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Dependence of the Variability of the HSP70 Gene on Adaptation to Heat Stress in Kalmyk and Kazakh White-Headed Cattle Breeds

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ABSTRACT

This study addresses an urgent problem in molecular genetics by presenting the first identification and classification of single-nucleotide polymorphisms in the 5'-untranslated region (5'-UTR) of the HSP70 gene in Kalmyk and Kazakh White-Headed cattle breeds bred in Kazakhstan. The purpose of this work was to investigate these SNPs in the 5'-untranslated part of HSP70 gene in cattle of Kalmyk and Kazakh white-headed breeds using direct Sanger sequencing. A 551bp fragment of the HSP70 gene was sequenced, and the results were analyzed with BioEdit software. This analysis identified 20 single-nucleotide polymorphisms common to both breeds, with an additional five characteristic only of the Kalmyk breed and four characteristic only of the Kazakh white-headed breed. The occurrence level of these identified single-nucleotide polymorphisms varied, with frequencies for nine ranging from 3.03% to 15.15%. These results indicate a high level of polymorphism in the 5'-untranslated part of the HSP70 gene in these cattle breeds. To identify alleles of these polymorphisms, PCR-RFLP analysis was employed, specifically determining the CCGTGAGAG/ACAGCTTCCGC single-nucleotide polymorphism in the 5'-untranslated part of the HSP70 gene using MwoI endonuclease. Genotyping revealed all three genetic variants of the CCGTGAGAG/ACAGCTTCCGC SNPs in the Kazakh white-headed breed. This study on single-nucleotide polymorphisms associated with high temperature tolerance in the 5'-untranslated part of the HSP70 gene in cattle of local breeds holds both theoretical and practical significance.

Key words: High temperature tolerance, Single-nucleotide polymorphism, Polymerase chain reaction, Sanger sequencing, Local breeds.

INTRODUCTION

Heat shock proteins (HSPs) are a critical group of proteins that safeguard cells from various forms of cellular stress, including heat stress. Among these, HSP70 is a highly conserved, sensitive, and widely expressed gene associated with physiological adaptation to HS, located on chromosome 23 of *Bos taurus*. Its central role in maintaining cellular thermal stability makes it a key candidate for studying heat tolerance (Hassan et al. 2019). HSP70 plays a vital role in protecting cells during HS by acting as a molecular chaperone, ensuring proteins maintain their correct structure and function under damaging conditions, and preventing the accumulation of harmful aggregates. HSP70 is recognized for its protective role during heat stress, regulating cell growth and proliferation, and is considered a marker for heat stress tolerance in various species (Kaushik et al. 2022).

The problem of heat stress in cattle is a growing concern globally, intensified by climate change, leading to significant economic losses in livestock production due to decreased performance, production, and reproduction (Sesay 2023; Wankar et al. 2024; Mićić et al. 2025). Dairy breeds are particularly susceptible, with estimates showing substantial milk production losses (Hutchins et al. 2025). Heat stress destabilizes acclimatization, negatively impacting homeostasis and productivity (Zhigerbaevich et al. 2023). The need to develop heat-tolerant dairy cattle is paramount for sustainable farming in changing climatic conditions (Habimana et al. 2023; Worku et al. 2023).

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Genetic variations within the HSP70 gene, particularly in its regulatory regions like the 5'-untranslated region (5'-UTR), have been reported to influence an animal's capacity to respond to HS (Deb et al. 2013; Öner et al. 2017; Mkize and Zishiri 2020). Numerous studies confirm a high level of polymorphism in the 5'-UTR of the HSP70 gene in cattle (Deb et al. 2013; Öner et al. 2017; Mkize and Zishiri 2020; Badri et al. 2021). For instance, Indonesian scientists identified 14 SNPs in the 5'-UTR of the HSP70 gene in various cattle populations, further describing genetic variation within the promoter and 5'-UTR that may influence gene regulation and expression (Prihandini et al. 2022). The HSP70 gene is considered highly sensitive to thermal signals, making its genetic variants important for understanding thermotolerance (Onasanya et al. 2021; Vijayakumar et al. 2025). Other research in Indonesian cattle breeds further described genetic variation within the promoter and 5'-UTR of the HSP70 gene, identifying specific nucleotide changes and insertions that may influence the gene's regulation and expression (Hecker and McGarvey 2011). Functional analyses have indicated that some SNPs in these regulatory regions, such as g.-69T>G, can affect transcriptional regulation of HSP70 (Hecker and McGarvey 2011). Additionally, an SNP at position 1128G>T in the 5'-UTR of HSP70 has been linked to a higher capacity of peripheral blood mononuclear cells to respond to heat shock (Basiricó et al. 2011). Evidence from livestock studies consistently shows that specific sequence variants in the HSP70 gene can modify how animals respond to high temperatures, with certain genotypes demonstrating improved thermal stability and production performance under hot conditions (Al Reyad et al. 2016; Muslimova et al. 2024).

