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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

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ABSTRACT

Heat shock proteins (HSPs) are a considerable family of evolutionarily conserved molecular chaperones that play an important role in protecting cells against cellular stressors. HSP70 is a sensitive, and superabundant gene associated with heat stress's physiological adaptability. This research aimed to identify the polymorphisms in 5′ - 3′ untranslated regions (5′ UTR and 3′ UTR) of the HSP70 gene in western Sudan indigenous cattle. The genomic DNA was extracted from blood samples of Sudanese Red Fulani, Nyalawi, and Messeri cattle, and analyzed using the Sangar sequencing method. The results showed that the existence of 5 SNPs of g.802C>T, g.895C/-, g.1125A>C, g.1128G>T, and g.1204T>C at 5′ UTR and one SNP of g.64T>G at 3′ UTR. The moderate polymorphic informative content (PIC) and highest effective number of allele values were detected in SNP of g.895C/- (1.97) and g.64T>G (1.88) in Sudanese Red Fulani and indicated that these SNPs are highly polymorphic. The deletion of Cytosine (g.895C/-) and G (g.64T>G) alleles were the most frequent while the (--) and (GT) genotypes were the most frequent among these cattle. The chi-square (χ^2) test revealed that the genotype frequency for the HSP70 gene disagreed with the Hardy–Weinberg equilibrium ($p>0.05$). This research is the first study concerned with HSP70 polymorphisms in Western Sudanese cattle. Thus, the results concluded that the HSP70 gene in (5′ UTR and 3′ UTR) in all studied cattle was polymorphic. The 2 SNPs of g.895C/- and g.64T>G are useful molecular markers to assist selection for thermal adaptation in Sudanese cattle breeds.

Introduction

Ecologically, Sudan covers different climatic regions that vary from desert and semi-desert in the northern areas to equatorial with a short rainy season in the center to equatorial with a long rainy and humid in equatorial southern areas (Khalid *et al*., 2012). This wide range of variation is a result of climatic factors, particularly rainfall and vegetation together leading to diversity in livestock population. Sudan is one of the most considerable livestock populations on the African continent. In 2009, the cattle population size was 41.653 million/heads (MARF, 2009). One of the largest and most important indigenous beef-producing cattle is Baggara ecotypes (Nyalawi, Messeri) and Sudanese Red Fulani cattle (Hamza and Fathi, 2020). Baggara ecotypes and Sudanese Red Fulani indigenous cattle are part of East African descendants of the humped Bos indicus (Tijjani *et al*., 2021). They are mainly found in the Darfur and Kordofan states, kept by nomadic and semi-nomadic pastoralist tribes. These pastoralists migrate with their herds from north to south according to the rainy season (Egemi, 2012). As African cattle, these indigenous cattle are widely acclimatized to the tropical environment due to their extreme degree of drought tolerant, resistance to tick-borne diseases, heat tolerance, and resistance to trypanosomiasis, also survive on poor quality pastures (Mattioli *et al*., 2000). In both Darfur and Kordofan states these indigenous cattle breeds are mainly considered as food security, store of value, wealth, power, and authority of these rearing tribes (Ahmed, 2014). Moreover, these two regions mainly play an important role in the source of beef for local consumption and contribute to the national economy as live cattle and beef export trade of Sudan (Bank, 2020). Furthermore, these indigenous cattle are considered a pool of genetic resources that account for the reserving genetic diversity (Nyamushamba *et al*., 2017). In the last few decades, selection pressures and breeding programs have been directed to improve livestock production. In recent decades, efforts have been made to enhance livestock production through the deliberate application of selective pressures and breeding programs. Consequently, there is a possibility of uncontrolled interbreeding occurring between indigenous and exotic breeds. Consequently, numerous indigenous cattle breeds have experienced complete extinction (Mwai *et al*., 2015).

Heat stress (HS) is the main factor that negatively affects the production and reproduction performance, health, and immune system of cattle (Thornton *et al*., 2022). Many genes belonging to the families of heat shock proteins (HSPs) regulate the physiological mechanism of adapting to heat stress at the cellular level (Kim *et al*., 2020). In cattle, the response of cells to heat stressors is controlled at the level of transcription by a group of genes known as heat shock factors (Good *et al*., 2021). Activated heat shock factors interact with the heat stress element, located in the promoter region of heat shock protein genes, leading to transcription enhancing of HSP mRNA. As a result, HSPs play an important role in protecting the cells during heat stress in cattle (Archana *et al*., 2017). Among these HSPs, HSP70 is a highly prominent and widely expressed heat shock protein that has been identified as an ideal genetic biomarker for measuring thermotolerance and quantifying heat stress in cattle (Kaushik *et al*., 2022). Therefore, a high expression of HSP70 mRNA acts as an indicator of a high level of thermotolerance (Hassan *et al*., 2019). Furthermore, these candidate genes can differentiate between individuals in heat stress response. The polymorphisms in the HSP70 gene influenced the performance of production and reproduction in cattle, according to Ramesha *et al*. (2016) and Gafer, *et al*. (2015) who found that the sperm motility in Egyptian bulls is influenced by these polymorphisms. Bhat *et al*. (2016) observed an impact on cellular thermotolerance traits in Tharparkar cattle. Turner *et al*. (2013) reported an association with horn fly response in Brahman and Angus cattle. Previous studies described several of these polymorphisms in the 5′ and 3′ untranslated regions of HSP70 gene (Grosz *et al*.,1994; Adamowicz *et al*., 2005; Basiricò *et al*., 2011; Öner *et al*., 2017). However, the linkage of HSP70 gene polymorphisms and thermotolerance, as well as environmental resistance in Sudanese indigenous cattle have not been studied at genetic characterization levels. Therefore, this research was conducted to evaluate the genetic polymorphisms in

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both 5′ and 3′ untranslated regions of the HSP70 gene in some Western Sudanese indigenous cattle.

