MOLECULAR CHARACTERIZATION OF *HSP* 70 GENE USING SINGLE NUCLEOTIDE POLYMORPHISM IN NIGERIAN BREEDS OF ZEBU CATTLE

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Abstract: Heat shock protein (*HSP*) 70 gene is a member of *HSPs* sub-family that act as molecular chaperons whenever animals come under thermal assault , they fulfill essential roles of providing cellular protection, immune response, protein synthesis, protein folding and unfolding, protection proteins from cellular stress, inhibitory apoptosis and adaptation during thermal assault. A total of ninety (90) adult bulls from across four extant breeds of Nigerian Zebu cattle comprising of White Fulani (25), Sokoto Gudali (21), Red Bororo (21) and Ambala (23) sampled from northern parts of Nigeria. Genomic DNA was extracted from 90 animal skin tissue samples and was subjected to polymerase chain analyses followed by sequencing of the PCR products for detection SNPs of *HSP* 70 gene in four Nigerian breeds of cattle. SNPs within the nucleotide sequences of four Nigerian bovine *HSP70* gene of were visualised, detected and bio-edited by chromatogram analyses using SeqMan Ngen Tool (DNASTAR®, Inc., Madison, Wisconsin, U.S.A). We pioneered a preliminary single nucleotide polymorphism study of *HSP* 70 gene for the first time in Nigerian zebu cattle. Sequence data detected 21 SNP loci within the coding region of exon 1 of *HSP* 70 gene which include: 1 Indel A7Del i.e. deletion of A at base position 7 (White Fulani), 10 transversions (White Fulani: C154G and G220T, Ambala: G220T, Sokoto Gudali: T198A, Red Bororo: C154G, A78T, G106C, T198A, G220T and T254A) and 10 transitions (White Fulani: C145T and G220A, Ambala: C154T and C244T, Sokoto Gudali: C184T and Red Bororo: G157A, C157T, G196A, C244T and G199A). We hypothesize that the detected SNPs should further be associated thermo-tolerance traits to unravel their possible effect on thermal-tolerance performance, adaptability and susceptibility of different Nigerian cattle breeds to environmental stress load and thermal assaults of tropical conditions.

Keywords: *HSP* 70 gene, thermal stress, PCR, single nucleotide polymorphism

Introduction

The heat stress (HS) has become a major issue in the era of climate change and it directly affects adaptability and survivability of livestock to thermal assault. It has been shown that animals can succumb to hyperthermia as they fail to abate the impact of HS load. Increased HS in cattle breeds and other livestock animals have been linked to poor food intake and slow metabolism, thereby affecting growth, milk production and reproductive efficiency with adverse economic loss. Several husbandry and management strategies have been employed to help mitigate effect of stress in dairy and beef animals however, this strategies is not far reaching (Kapila *et al.*, 2013). It is believed that enhanced thermo-tolerance performance in zebu cattle could be due to their emergence and natural selection through generations. *HSP* 70 is a member of *HSPs* sub-family (molecular chaperone families) known to be highly expressed under stressful thermal and physiological conditions. These motivate responses to environmental heat loads above thermo-neutral zones in animals through intra and extracellular signals that coordinate cellular and whole animal metabolism (Collier *et al.*, 2008). For instance, single nucleotide polymorphisms (SNPs) have been identified in *HSP* 70 locus and were linked to diseases susceptibility or HS tolerance in zebu cattle (Jian-Bo *et al.*, 2009).