Despite the recognized importance of HSP70 polymorphisms in heat tolerance, a notable gap exists in the study of these genetic variations within indigenous cattle breeds in Kazakhstan. While Kazakh scientists have explored genetic factors related to reproductive function and disease resistance (Bimenova et al. 2019; Beishova et al. 2023) and recent whole-genome resequencing efforts are shedding light on ancestral contributions and selection signatures in Kazakh White-Headed cattle (Niyazbekova et al. 2025; Khamzina et al. 2025), there is a lack of specific data concerning the genetic polymorphism of the HSP70 gene, particularly in relation to heat tolerance, in local Kazakhstani breeds such as the *Kalmyk* and *Kazakh White-Headed*. These breeds are raised in regions characterized by pronounced continental climates with significant temperature extremes, making the study of HS-associated SNPs an urgent problem with substantial practical importance for local livestock farming (Igoshin et al. 2021; Zhigerbaevich et al. 2023).

Therefore, the aim of this study was to investigate single-nucleotide polymorphisms in the 5'-UTR region of the HSP70 gene in Kalmyk and White-Headed cattle breeds of Kazakh from Kazakhstan. This involved the discovery of novel SNPs using direct Sanger sequencing and the subsequent development and application of a genotyping assay, specifically PCR-RFLPs analysis, to assess the distribution of these identified variants within the studied populations. This research contributes to establishing a molecular basis for selecting cattle with enhanced tolerance to high ambient temperatures in the local climatic conditions.

MATERIALS AND METHODS

Collection of sample and extraction of DNA

Frozen blood samples from a total of 33 animals (16 Kalmyk and 17 Kazakh White-Headed cattle) were used in this study. The sampled animals represented a mixed sex and age structure, including cows, breeding bulls, and young animals. Sampling was conducted across multiple farms located in the Almaty and Jetisu regions of the Republic of Kazakhstan, forming a broad regional sampling frame. The inclusion of animals from different farms and herds was intended to reduce potential bias associated with a single herd and to limit the likelihood of close relatedness among sampled individuals.

Blood samples for DNA extraction were collected from each animal from the jugular vein and, in some cases, from the caudal vein. Two milliliters of blood were taken into vacuum tubes containing EDTA to prevent clotting. The samples were frozen and then processed at the Green Biotechnology and Cellular Engineering Laboratory of the Kazakh-Japanese Innovation Center, Kazakh National Agrarian Research University. Genomic DNA was isolated in two ways: by the classical phenolic extraction method and by using the PureLink™ Genomic DNA Mini Kit, strictly following the manufacturer's protocol.

To minimize potential variability associated with the use of two DNA extraction methods, all extracted DNA samples were assessed for quality and suitability prior to downstream analyses. DNA concentration and purity were evaluated spectrophotometrically, and only samples meeting standard quality criteria were used for PCR amplification and sequencing. The same PCR conditions, reagents, and downstream protocols were applied uniformly to all samples, regardless of the extraction method, ensuring comparability of results.

PCR amplification

A 551bp fragment of the HSP70 gene was amplified by PCR using a specific primer pair. The forward primer F had the sequence 5'-GTCGCCAGGAAACCAGAGAC-3', and the reverse primer R had the sequence 5'-GGAACACCCCTACGCAGGAG-3'. The PCR was performed in a thermocycler with a five-minute initial denaturation at 95°C. 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 60 seconds were performed after this step. At the end of the cycling, a final extension at 72°C for 5minutes was included to complete synthesis of any remaining incomplete products.

The resulting amplicons were separated on a 1.5% agarose gel. This electrophoresis step was used to verify that a clear band of the expected size (551bp) had been obtained and to confirm the specificity of the amplification (Schwerin et al. 2003).

DNA sequencing and sequence analysis

For subsequent analysis, the sequencing results for the HSP70 gene were imported into BioEdit, where they were organized, checked, and edited where necessary, and then aligned. This formed the basis for the downstream statistical evaluation of variation within the amplified region (Hall 1999).

A fragment of the HSP70 gene was sequenced by direct Sanger sequencing. The amplified fragment was first sequenced, and the resulting chromatograms were checked and edited. To increase accuracy, each fragment was sequenced in both forward and reverse directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Hu et al. 2019). This bidirectional approach helped confirm base calls and reduce sequencing errors.

Before sequencing, the PCR products were purified. Enzymatic cleanup with ExoSAP-IT (Thermo Fisher Scientific) was performed according to the manufacturer's protocol. The purified amplicons were then used directly as templates in the sequencing reactions.

Sanger sequencing was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Each reaction had a final volume of 10μL and contained 4μL of BigDye Terminator v3.1 mix, 1μL of primer (3.2pmol), 1–3μL of purified PCR product (10–40ng of template DNA), and nuclease-free water to bring the volume to 10μL. This setup ensured an appropriate ratio of dye terminators, primer, and template DNA.