Materials and methods

Description of the Study Area

This research was carried out in two different ecological areas as below:

South Darfur State

The South Darfur State is situated in the southwestern region of Sudan (Fig. 1). It has covered an area extending 139800 km2, spanning from latitude 13–9.30° north and longitude 27–24.30° east. This state shares a border with three neighboring states, namely North, Central, and East Darfur. Moreover, it also shares boundaries with South Sudan and the Central African Republic (Ismail *et al*., 2016). South Darfur State's geographical location is characterized by a savannah climate, with the presence of mud sandy soil in its southern regions, while the northern areas exhibit characteristics of a semi-desert, and sandy soil is prevalent. The temperature ranges within this state vary from 20.98 ºC to 35.14 ºC, with an average annual relative humidity of 35.58% and a yearly average rainfall of 402 mm. The dry period in this region occurs from June to October, with the majority of the precipitation concentrated from July to September (Ismail *et al*., 2016).

West Kordofan State

West Kordofan State lies in the southern part of Sudan (Fig.1), between a latitude of 9˚ 12´ - 12˚ 30´ North and a longitude of 15˚ 27´ and 18˚ 30´ East. It shares common borders with the states of East Darfur, North Darfur, and North Kordofan. Additionally, it has an international border with South Sudan. A semi-desert and savanna climate is prevalent in this state. The annual temperature in West Kordofan State exhibits a range of 23.13ºC to 35.33ºC, while the annual rainfall varies from 450 to 650 mm. (Bashir and El Zubeir, 2013).

Data Collection

Ethical approval: This experiment was approved according to the Committee on Ethics of Animal Experimentation from Alexandria University, Alexandria, Egypt (Code ID: AU-08-21-03-1-2-74).

Blood sample and DNA extraction

A total of 60 unrelated indigenous breeds, Sudanese Red Fulani (n = 20), Nyalawi (n = 20), and Messeri (n = 20) cattle were randomly selected. The blood samples of Nyalawi and Sudanese Red Fulani cattle were collected from Nyala city in South Darfur State, while the blood samples of Messeri ecotype cattle were collected from Elfoula city and around areas located in Western Kordofan State, during the period from July to September 2021. 5 ml of blood samples were collected from each animal in a sterile EDTA tube, and kept at -20°C. After being transported to the laboratory of the Institute of Molecular Biology, University of Nyala, Sudan, isolation of genomic DNA from the blood samples was performed according to the manufacturer's instruction (GB100/300, Genomic DNA Mini Kit (Blood/Cultured Cell) Kit, Geneaid Biotech Ltd, China). DNA was extracted from fresh samples and stored at -20°C for further analysis. Estimation of the quality and purity of DNA was evaluated using gel electrophoresis and Nanodrop.

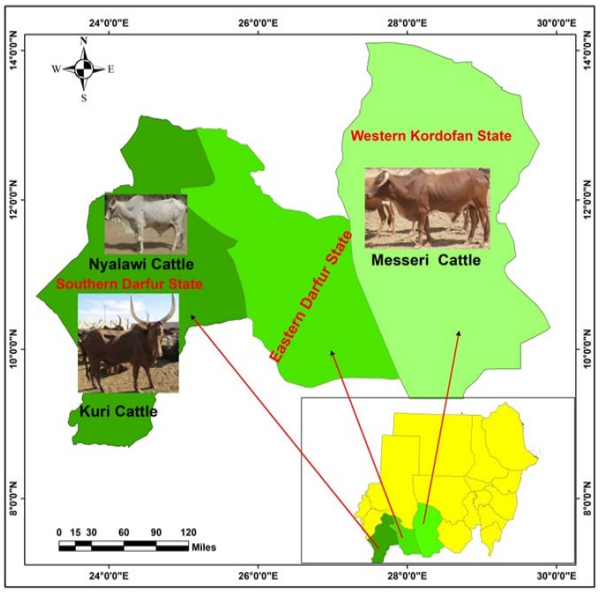

Fig. 1. Geographical location of the study area, South Darfur and West Kordofan States.

PCR amplification and DNA sequencing

The untranslated regions (UTRs) of the HSP70 gene, specifically the 5′ and 3′ UTRs, were amplified using two sets of primers as mentioned in Table 1. The polymerase chain reactions (PCR) analyses were performed in a total volume mixture of 25 µL carried out using (R1211119, Rotor-Gene Q, QIAGEN Hilden, Germany) thermal cycler. Each reaction mixture contained 1 µM of each primer pair and 25 ng of DNA. The conditions of the thermal cycle for PCR were as follows; 94°C for 5 min was followed by 30 cycles at 94°C for 45 s, T°C (T = 59 for 5′and 3′ UTR, respectively) for 30 s, and 72°C for 60s, followed by a final extension at 72°C for 5 min. The PCR amplicons were then separated through a 1% (w/v) agarose gel in 1× TBE (tris-borate-EDTA) buffer stained with 0.2 µl ethidium bromide (10 mg/mL) at 80 volts until complete separation of the bands, parallel with 5 µl of the100 bp DNA ladder. Then, amplification products were visualized and documented under ultraviolet (UV Transilluminator) light to assess successful amplification. After PCR amplification, the 20 µL PCR products were sent to Macrogen Company, (API3730XL DNA Analyzer, Applied Biosystems) for sequencing in two directions. Gene bank accession numbers of samples for 3′ UTR region sequence were from OR906096 to OR906127, and Gene bank accession numbers of samples for 5′ UTR region sequence were from OR946283 to OR946319.