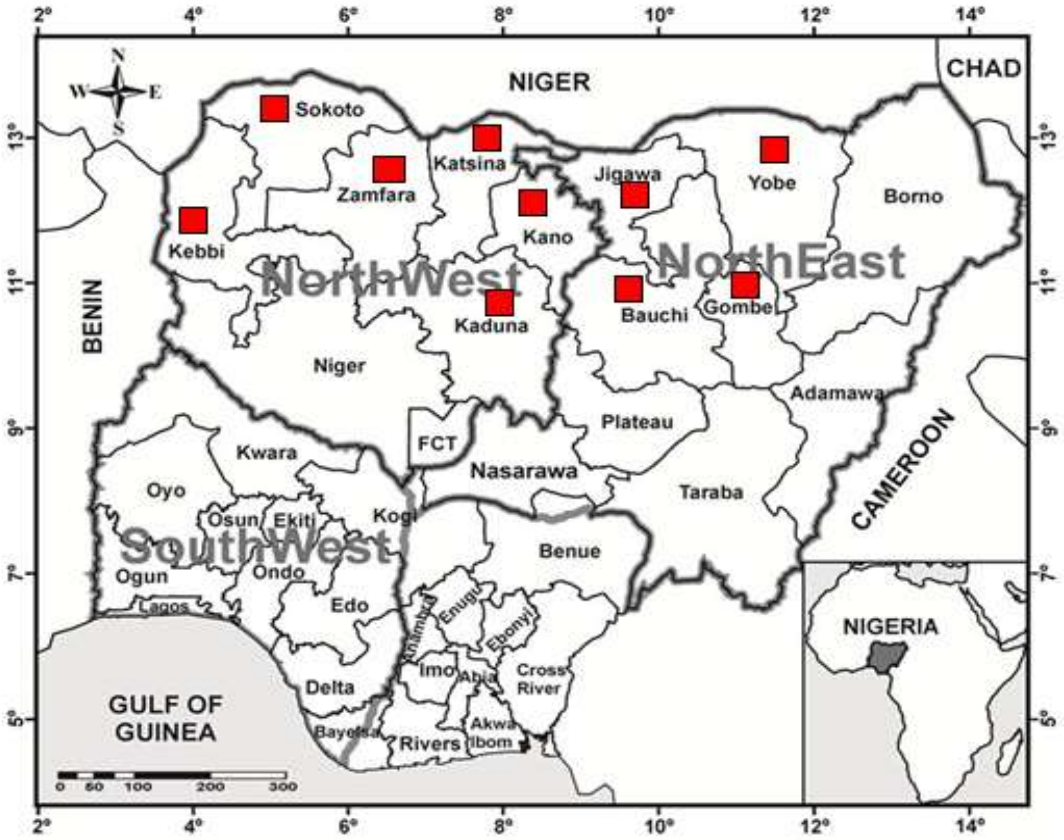
In addition, detection of SNPs of *HSP* 70 gene might be exploited for the identification of thermo-tolerant animals through thermo-tolerance traits association studies so as to drift herds toward superior thermo-tolerant ability through the improvement of thermal-vulnerable animals with thermo-tolerant ones. Nigeria is a country with thermally harsh climate with severe influence of thermal stress which significantly lowers production performance of livestock animals. As far as we know, no feasible effort has been made to study the polymorphism of *HSP* genes in Nigeria livestock especially cattle for possible characterization of these candidate genes. To the best of our knowledge this study attempts to pioneer characterization of *HSP* 70 gene using SNPs in Nigerian zebu cattle breeds towards better understanding of thermo-regulation of Nigerian cattle under assault of thermal condition.

Materials and Methods

Sampling regions and experimental animals

A total of ninety (90) adult bulls from across four extant breeds of Nigerian Zebu cattle comprising of White Fulani (25), Sokoto Gudali (21), Red Bororo (21) and Ambala (23) were sampled across ten (10) states in the Northern parts of Nigeria (Fig. 1).The consent of the herd owners were granted before the commencement of the sampling process. The animals originated from different herds and were reared under

the traditional system of cattle rearing where they grazed during the day on natural pasture containing forages such as Stylo (*Stylosanthes gracilis*), Leucaena (*Leucaena leucocephala*) and Guinea grass (*Panicum maximum*), crop residues and scavenged on kitchen wastes whenever available.



Source: Onasanya *et al*. (2018)

Fig. 1: Map of Nigeria with red boxes showing the regions of sampling

Sample collection and genomic DNA extraction

From each of the 90 samples, 200 g of skin tissue sample was excised from each the animals prior to bleeding in the abattoir/slaughter house and same were sliced into less than 0.5 cm (1 mg) weight and was quickly submerged into 0.5 ml Eppendorf tubes containing RNA*later* reagent. Thereafter, they were quickly transported in iced packed box to laboratory and stored at -20ºC until further analyses.

DNA extraction

The protocol for DNA extraction employed for this study was according to HiPurA™ Multi-Sample DNA Purification procedure, MolBio™Himedia®, Mumbai, India. 25 mg of skin tissue was fetched from each of the 90 samples of skin tissue preserved in RNA*later*. Samples were weighed using sensitive scale, then shredded into smaller pieces and transferred into 2 ml collection tube. 180 µl of resuspension buffer was added into the 2 ml collection tube containing the shredded skin tissue. Thereafter, 20 µl of proteinase K solution was added and thoroughly mixed by vortexing for proper tissue digestion. Incubation of the samples was done using ACCUBLOCK™ digital dry bath at 55ºC for 2–4 h until the tissue was completely digested with no residues. During incubation, the samples were mixed occasionally by vortexing. After digestion, the samples were vortexed briefly for 30 seconds.