Cycle sequencing was performed in a thermal cycler. The program began with a one-minute first step at 96°C. Twenty-five cycles of denaturation at 96°C for ten seconds, annealing at 50°C for five seconds, and extension at 60°C for four minutes were then performed. This protocol generated a series of labeled extension products suitable for downstream sequence analysis. Under these conditions, the DNA polymerase progressively incorporated labeled terminator nucleotides, generating a series of extension products of different lengths that are suitable for subsequent sequence analysis. Purification of sequencing products and capillary electrophoresis. Purification of sequencing products. Following the cyclic reaction, the BigDye™ XTerminator™ Purification Kit (Thermo Fisher Scientific) was used to purify the products in compliance with the manufacturer's instructions. 45mL of SAM Solution and 10mL of XTerminator Bead Solution were added to each sample, then they were mixed (vortexed) for 20minutes at 1,800rpm, after which the samples were centrifuged at 1,000g for 2minutes. An ABI 3500xl Genetic Analyser automated sequencer (Applied Biosystems) was used for capillary electrophoresis. Sequencing Analysis Software v5.4 (Applied Biosystems) was used for the sequence analysis.

SNP identification and genotyping

The obtained sequencing results of the 5'-UTR part of the HSP70 gene were analyzed using the BioEdit software, and the number of SNPs was determined in the studied cattle population of Kazakh local breeds, the Kalmyk breed (n=16), and the Kazakh white-headed breed (n=17). A search for restriction enzymes was performed to identify the SNPs in the 5'-UTR part of the HSP70 gene using the Nebcutter software (https://nc3.neb.com/NEBcutter /). Two single-nucleotide polymorphisms located in the 5′-untranslated region of the HSP70 gene were selected for genotyping: g.319G>A (corresponding to the CCGTGAGAG/ACAGCTTCCGC sequence variation), detected using the MwoI restriction enzyme, and a second site within the amplified 5′-UTR fragment assessed using the DdeI endonuclease (C/TNAG recognition site), which did not reveal allelic variation in the studied samples. The BclI endonuclease with the T/GATCA restriction site was used as a negative control to identify the absence of identified SNPs. PCR restriction of the product was carried out according to the instructions provided by the manufacturer, then horizontal electrophoresis was performed in agarose gel, and the resulting electropherograms were analyzed for the presence of genetic polymorphism.

RESULTS

Amplification of the 5'-UTR region of the HSP70 gene was carried out using a specific primer pair designed to flank this segment. The forward primer F had the sequence 5'-GTCGCCAGGAAACCAGAGAC-3', and the reverse primer R had the sequence 5'-GGAACACCCCTACGCAGGAG-3'. Under the applied amplification conditions, this primer set yielded a single product with an expected length of 551bp (Fig. 1), confirming that the reaction was specific for the targeted region of the HSP70 gene.

The amplified PCR product was then subjected to Sanger sequencing to determine the nucleotide sequence of the 5'-UTR fragment. Sequencing was performed using standard cycle sequencing chemistry, and the resulting chromatograms were processed and examined in the BioEdit software environment. Within BioEdit, raw sequence traces were visualized, low-quality bases were checked and, where necessary, edited, and the final sequences were aligned with the reference HSP70 gene sequence. This analysis allowed the identification and comparison of sequence variants present in the amplified region.

The results of direct sequencing of the 5'-UTR part of the HSP70 gene of 16 DNA samples of the Kalmyk breed and 17 samples of the Kazakh white-headed breed were analyzed. Only 20 SNPs in the 5'-UTR part of the HSP70 gene were found to be common to two Kazakh local breeds, of which 5 SNPs are characteristic only of the Kalmyk breed and 4 SNPs are characteristic of the Kazakh white-headed breed (Table 1).

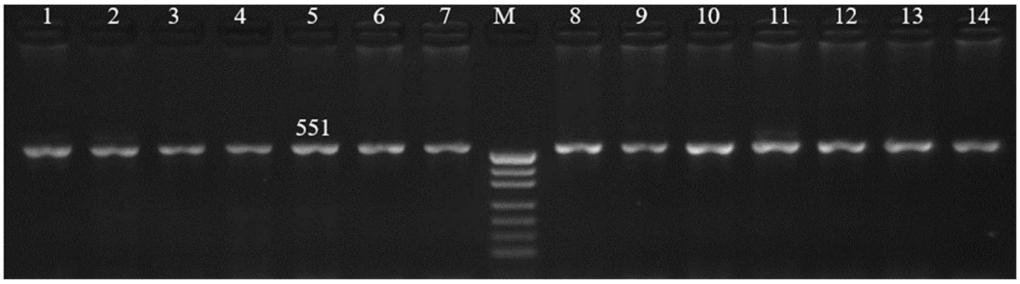

Fig. 1: An electropherogram of the HSP70 gene amplification, 2.0% agarose gel, wells 1-7, 8-14: 551bp PCR product, M-DNA marker pUC19/MspI.