Identification of polymorphisms in 5′UTR and 3′ UTR regions of the HSP70 gene

PCR products, resulting from the amplification of DNA samples of

Table 1. Description of the primers pairs applied for amplification of certain sequences of heat shock protein 70 (HSP70) genes.

Primer	Primers (5´- 3´ sequence)	GenBank accession No.	Gene region	Amplicon size (bp)	Reference
HSP70A	F:5′-GCCAGGAAACCAGAGACAGA-3′ R:5′-CCTACGCAGGAGTAGGTGGT-3′	M98823.1	5′-UTR	539 bp	Basiricò *et al*. (2011)
HSP70-1	F:5′-GGATTGCTCATGTTTGTTATGG-3′ R:5′-CTTGGAAGTAAACAGAAACGGG-3′	AY626950	3′-UTR	253 bp	Grosz *et al*. (1994)

*F: forward; R: reverse; HSP70: Heat shock protein 70.

Sudanese Red Fulani, Nyalawi, and Messeri indigenous cattle breeds, were subjected to sequencing analysis to determine single nucleotide polymorphism in 5'UTRof hsp70A gene and 3'UTR of hsp70-1 gene. Sample sequences were aligned with the reference gene sequences of HSP70 via the NCBI database. The 5′ UTR sequence has a GenBank accession number of M98823.1, while the 3′ UTR sequence has an accession number of AY626950.1, as indicated in Table 1. Multiple sequence alignments of the sequenced samples from the three breeds were achieved using the tool provided by the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/). Additionally, the MEGA program (https://www.megasoftware.net/) was utilized to construct a phylogenetic tree to analyze the genetic variation in this gene among the three breeds.

Statistical Analysis

Data on the HSP70 gene sequence was analyzed using PopGene32 (version 1.31) software according to (Yeh, 1999). The Data presented as genotype and allele frequencies, expected and observed heterozygosity, number of effective alleles, and polymorphic informative content (PIC) was calculated. Then, the Hardy-Weinberg equilibrium was estimated.

Results

PCR amplification of 3′ and 5′ untranslated regions (5′, 3′ UTRs) of HSP70 gene

The 5′UTR of the HSP70A gene of three studied breeds, Sudanese Red Fulani, and two Western Baggara ecotypes (Nyalawi and Messeri) cattle were effectively amplified using a pair of primers (Table 1). The electrophoretic patterns (Fig. 2) showed that the amplified PCR products had the same length (539 bp) as the 5′UTR of the Bos taurus HSP70A gene previously reported by Basiricò *et al*. (2011). The 3′ UTR of the HSP70-1 gene of all studied cattle breeds was successfully amplified by using a specific primer pair (Table 1). Gel electrophoresis (Fig. 2) revealed the amplified PCR products at the appropriate amplicon length (253 bp), which is the same length as the 3′ UTR region of the HSP70-1 gene previously reported by Grosz *et al*. (1994).

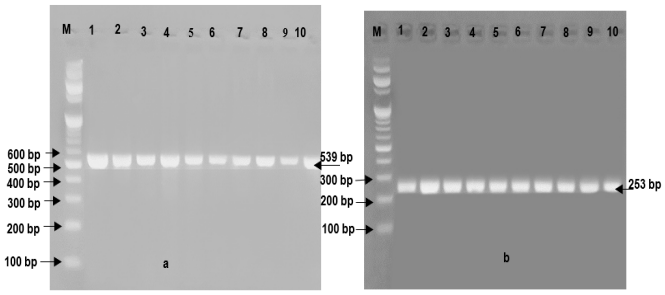

Fig. 2. The electrophoretic pattern of PCR amplification of 5′UTR, 3′ UTR regions of the HSP70 gene. (A) estimated amplicon of 539bp of HSP70A-5′UTR (B) estimated amplicon of 253bp of HSP701- 3′ UTR, Lane M: a molecular marker of 100 bp Pattern. Lane 1- 10 representative number samples of the studied breed.

Sequencing and Polymorphisms in 5′UTR and 3′ UTR regions of the HSP70A gene

The heat shock protein (HSP) family's molecular chaperones play an important role in high production and reproduction rates in cattle. Our study included two regions of the HSP70gene (5'UTR of the hsp70A and

Table 2. The frequencies of genotypes and alleles of (SNPs) within the polymorphic sites at 5'UTR of the HSP70A and 3'UTR of HSP70.1 in Sudanese Red Fulani cattle.

