *Animal Frontiers, 9*(1), 39-46.

Taborda-Charris, J. C., Rodríguez-Hernández, R., Herrera-Sánchez, M. P., Uribe-García, H. F., Otero-Arroyo, R. J., Naranjo-Gomez, J. S., . . . Rondón-Barragán, I. S. (2023). Expression profiling of heat shock protein genes in whole blood of Romosinuano cattle breed. *Veterinary World, 16*(3), 601.

Tesema, Z., Taye, M., & Ayichew, D. (2019). The role of phenotypic and genetic basis of livestock selection for climate change adaptation and mitigation: A review. *J Appl Adv Res, 4*(2), 66-77.

UniProt Consortium, T. (2018). UniProt: the universal protein knowledgebase. *Nucleic acids research, 46*(5), 2699-2699.

Varadi, M., Bertoni, D., Magana, P., Paramval, U., Pidruchna, I., Radhakrishnan, M., . . . Yeo, J. (2024). AlphaFold Protein Structure Database in 2024: providing structure coverage for over 214 million protein sequences. *Nucleic acids research, 52*(D1), D368-D375.

Velayudhan, S. M., Brügemann, K., Alam, S., Yin, T., Devaraj, C., Sejian, V., . . . König, S. (2022). Molecular, physiological and hematological responses of crossbred dairy cattle in a tropical savanna climate. *Biology, 12*(1), 26.

Venselaar, H., Te Beek, T. A., Kuipers, R. K., Hekkelman, M. L., & Vriend, G. (2010). Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC bioinformatics, 11*, 1-10.

Wang, L., Yan, X., Wu, H., Wang, F., Zhong, Z., Zheng, G., . . . Na, W. (2024). Selection Signal Analysis Reveals Hainan Yellow Cattle Are Being Selectively Bred for Heat Tolerance. *Animals, 14*(5), 775.

Xu, L., Bickhart, D. M., Cole, J. B., Schroeder, S. G., Song, J., Tassell, C. P. V., . . . Liu, G. E. (2015). Genomic signatures reveal new evidences for selection of important traits in domestic cattle. *Molecular biology and evolution, 32*(3), 711-725.

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