Table 1: The number of identified SNPs in the 5'-UTR part of the HSP70 gene in Kalmyk and Kazakh white-headed cattle, identified by direct sequencing

Number of identified SNPs	Location of the identified SNPs in the 5'-UTR part of the HSP70 gene
Total number of identified SNPs (n=20)	GAGACAGT/AGTGGGTG, GACCTTCA/CCAGCCCC, CCCCtCTA/CCTTAGGA, ACACAGAG/ ACCGCCTGA CTTAGTCT/CGTGAGAG/ACAGCTTC, ACCCGCTA/GTCTCCAA, CCGAGGA/ GGCACCA, GCGCG/TTTCAGTT, AGCTTATC/TTCGGAG/TCCGGAAA, GAAAAACA/ CCaGCTAT, CCGTGAGAG/ACAGCTTCCGC, CCCCTCTC/ACTTAGGA, CCCCGAGGA/ GGCACCAGcGCG/TTTCAG, AGCTTATC/TTCGGA, CGTTCAGT/CTTTCGGG, GCTTAGTCT/ CGTGAGA, GGAGAGAG/ACTGATAA
Kalmyk breed (n=5)	CCGAGGA/GGCACCA, AGCTTATC/TTCGGAG/TCCGGAAA, CCCCTCTC/ACTTAGGA, CCCGAGGA/GGCACCAG
Kazakh white-headed breed (n=4)	AGCTTATC/TTCGGA, CGTTCAGT/CTTTCGGG, GCTTAGTCT/CGTGAGA, GGAGAGAG/ACTGATAA

Thus, as a result of direct sequencing, 20 SNPs were identified in the studied population of Kalmyk and Kazakh white-headed cattle, and the locations of these SNPs were determined. The Nebcutter software was used to search for appropriate restriction enzymes to identify 20 SNP alleles. To identify the alleles of this CCGTGAGAG/ACAGCTTCCGC SNP, the MwoI endonuclease with the GCNNNNN/NNGC restriction site was used. Primers F – 5' – GTCGCCAGGAAACCAGAGAC-3' and R, 5' - GGAACACCCCTACGCAGGAG-3' were used to obtain an amplification, a region of the HSP70 gene where the newly identified CCGTGAGAG/ACAGCTTCCGC SNP was localized. Analysis of the sequence of the 5'-UTR part of the HSP70 gene indicates that there are two restriction sites of the MwoI endonuclease. The first restriction site is common to all samples (highlighted in red), and the second restriction site allows for genotyping of DNA samples (highlighted in red, indicated by the nucleotide substitution G>A). Thus, no genetic polymorphism (CCGTGAGAG/ACAGCTTCCGC) of the SNP was detected on the electropherogram of the Kalmyk breed. As a result of restriction by MwoI endonuclease, the following fragments were formed: 212bp, 215bp, and 122bp (Fig. 2).

Genetic polymorphism was revealed in cattle of the Kazakh white-headed breed, and three genetic variants (CCGTGAGAG/ACAGCTTCCGC) were identified in the SNP of the studied animals. The following genotypes were identified: a homozygous GG genotype (212bp, 215bp, and 122bp fragments), a homozygous AA genotype (428bp and 122bp fragments), and a heterozygous GA genotype (428bp, 212bp, 215bp, and 122bp fragments) (Fig. 3).

A fragment of the HSP70 gene and the location of the point mutation G>A (CCGTGAGAG/ACAGCTTCCGC) in the 5'-UTR part of the HSP70 gene (sample 5 of the Kalmyk breed):

GCCAGGAAACCAGAGACAGAGTGGGTGGACCTTCCCAGCCCCTCTCCCCCTCTCCTTAGGACTCCTGTTTC
CTCCAGCGAATCCTAGAAGAGTCTGGAGAGTTCTGGGAGGAGAGGCATCCAGGGCGCTGATTGGTTCCAG
AAAGcCAGGGGGCAGGACTTGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCCCACTGAGCTCGGT
CATTGGCTGACGAGGGAAAAGGCGGGGCTTGATGAAGAATTATAAACACAGAGCCGCCTGAGGAGAAAC
AGCAGCCTGGAGAGAGCTGATAAAACTTACGGCTTAGTCCGTGAGAG/ACAGCTTCCGCAGACCCGCTATC
TCCAAGGACCGCCCCGAGGGGCACCAGCGCTTTCAGTTTTCGGGTTCCGAAAAGCCCGAGCTTCTCGTCGC
AGATCCTCTTCACCGATTTCAGGTTTGAAGCTTATCTCGGAGCCGGAAAAGCAGGGCACCGGCATGGCGAA
AAaCCCCGCTATCGGCCTCGACCTGGGCACCACCTACTCCTGCGTAGGGGTGTTCC

Preliminary sequence analysis in the 5'-UTR part of the HSP70 gene showed that this part of the gene had a restriction site for DdeI endonuclease, and for identification of various genetic variants of this (CCGTGAGAG/ACAGCTTCCGC) SNP, the obtained PCR product was restricted by DdeI restriction enzyme with the C/TNAG recognition site. The analysis of the obtained electropherogram shows that the studied animals of the Kalmyk and Kazakh white-headed breeds have the same genotype (Fig. 4).