SNP	Genotype frequency			Allele frequency		H_o	H_e	ne*	PIC	χ^2
5′-UTR g.802C>T	CC (0.12)	TC (0.67)	TT (0.1)	C (0.31)	T (0.69)	0.32	0.44	1.76	0.34	1.70 ^{ns}
5′-UTR g. 895C/-	CC (0.02)	C- (0)	-- (0.98)	C (0.01)	- (0.99)	0	0.47	1.97	0.01	20. 38^{ns}
5′-UTR g.1125A>C	AA (0.20)	AC (0.6)	CC (0.2)	A (0.79)	C (0.21)	0.1	0.33	1.4	0.28	10.18^{ns}
5′-UTR g.1128G>T	GG (0.15)	GT (0.65)	TT (0.2)	G (0.76)	T (0.24)	0.16	0.37	1.57	0.3	6.93^{ns}
5′-UTR g.1204T>C	TT (0.7)	TC (0.2)	CC (0.1)	T (0.79)	C (0.21)	0.21	0.33	1.5	0.28	3.13^{ns}
3′-UTR g.64T>G	GG (0.05)	GT (0.95)	TT (0.1)	G (0.97)	T (0.3)	0	0.47	1.88	0.56	20.23^{ns}

SNP: single nucleotide polymorphism; He: expected heterozygosity; Ho: observed heterozygosity; ne*: effective number of alleles; PIC: polymorphic informative content; χ2: chi-square values for Hardy-Weinberg equilibrium test.; *under Hardy-Weinberg equilibrium (p<0.05) ns: not significant; (-): any nucleotide except Cytosine (A, T, or G).

Table 3. The frequencies of genotypes and alleles of (SNPs) within the polymorphic sites at 5'UTR of the HSP70A and 3'UTR of HSP70.1 in Nyalawi cattle.

SNP	Genotype frequency			Allele frequency		H_o	H_e	ne*	PIC	χ^2
5′-UTR g.802C>T	CC (0.25)	TC (0.65)	TT (0.1)	C (0.34)	T (0.66)	0.26	0.45	1.89	0.35	3.77 ^{ns}
5′-UTR g. 895C/-	CC (0.02)	C- (0)	-- (0.98)	C (0.01)	- (0.99)	0	0.47	1.97	0.01	20. 38^{ns}
5′-UTR g.1125A>C	AA (0.84)	AC (0)	CC (0.16)	A (0.84)	C (0.16)	0.1	0.26	1.43	0.25	8.42^{ns}
5′-UTR g.1128G>T	GG (0.16)	GT (0.73)	TT (0.11)	G (0.82)	T (0.18)	0.16	0.3	1.43	0.25	5.18^{ns}
5′-UTR g.1204T>C	TT (0.8)	TC (0.04)	CC (0.16)	T (0.84)	C (0.16)	0.1	0.26	1.36	0.25	8.42^{ns}
3′-UTR g.64T>G	GG (0.78)	GT (0.11)	TT (0.11	G (0.81)	T (0.18)	0.16	0.3	1.43	0.25	5.18^{ns}

SNP: single nucleotide polymorphism; He: expected heterozygosity; Ho: observed heterozygosity; ne*: effective number of alleles; PIC: polymorphic informative content; χ^2: chi-square values for Hardy-Weinberg equilibrium test.; *under Hardy-Weinberg equilibrium (p<0.05) ns: not significant; (-): any nucleotide except Cytosine (A, T, or G).

3'UTR of hsp70.1) were studied. Based on the obtained sequencing data of the HSP70 gene. Among the two regions, the sequencing screening in a 539 bp region of 5′-UTR was found to be more variable than the 3′-UTR as shown in Fig. 3.

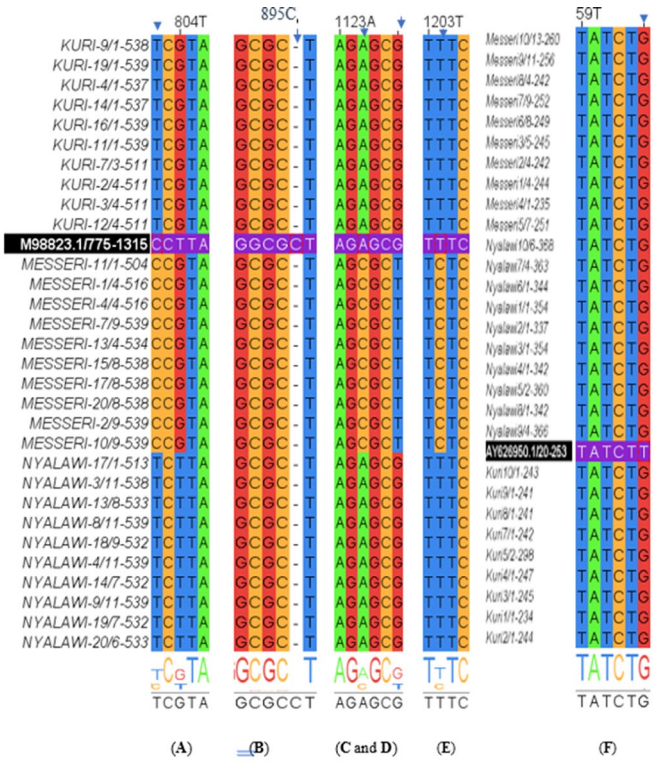

Fig. 3. Multiple sequence alignment of HSP70A- 5'and HSP70.1-3' UTR using Clustal Omega: Reference sequences (Dark Violet color in a row) from GenBank database. Accession number M98823.1 for the 5' UTR and AY626950.1 for and 3' UTR. (a): 5′-UTR g.802C>T(b) 5′-UTR g.895C/- (c)5′-UTR g.1125A>C (d) 5′-UTR g.1128G>T (e) 5′-UTR g.1204T>C (f) 3′-UTR g.64T>G.