A fragment of the HSP70 gene and the location of the DdeI endonuclease restriction site with the C/TNAG restriction site in the 5'-UTR part of the HSP70 gene:

GCCAGGAAACCAGAGACAGAGTGGGTGGACCTTCCCAGCCCCTCTCCCCCTCTCCTTAGGACTCCTGTTTC
CTCCAGCGAATCCTAGAAGAGTCTGGAGAGTTCTGGGAGGAGAGGCATCCAGGGCGCTGATTGGTTCCAG
AAAGcCAGGGGGCAGGACTTGAGGCGAAACCCCTGGAATATTCCCGACCTGGCAGCCCCACTGAGCTCGGT
CATTGGCTGACGAGGGAAAAGGCGGGGCTTGATGAAGAATTATAAACACAGAGCCGCCTGAGGAGAAAC
AGCAGCCTGGAGAGAGCTGATAAAACTTACGGCTTAGT/CCCGTGAGAG/ACAGCTTCCGCAGACCCGCTAT
CTCCAAGGACCGCCCCGAGGGGCACCAGCGCTTTCAGTTTTCGGGTTCCGAAAAGCCCGAGCTTCTCGTCG
CAGATCCTCTTCACCGATTTCAGGTTTGAAGCTTATCTCGGAGCCGGAAAAGCAGGGCACCGGCATGGCGA
AAAaCCCCGCTATCGGCCTCGACCTGGGCACCACCTACTCCTGCGTAGGGGTGTTCC

To identify a possible SNP in the 5'-UTR part of the HSP70 gene in cattle of Kalmyk and Kazakh white-headed breeds, the obtained amplification was restricted by BclI endonuclease with the T/GATCA restriction site. However, the analysis of the obtained electropherograms shows that the studied population lacks genetic polymorphism (CCGTGAGAG/ACAGCTTCCGC) in SNP (Fig. 5).

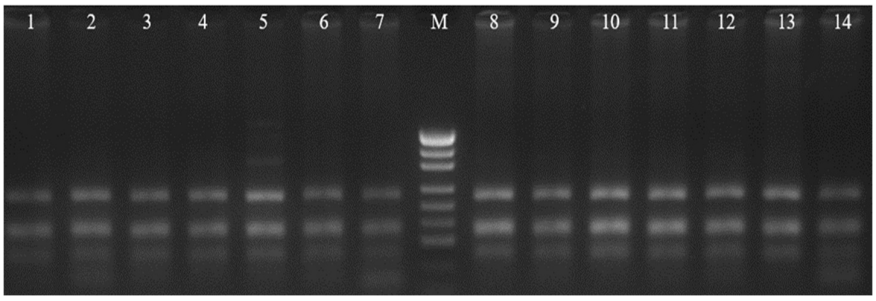

Fig. 2: An electropherogram of HSP70 gene amplification in Kalmyk cattle after restriction by MwoI endonuclease with GCNNNNN/NNGC restriction site, 3.0% agarose gel, wells 1-7, 8-14: homozygous GG genotype, 212bp, 215bp, and 122bp fragments, M-DNA marker pUC19/MspI.

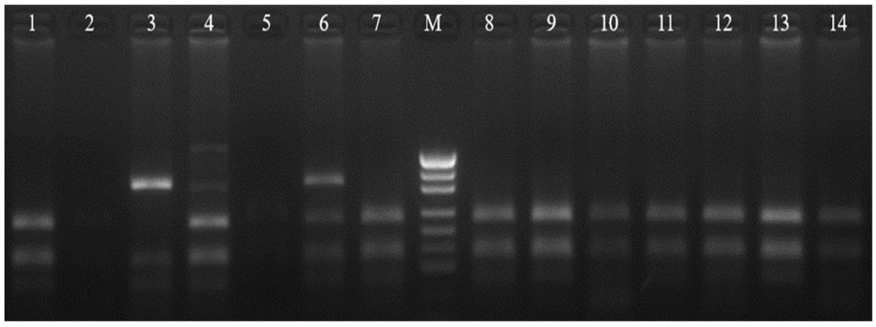

Fig. 3: An electropherogram of HSP70 gene amplification in Kazakh white-headed cattle after restriction by MwoI endonuclease with GCNNNNN/NNGC restriction site, 3.0% agarose gel, wells 1-2, 5, 7, 8-14: homozygous GG genotype, 212bp, 215bp, and 122bp fragments, well 3: homozygous AA genotype, 428bp and 122bp fragments, well 6: heterozygous GA genotype, 428bp, 212bp, 215bp, and 122bp fragments, M-DNA marker pUC19/MspI.

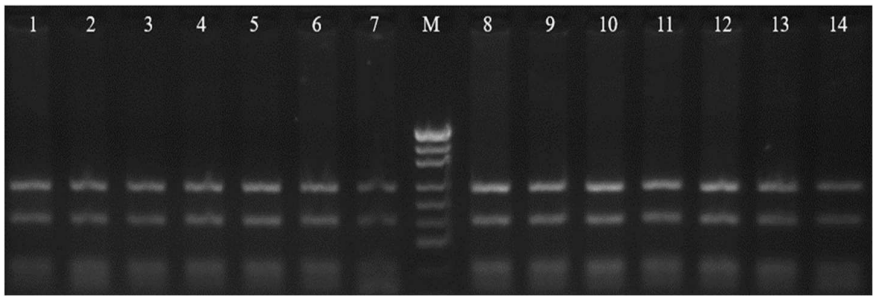

Fig. 4: An electropherogram of the HSP70 gene amplification after restriction by DdeI endonuclease with the C/TNAG restriction site, 3.0% agarose gel, wells 1-7, 8-14: homozygous genotype, 234bp, 147bp, 55bp, 68bp, and 44bp fragments, M-DNA marker pUC19/MspI.