Multiple sequence alignment of Sudanese Red Fulani, Nyalawi, and Messeri breeds, and reference sequences in the hsp70A gene at 5'UTR region showed that the identities were 99% in Nyalawi whereas the identities in both Sudanese Red Fulani and Messeri sequences were 97%. in contrast, reference sequences in HSP70.1 gene at 3'UTR region showed that the identities were 99% in both Sudanese Red Fulani and Nyalawi cattle, whereas the identities were 98% in Messeri cattle sequences. Analysis of single nucleotide polymorphisms (SNPs) was performed at 5'UTRof the HSP70A and 3'UTR of HSP70.1 regions of Sudanese Red Fulani (Kuri), Nyalawi, and Messeri indigenous cattle breeds as shown in (Fig 3; a, b, c, d, e and f).

In accordance with the polymorphism estimated results of the HSP70 gene, five polymorphic SNPs were identified in 5' untranslated region (5' -UTR) of HSP70A: g.802C>T, g.895C/-, g.1125A>C, g.1128G>T and g.1204T>C and only one polymorphic SNP of g.64T>G in HSP70.1-3' UTR, as shown in (Fig. 3). Three transition mutations, four transversion mutations, and one deletion mutation, were detected based on sequence alignment with the reference sequence (Fig. 3). The frequencies of genotypes and alleles for the nucleotide sequence polymorphism at the 5'-UTR HSP70.1 and HSP70.1-3' UTR region of this gene have been recorded in Tables 2, 3, and 4, as well as Fig. 4. SNPs analysis, in a 539 base pair in 5′ untranslated region (UTR) of this gene, detected a new synonymous SNP C>T transition of cytosine to thymine at nucleotide base position g.802C>T in Sudanese Red Fulani, Nyalawi and Messeri cattle respectively. As shown in Tables 2, 3, and 4; the TC genotype had a high frequency in both Sudanese Red Fulani (0.67) and Nyalawi (0.65) cattle, while the genotype CC had a high frequency (0.70) in Messeri cattle. As shown in (Fig. 4) the T allele reported a higher frequency in both Sudanese Red Fulani (0.69) and Nyalawi (0.66) cattle than in Messeri (0.27) cattle. In our study, the most thoroughly investigated mutation was detected as a single mutation deletion of cytosine at nucleotide base position 895C/-. The detected mutation of cytosine deletion at nucleotide base g.895C/- was observed; the genotype (--) recorded the highest frequency (0.98) in all cattle. In addition, our results detected two transversion mutations; the first transversion mutation occurred at nucleotide position g.1125A>C, the highest genotype frequencies were genotype (AA) in Nyalawi (0.84), genotype (CC) in Messeri (0.70) and genotype (AC) in Sudanese Red Fulani cattle (0.60). The allele A had the highest frequencies were 0.84 and 0.79 Nyalawi and Sudanese Red Fulani respectively, however, the allele C had the highest frequency (0.73) in Messeri cattle. The second transversion mutation g.1128G>T was detected in all studied cattle. The most allele frequent was G in Nyalawi (0.82) and Sudanese Red Fulani (0.76), and allele T in Messeri cattle (0.77). Moreover, transition mutation occurred at nucleotide position g.1204T>C which is presumed to be fixed across the whole studied breeds. The T allele showed higher frequencies in both Sudanese Red Fulani (0.79) and Nyalawi (0.84) cattle than in Messeri cattle (0.20). The C allele revealed higher frequencies (0.8) in Messeri cattle than in Sudanese Red Fulani (0.21) and Nyalawi (0.16) as shown in Tables 2, 3, and 4 and Fig. 4.

Sequencing analysis of 253 base pair fragments of the HSP70.1-3' UTR showed few variabilities within sequenced samples sequences as shown (Fig. 3). Our findings at the genetic polymorphism level concerning 3′-untranslated region of HSP70.1 gene in all studied breeds indicated the presence of monomorphic alleles for the transversion mutation from (T) to Thymine (G) at position g.64 T > G. In addition, the G allele illustrated a greater occurrence in all investigated breeds compared to the T allele. The G allele frequencies were 0.83, 0.82, and 63 in Messeri, Nyalawi, and Sudanese Red Fulani cattle, respectively (Tables 2, 3, 4, and Fig. 4).

The range of polymorphic informative content (PIC) values was from

Table 4. The frequencies of genotypes and alleles of (SNPs) within the polymorphic sites at 5'UTR of the HSP70A and 3'UTR of HSP70.1 in Messeri cattle.

SNP	Genotype frequency			Allele frequency		H_o	H_e	ne*	PIC	χ^2
5′-UTR g.802C>T	CC (0.7)	CT (0.2)	TT (0.1)	C (0.73)	T (0.27)	0.15	0.4	1.66	0.32	8.68^{ns}
5′-UTR g. 895C/-	CC (0.02)	C- (0)	-- (0.89)	C (0.01)	- (0.99)	0	0.47	1.97	0.01	20.40^{ns}
5′-UTR g.1125A>C	AA (0.00)	AC (0.3)	CC (0.7)	A (0.27)	C (0.73)	0.15	0.4	1.66	0.32	8.09^{ns}
5′-UTR g.1128G>T	GG (0.17)	GT (0.08)	TT (0.75)	G (0.23)	T (0.77)	0.15	0.35	1.54	0.29	7.45^{ns}
5′-UTR g.1204T>C	TT (0.22)	TC (0)	CC (0.78)	T (0.2)	C (0.8)	0.1	0.32	1.47	0.27	10.84^{ns}
3′-UTR g.64T>G	GG (0.8)	GT (0)	TT (0.2)	G (0.83)	T (0.17)	0.05	0.29	1.4	0.25	15.79^{ns}

SNP: single nucleotide polymorphism; He: expected heterozygosity; Ho: observed heterozygosity; ne*: effective number of alleles; PIC: polymorphic informative content; χ^2: chi-square values for Hardy-Weinberg equilibrium test.; *under Hardy-Weinberg equilibrium ($p<0.05$) ns: not significant; (-): any nucleotide except Cytosine (A, T, or G).

0.56 to 0.23, showing that the single nucleotide polymorphisms in the HSP70 gene revealed a relatively low to moderate level of genetic variation in the breeds under investigation. Furthermore, the highest estimated values were reported in SNP of g.895C/- (1.97) and g.64T>G (1.88) in Sudanese Red Fulani and indicated that these SNPs are highly polymorphic. The C deletion and G alleles were the most frequent then (--) and GT genotypes were the most frequent genotypes among Sudanese Red Fulani cattle.

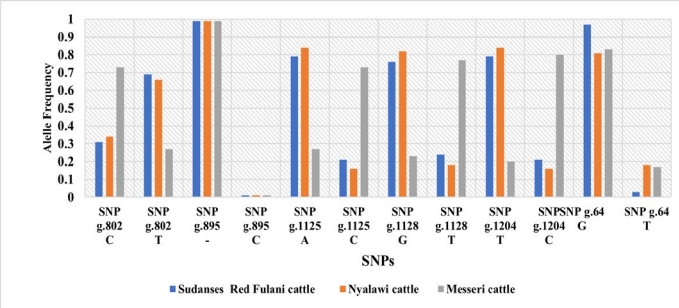

Fig. 4. Distribution of allele frequencies of single nucleotide polymorphisms (SNPs) within the polymorphic sites at 5'UTR of the HSP70A and 3'UTR of HSP70.1.

The results presented in Tables 2, 3, and 4, indicate that Chi-square (χ^2) tests demonstrated disagreement between the frequencies of genotypes and alleles of the HSP70 gene, and Hardy-Weinberg equilibrium ($p>0.05$). Neighbor-joining tree analyses among breeds were obtained as shown in (Fig. 5). The phylogenetic relationship tree was constructed using the alignment of the M98823.1 for 5′UTR (Fig. 5a) and AY626950.1 for 3′UTR (Fig. 5b) sequences and sequences of the same gene of the three studied cattle ecotypes. The constructed phylogenetic tree showed that (Fig. 5a and b) Nyalawi and Messeri cattle were more similar than (Kuri) Red Fulani for both 3′UTR and 5′UTR sequences.

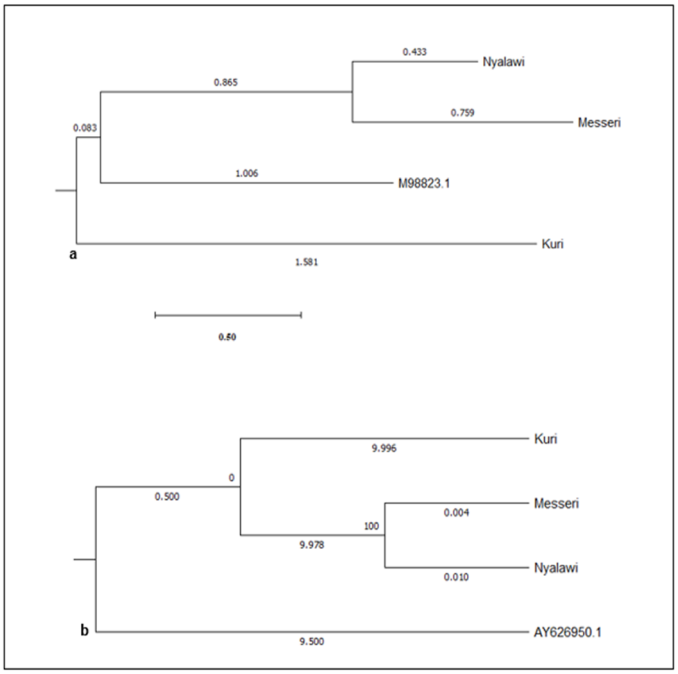

Fig. 5. Phylogenetic relationship of the HSP70 (5'UTR of the HSP70A and 3'UTR of HSP701) gene nucleotide sequences from Sudanese Red Fulani (Kuri), Nyalawi and Messeri cattle: the phylogenetic tree constructed by using Neighbor-joining method. (a): 5'UTR of the HSP70A; (b): 3'UTR of HSP701.