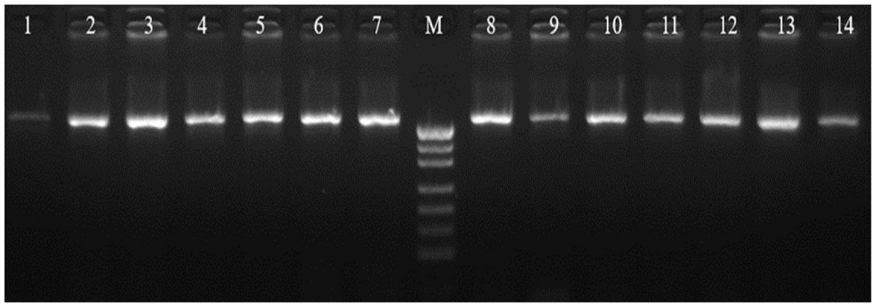

Fig. 5: An electropherogram of the HSP70 gene amplification after restriction by BclI endonuclease with the T/GATCA restriction site, 3.0% agarose gel, wells 1-7, 8-14: homozygous genotype, 551bp fragments, M-DNA marker pUC19/MspI.

A fragment of the HSP70 gene and the absence of localization of the BclI endonuclease restriction site in the 5'-UTR part of the HSP70 gene:

GCCAGGAAACCAGAGACAGAGTGGGTGGAC
CTTCCCAGCCCCTCTCCCCCTCTCCTTAGGACTCC
TGTTTCCTCCAGCGAATCCTAGAAGAGTCTGGAG
AGTTCTGGGAGGAGAGGCATCCAGGGCGCTGATT
GGTTCCAGAAAGcCAGGGGGCAGGACTTGAGGC
GAAACCCCTGGAATATTCCCGACCTGGCAGCCCC
ACTGAGCTCGGTCATTGGCTGACGAGGGAAAAG
GCGGGGCTTGATGAAGAATTATAAACACAGAGC
CGCCTGAGGAGAAACAGCAGCCTGGAGAGAGCT
GATAAAACTTACGGCTTAGTCCGTGAGAGACAGC
TTCCGCAGACCCGCTATCTCCAAGGACCGCCCCG
AGGGGCACCAGCGCTTTCAGTTTTCGGGTTCCGA
AAAGCCCGAGCTTCTCGTCGCAGATCCTCTTCAC
CGATTTCAGGTTTGAAGCTTATCTCGGAGCCGGA
AAAGCAGGGCACCGGCATGGCGAAAAaCCCCGCT
ATCGGCCTCGACCTGGGCACCACCTACTCCTGCG
TAGGGGTGTTCC

The results of the analysis of the occurrence of the identified SNPs in the studied local cattle breeds bred in Kazakhstan were interesting (Table 2). A total of 33 DNA samples of Kalmyk and Kazakh white-headed breeds were sequenced, and 26 of them showed ACCCGCTA/ GTCTCCAA SNP which had a high frequency of 78.78%, followed by AGCTTATC/ TTCGGA SNP with a prevalence of 57.57%. The occurrence of CTTAGTCT/ CGTGAGAG/ACAGCTTC and CCGAGGA/ GGCACCA SNPs in the studied animals was the same and amounted to 36.36%, while the frequency of the GACCTTCA/ CCAGCCCC SNP was 30.3%. The incidence of other SNPs ranged from 3.03% to 24.24%. The CCCGAGGA/ GGCACCAG SNP was highly common in Kalmyk cattle (27.27%), and GCTTAGTCT/ CGTGAGA was highly common in Kazakh white-headed cattle (15.15%).

Table 2: The occurrence of identified SNPs in the 5'-UTR part of the HSP70 gene in Kalmyk and Kazakh white-headed cattle