Discussion

Many cattle breeds, including Holstein-Friesian, Chinese Holstein cattle, Chinese Simmental, Hereford, Hariana, Xinjiang Brown, and Sanhe cattle, have been shown to contain variations in the 5′-UTR region of the hsp70 gene (Hu *et al*., 2019; Abbas *et al*., 2020). In contrast, several promoters and 3′ UTR of HSP70-1gene polymorphisms were reported (Grosz *et al*., 1994; Adamowicz *et al*., 2005; Rosenkrans Jr *et al*., 2010; Basiricò *et al*., 2011). Nevertheless, only a few variants have been screened in the coding and 3′-UTR of the HSP70.1 (Basiricò *et al*., 2011). Five SNPs were detected in a region of 539 base pairs in HSP70.1-5' untranslated region, and new synonymous SNP g.802C>T was detected in all tested breeds as shown in Tables 2, 3 and 4; both Sudanese Red Fulani and Nyalawi cattle have high frequency for TC genotype and T allele compared to Messeri cattle, while Messeri cattle has a high frequency for CC genotype compared to Red Fulani and Nyalawi cattle. The present results demonstrated that the fragment of the HSP70 gene in a cross-all studied breeds was polymorphic because one of the alleles was more frequent than the other. This is to the finding of Beckham *et al*. (2004) who reported that the gene is considered polymorphic when one of the allele's frequency is less than 99 percent. These new synonymous SNP 802C>T could presumably explain the relative heat adaptation exhibited by Sudanese Red Fulani and Nyalawi cattle. The currently detected mutations have not been reported previously in Sudanese indigenous cattle and need further studies. Many investigators suggested that synonymous single nucleotide polymorphisms may potentially impact the resulting protein by altering the process of transcription, influencing the accuracy of mRNA splicing, and controlling transcription (Cartegni *et al*., 2002; Hunt *et al*., 2014 ; Gafer *et al*., 2015). A similar observation was reported earlier by Han *et al*.(2009). Moreover, Gafer *et al*. (2015) demonstrated a highly significant association between cattle bull-carrying genotypes (TT) with traits of semen quality. Thus, the T allele appeared to be a more favorable allele than the C allele. The present results are in agreement with earlier results of authors (Nikbin *et al*., 2014) for goats.

In addition, one single mutation was detected as a Cytosine deletion at nucleotide position g.895C/-, it was noticed that all breeds revealed the highest frequency (0.98) for the genotype (--). The genotypic variants of the HSP70.1 gene region, especially the deletion of Cytosine base (base position g.895) is associated with decreasing cellular thermo-tolerance in cattle. This result agrees with the previous results by many investigators (Schwerin *et al*., 2003;Banks, 2007; Rosenkrans Jr *et al*., 2010; Deb *et al*., 2013).

Subsequently, thermo-tolerability was additionally revealed in all studied breeds. This deletion is found in the AP2 box transcription binding site led to decreased transcription binding function (Schwerin *et al*., 2002). While previous study documented high cell survival and thermal tolerance levels in cattle carrying C deletion (Basiricò *et al*., 2011). Moreover, two transversion mutations were observed; the first detected at nucleotide position g.1125A>C, and the highest genotype frequencies were genotype (AA) in Nyalawi, genotype (CC) in Messeri, and genotype (AC) in Sudanese Red Fulani cattle. Red Fulani and Nyalawi had the highest frequencies for allele A, however, allele C had the highest frequency in Messeri cattle. The second transversion mutation g.1128G>T was detected in all studied cattle. Nyalawi and Sudanese Red Fulani had the most frequent allele G, and Messeri cattle had a higher frequency of allele A than the other breeds. Furthermore, transition mutation g.1204T>C was noticed in all breeds, The higher frequent allele in Messeri cattle was the C allele, whereas the higher frequent allele in both Sudanese Red Fulani and Nyalawi cattle was the T allele as shown in Tables 2, 3 and 4 and Fig. 4. Based on that, it is worthy to mention that the Messeri cattle carry three previous mutant alleles at g.1125A>C, g.1128G>T and 1204T>C in high frequencies, whereas these alleles revealed in very low frequencies in Sudanese Red Fulani and Nyalawi breeds. These single nucleotide variants observed in Messeri cattle may be a result of the biological mechanisms related to heat stress response, which improves the heat adaptation of this breed compared to Sudanese Red Fulani and Nyalawi. This mutation also distinguishes the studied breeds according to geographical location (Fig. 1). The findings presented in this study are in line with the research conducted by Basiricò *et al*. (2011), which demonstrated a correlation between the occurrence of Cytosine deletion at nucleotide base 895C/- and a transversion from (G) to (T) at base 1128G>T of this gene, and the increase in gene expression and synthesis of HSP70.1. Additionally, these modifications enhance the capacity of cattle mononuclear cells to respond to heat stress by increasing cell viability. Furthermore, the Genotype GT for (SNP) of g.1128G>T has been recorded as the superior genotype for the immune responses in Friesian Holstein cattle (Basiricò *et al*., 2011). As previously documented, Banks (2007) mentioned that two (SNPs) at g.1125A>C and g.1128G>T significantly affect the calving percentage in Brahman cattle, carrying the genotypes AA (g.1125A>C) and GG (g.1128G>T). These two genotypes were reported as the most preferred genotypes. A previous investigation conducted on Brahman and Angus cattle revealed that (SNP) located at position g.1128G>T exhibits a noteworthy correlation with the response to infestation by horn-flies. The related genotypes GG and TT were designated as the best genotypes (Turner *et al*., 2013). Similarly, numerous researchers have documented these mutations at various locations within the HSP70 gene in cattle, revealing variations in thermo-tolerance. Nonetheless, they were at different loci; they support our study results that the gene is an important

molecular marker for thermotolerance in cattle (Li *et al*., 2011; Xiong *et al*., 2013).

On the other hand, the obtained results at the genetic polymorphism level in 3′-UTR of the HSP70.1 gene in all tested breeds showed one transversion mutation at position g.64T>G. This monomorphic mutant allele G showed higher frequencies in all studied breeds than the T allele. (Tables 2, 3, and 4 and Fig. 4). This result agrees with the previous result by Basiricò *et al*. (2011), who reported a monomorphic site for the same SNP in Italian Holstein cattle. Furthermore, numerous researchers have documented that 253 base pair segment of HSP70.1 3'-untranslated region exhibited a monomorphism in both the Bos taurus and Bos indicus bovine breeds (Basiricò *et al*., 2011; Sodhi *et al*., 2013).

The estimation of genetic diversity in a population is achieved by employing the calculation of heterozygosity values (Marson *et al*., 2005). Thus, the heterozygosity value is employed to ascertain the polymorphism proportion related to an allele, and the potentiality of preserving the genetic variation within the population for forthcoming generations (Falconer and Mackay,1996). Genetic diversity represents a fundamental element of population selection. Cattle's ability to tolerate heat can be bred from populations that show considerable genetic variation that reveal different responses to adapt to the environment (Suhendro *et al*., 2021). Heterozygosity illustrates the presence of genetic diversity within a given population. Correspondingly, a population with a greater magnitude of heterozygosity will exhibit a correspondingly higher level of genetic variation (Haddar and Noor, 2022). The calculated and observed values of heterozygosity (He and Ho) in all studied cattle were observed to be less than 0.5. These findings propose that the heterozygosity value was relatively low across all populations. As per the results obtained by Botstein *et al*. (1980), values of heterozygosity were greater than 0.5 indicating a lower variation of allele in the population. The diminished level of heterozygosity could be due to the inbreeding procedure adopted by owners. This finding can be used for future conservation and breeding programs in the Sudanese indigenous cattle population.

Regarding to 6 identified SNPs, the Polymorphic Information Content (PIC) value for each locus was calculated based on the following classification: a PIC value less than 0.25 indicates low polymorphism, a PIC value between 0.25 and 0.5 suggests an intermediate level of polymorphism, and PIC value greater than 0.5 signifies a high degree of polymorphism (Botstein *et al*., 1980). In this study, the range of observed PIC values was from 0.56 to 0.23, demonstrating that the SNPs within the HSP70 gene revealed a level of polymorphism that falls within the low to moderate range among the investigated populations. The SNP of g.895C/- (1.97) and g.64T>G (1.88) exhibited the highest value in Sudanese Red Fulani, suggesting a high level of polymorphism in these SNPs. The C deletion and G alleles were the most frequent then (--) and GT genotypes were the most frequent genotypes among Sudanese Red Fulani cattle. Therefore, molecular selection based on SNPs of g.895C/- (1.97) and g.64T>G (1.88) can be used for selecting and breeding thermotolerants and matching to appropriate environments to mitigate the consequences of heat stress on sustainable Sudanese indigenous cattle genetic resources.

As shown in Tables 2, 3, and 4; the Chi-square (χ^2) tests revealed that all genotypes and alleles frequencies of the HSP70 gene disagreed with the Hardy–Weinberg equilibrium ($p>0.05$). This disequilibrium may be due to inbreeding, selection, or random genetic drift. A population is in equilibrium if the genotype and allele frequencies are constant from generation to generation. Our present observations demonstrated that the HSP70 gene fragment in Sudanese Red Fulani, Nyalawi, and Messeri cattle exhibited polymorphism representing a perfect chance to select indigenous cattle that are more tolerant to heat stress. Cattle with genetic variation are more adaptive to environmental conditions change (Haddar and Noor, 2022). These current results could be used as a reference in controlling the breeding goals for Sudanese cattle for producing indigenous cattle that have more efficient heat tolerability.

The recent phylogenetic tree indicated that the gene sequences of two Baggara ecotypes (Nyalawi and Messeri) were closer and evolutionarily shared the same ancestor, however, the gene sequences of two Baggara ecotypes were closer to M98823.1 for 5′UTR sequence than the same sequence of Sudanese Red Fulani (Kuri) (Fig. 5a). On the other hand, and regarding the sequence 3′-UTR; the two sequences of Baggara ecotypes were closer to the sequence of Sudanese Red Fulani (Kuri) than the sequence of AY626950.1 for 3′-UTR (Fig. 5 b). Generally, the two Baggara ecotypes had the same ancestor, were closer to each other, and exhibited a greater similarity when compared with Sudanese Red Fulani cattle. This finding is dissimilar to that reported by Hamza and Fathi, 2020, they showed phenotypical differences between two Baggara ecotypes (Nyalawi and Messeri). Based on morphological phenotypic and geographically distinct populations of this Western Baggara cattle, Nyalawi and Messeri and Sudanese Red Fulani ("Kuri" local name) which are well-known for their unique meat production capabilities and adaptation attributes (Hamza, 2021). Western Baggara cattle are classified and named according to their ecological, geographic location, and tribal ownership. The Sudanese Red Fulani cattle had superior phenotypic beef characteristics over Nyalawi and Messeri ecotype cattle (Hamza, 2021). The common phenotypic traits in most Sudanese Red Fulani cattle differ from those of Senegalese and White Fulani (Mason and Maule, 1960).

Conclusion

The newly synonymous SNP of g.802C>T transition is not previously documented in Sudanese indigenous cattle. Further studies are needed in a sizable population to detect the association between the SNPs and thermotolerance. The SNPs of g.895C/- and g.64T>G can be used as molecular markers to assist selection for heat tolerance in Sudanese cattle breeds.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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