SNP Designation	Variant	Breed	Number of Samples with SNP (out of total 33)	Percentage Frequency of SNP
Common SNPs (identified in both breeds)				
GAGACAGT/AGTGGGTG	Motif	Both	5	15.15
GACCTTCA/CCAGCCCC	Motif	Both	10	30.3
CCCCTCTA/CCTTAGGA	Motif	Both	8	24.24
ACACAGAG/ACCGCCTGA	Motif	Both	2	6.06
CTTAGTCT/CGTGAGAG/ACAGCTTC	Motif	Both	12	36.36
ACCCGCTA/GTCTCCAA	Motif	Both	26	78.78
GCGCG/TTTCAGTT	Motif	Both	4	12.12
AGCTTATC/TTCGGAG/TCCGGAAA	Motif	Both	2	6.06
GAAAAACA/CCAGCTAT	Motif	Both	8	24.24
CCGTGAGAG/ACAGCTTCCGC (g.319G>A)	G>A	Both	7	21.21
CCCCTCTC/ACTTAGGA	Motif	Both	2	6.06
CCCCGAGGA/GGCACCAGcGCG/TTTCAG	Motif	Both	1	3.03
AGCTTATC/TTCGGA	Motif	Both	19	57.57
CGTTCAGT/CTTTCGGG	Motif	Both	2	6.06
GCTTAGTCT/CGTGAGA	Motif	Both	5	15.15
GGAGAGAG/ACTGATAA	Motif	Both	2	6.06
Breed-Specific SNPs				
CCGAGGA/GGCACCA	Motif	Kalmyk	2	6.06
AGCTTATC/TTCGGAG/TCCGGAAA	Motif	Kalmyk	2	6.06
CCCCTCTC/ACTTAGGA	Motif	Kalmyk	2	6.06
CCCCGAGGA/GGCACCAG	Motif	Kalmyk	9	27.27
AGCTTATC/TTCGGA	Motif	Kazakh White-Headed	1	3.03
CGTTCAGT/CTTTCGGG	Motif	Kazakh White-Headed	2	6.06
GCTTAGTCT/CGTGAGA	Motif	Kazakh White-Headed	5	15.15
GGAGAGAG/ACTGATAA	Motif	Kazakh White-Headed	2	6.06

DISCUSSION

The results of direct sequencing of the 5'-UTR part of the HSP70 gene in cattle of *Kalmyk* and *Kazakh White-Headed* breeds indicate that both breeds have revealed a genetic polymorphism in the 5'-UTR part of this gene. Most authors claim that the 5'-UTR part of the HSP70 gene in cattle has from 14 to 20 SNPs. Thus, according to Indonesian scientists (Prihandini et al. 2022), 102 specimens from seven different populations of Indonesia were found to have 14 SNPs in the 5'-UTR part of the HSP70 gene (Prihandini et al. 2022). Among the SNPs previously described in the HSP70 gene, only three variants—1117G>A, 1125A>C, and 1204T>C—were consistently detected across all breeds examined by Prihandini et al. (2022), indicating that these positions represent common polymorphic sites shared among diverse cattle populations. In contrast, other studies have reported a broader range of variation in this region. For example, Suhendro et al. (2021) identified seven SNPs in the 5'-UTR region of the HSP70 gene in several Indonesian cattle breeds, suggesting that the regulatory segment upstream of the coding sequence can harbor substantial breed-specific or population-specific diversity (Freitas et al. 2021; Onasanya et al. 2021; Sikiru et al. 2021).

In line with these observations, the results of our work showed that the 5'-UTR region of the HSP70 gene in Kalmyk and Kazakh white-headed cattle is also polymorphic. Sequence analysis revealed a set of variants in this regulatory region, and in total, 20 SNPs that were shared between Kalmyk and Kazakh white-headed cattle bred in the Republic of Kazakhstan were identified. The presence of this number of common SNPs indicates a considerable level of genetic variation within the 5'-UTR of HSP70 in these breeds, which may contribute to differences in gene regulation and, potentially, to variation in stress response and adaptation (Vijayakumar et al. 2022; Hariyono and Prihandini 2022). We identified several SNPs that appear to be breed specific. Five SNPs were detected only in animals of the Kalmyk breed, and four SNPs were found only in cattle of the Kazakh white-headed breed. In total, sequences of the 5'-UTR region of the HSP70 gene were obtained for 16 Kalmyk cattle and 17 Kazakh white-headed cattle. The blood samples were collected in a representative way. Animals were selected from different regions and from different farms to better reflect the genetic structure of the populations and to avoid bias from a single herd. The occurrence of breed-specific polymorphisms underscores the unique genetic architecture within different cattle populations and their adaptation mechanisms (De Campos et al. 2024; Nayak et al. 2024; Vijayakumar et al. 2025).

In the Republic of Kazakhstan, the Kalmyk breed is mainly kept in the Jambyl, Almaty, and Jetisu regions. These areas are characterized by pronounced continental climate. Winters are cold, with long periods of low temperature. Summers are hot, and ambient temperature can reach 40°C. Under such conditions, cattle are regularly exposed to temperature extremes and heat load. The detection of specific SNPs in the HSP70 gene in these breeds is therefore of particular interest, as these variants may be associated with adaptation to harsh climatic conditions and improved tolerance to thermal stress (Habib et al. 2022; Hariyono and Prihandini 2022; Zayas et al. 2025). Understanding these genetic links is crucial for developing resilient livestock in the face of global climate change (Strandén et al. 2022; Cartwright et al. 2023; Gujar et al. 2023).

Therefore, the study of SNPs associated with HS is an urgent problem and is of great practical importance. A

search was carried out for restriction enzymes to identify alleles of newly identified SNPs in Kalmyk and Kazakh white-headed breeds. The Kalmyk breed (sample 5) revealed a new SNP CCGTGAGAG/ACAGCTTCCGC, the occurrence of which was 21.21% in the studied population of Kalmyk and Kazakh white-headed breeds (n=7). The presence of the identified CCGTGAGAG/ACAGCTTCCGC SNP was additionally proved by the PCR-RFLP analysis. The PCR product obtained was restricted by the MwoI endonuclease with the GCNNNNN/NNGC restriction site, and only the homozygous GG genotype (212bp, 215bp, and 122bp fragments) was detected in the Kalmyk breed, while all three genetic variants were found in the Kazakh white-headed breed: the homozygous GG genotype (212bp, 215bp, and 122bp fragments), the homozygous AA genotype (428bp and 122bp fragments), and the heterozygous GA genotype (428bp, 212bp, 215bp, and 122bp fragments). A total of 17 DNA samples of the Kazakh white-headed breed were genotyped at the HSP70 gene locus (CCGTGAGAG/ACAGCTTCCGC SNP), and of these, one specimen turned out to be homozygous with the AA genotype (5.88%), one specimen had the heterozygous GA genotype (5.88%), and the remaining 15 samples turned out to be homozygous with the GG genotypes (88.24%). The studied Kazakh white-headed breed had an excessive occurrence of the homozygous GG genotype compared to other AA and GA genotypes. Besides, to identify SNPs in the 5'-UTR part of the HSP70 gene (CCGTGAGAG/ACAGCTTCCGC) in Kalmyk and Kazakh white-headed cattle, DdeI endonuclease with a C/TNAG restriction site was used, but genotyping results did not reveal the presence of a genetic polymorphism in the studied animals; all specimens turned out to be homozygous. Currently, scientists are conducting in-depth functional studies on the expression level of the HSP70 gene in dairy cows. The prevalence of genetic variants of this gene has been studied, and the effect of HSP70 gene alleles on HS tolerance has been determined (Sai Prasanna et al. 2022; Guzmán et al. 2023; Kim et al. 2025). Thus, the study of SNPs associated with high temperature tolerance in cattle of local breeds of the Republic of Kazakhstan is of great practical importance (Kaushik et al. 2022; Al-Jaryan et al. 2023).

Despite these revelations, there are a number of limitations to this study that should be taken into account. Firstly, the relatively small sample size (n=33) may limit the generalizability of the findings and the statistical power to detect all existing polymorphisms or their associations, as larger sample sizes are required to detect causal variants with very small effects in complex traits (Cheruiyot et al. 2021, 2022). Secondly, this research focused solely on the identification and genotyping of SNPs without associating them with phenotypic traits related to heat tolerance, such as body temperature, respiration rate, or milk production. Future studies should aim to link identified polymorphisms with specific physiological and physical thermotolerance traits (Hariyono and Prihandini 2022; Osei-Amponsah et al. 2023; Suhendro et al. 2024). Consequently, the functional implications of the identified polymorphisms remain to be elucidated. Thirdly, there was no experimental validation of the expression levels or functional impact of these genetic variants on HSP70 protein activity, which studies employing functional assays have shown to be crucial (Badri et al. 2021; Kim et al. 2025). Lastly, the study did not include direct measurements of environmental Temperature Humidity Index or other specific environmental stressors experienced by the animals, which would have provided a more direct link between genetic variation and local adaptation to heat stress. Integrating environmental parameters, such as ambient temperature and relative humidity, alongside physiological data is a crucial strategy for understanding heat tolerance mechanisms (Habimana et al. 2023; Shu et al. 2023; Chen et al. 2024). Future studies should integrate these aspects—larger sample sizes, phenotypic data, functional validation, and environmental monitoring—to comprehensively clarify the role of HSP70 gene polymorphisms in heat adaptation.

Conclusion

In this study, new SNPs were identified in the 5'-UTR region of the HSP70 gene in Kalmyk and Kazakh white-headed cattle. These variants are candidate markers related to thermotolerance, requiring validation in phenotype-association studies. Their presence was demonstrated for the first time in these breeds using direct sequencing. This result suggests that specific sequence changes in the regulatory region of HSP70 may contribute to the ability of these animals to cope with heat and may be useful for further genetic and breeding studies on heat resistance. In total, 20 common SNPs were identified for the two breeds. Additionally, five SNPs were characteristic only of the Kalmyk breed, and four SNPs were found only in the Kazakh White-Headed breed. The occurrence of identified SNPs in cattle of the Kalmyk and Kakhzakh white-headed breeds ranged from 3.03% to 78.78%, and the following SNPs had a high prevalence: ACCCGCTA/GTCTCCAA and AGCTTATC/TTCGGA. The PCR-RFLP analysis method was successfully used to identify CCGTGAGAG/ACAGCTTCCGC SNP alleles.

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12/2025 date 3rd August, 2025). Blood sampling was carried out in accordance with national and institutional guidelines for the care and use of animals in research, as part of routine veterinary procedures, and did not cause harm or distress to the animals.

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