The preparation of lysate was done by adding 200 µl of lysis solution to 2 ml collection tube containing digested tissue, vortexing thoroughly for 15 seconds and subsequently incubated using ACCUBLOCK™ digital dry bath at 70ºC for 10 minute to generate lysate. Regarding the binding lysate to the spin column, 200 µl of ethanol (100%) was added to the lysate obtained and mixed thoroughly by gentle pipetting and transferred into HiElute Miniprep spin column and centrifuged at 10000 rpm for 1 minute using Thermo Scientific Nanofuge (MCROCL 21/21R) micro-centrifuge. The flow–through liquid was discarded and the column was placed into a fresh 2 ml collection tube. 500 µl of dilute pre-wash solution (12 ml pre-wash + 18 ml ethanol) was added to the column containing the lysate and was centrifuged at 10, 000 rpm for 1 minute. The flow-through liquid was subsequently discarded. In a separate step, 500 µl of diluted wash solution (8 ml of wash solution + 24 ml of ethanol) was added to the column containing the lysate and was centrifuged at 13,000 rpm for 3 min and the flow through liquid was discarded. The column was further centrifuged at 13, 000 rpm for 1 minute using and the collection tube containing flow–through liquid was discarded. The column was then placed into a new 2 ml collection tube and 100 µl of elution buffer added directly into the column and incubated for 5 minutes at room temperature (15-25ºC) and then centrifuged at 10, 000 rpm for 1 minute to elute DNA into collection tube after which the column was discarded. Eluted DNA was briefly incubated at 60ºC to free the DNA of any contamination and was subsequently stored at -20ºC for further analyses. The quality and quantity of DNA was estimated using Thermo Scientific-NanoDrop 2000 spectrophotometer (Shimadzu co-operation, Japan). The absorbance ratio between OD_{260} and OD_{280} ($OD_{260/280}$ DNA purity) was observed for each sample. DNA sample with absorbance ratio of 1.6-1.9 was considered good and taken for further analysis.

Primer sequence and target regions

The *HSP70* gene primers set (Table 1) used for this study was obtained from the earlier works of Bhat *et al.* (2016) and were optimized for primer specificity. The fragment size for *HSP*70 gene was 295 bp covering coding region in exon 1.

Polymerase chain reaction and amplification condition

The PCR reactions were carried out in a total volume of 15 µl containing template DNA of 1.0, 1.0 µl of each of forward and reverse primers, 7.5 µl PCR Master Mix (2x) (GeNei™ Red Dye PCR Master Mix) and 4.5 µl of nuclease free water. PCR amplification was performed in a TaKaRa Thermal Cycler Dice™ version III (Takara Bio Inc., Japan). The amplification condition involved initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 60 seconds, annealing temperature of 65°C for 45 seconds, extension at 72°C for 1 minute followed by final extension at 72°C for 7 min. PCR products were evaluated using 2% agarose gel electrophoresis after staining with 1 ug/ml of ethidium bromide and visualized under Bio-RAD Gel Doc™ XR+ Imaging System version 5.1 (Gel Documentation Molecular Imager, Bio-Rad Laboratories, Inc., USA).

Table 1: Heat Shock protein (HSP) 70 gene primer sequence and target region (181...475 bp)

***HSP* 70 Sequence (5'-3')**	**No. of bases**	**Targeted region**	**Amplicon Size**
F-AAACATGGCTATCGGCATCGACCT	24	Exon 1	295bp
R-AGGCTTGTCTCCGTCGTTGATGA	23		

Polymerase chain reaction and amplification condition for HSP 70 gene

The PCR reactions were carried out in a total volume of 15 µl containing template DNA of 1.0, 1.0 µl of each of forward and reverse primers, PCR Master Mix (2x) (GeNei™ Red Dye PCR Master Mix) of 7.5 µl, and 4.5 µl of nuclease free water. PCR amplification was performed in a TaKaRa Thermal Cycler Dice™ version III (Takara Bio Inc., Japan). The amplification condition involved initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94 °C for 60 seconds, annealing temperature of 65°C for 45 seconds, extension at 72°C for 1 minutes followed by final extension at 72°C for 7 min. PCR products were evaluated using 2% agarose gel electrophoresis after staining with 1 ug/ml of ethidium bromide and the fragments were visualised under Bio-RAD Gel Doc™ XR+ Imaging System version 5.1 (Gel Documentation Molecular Imager, Bio-Rad Laboratories, Inc., USA). Subsequently the PCR products for *HSP* 70 gene were sequenced using an automated ABI DNA Sequencer (Eurofins Genomics Pvt. Ltd., Bangalore, India).

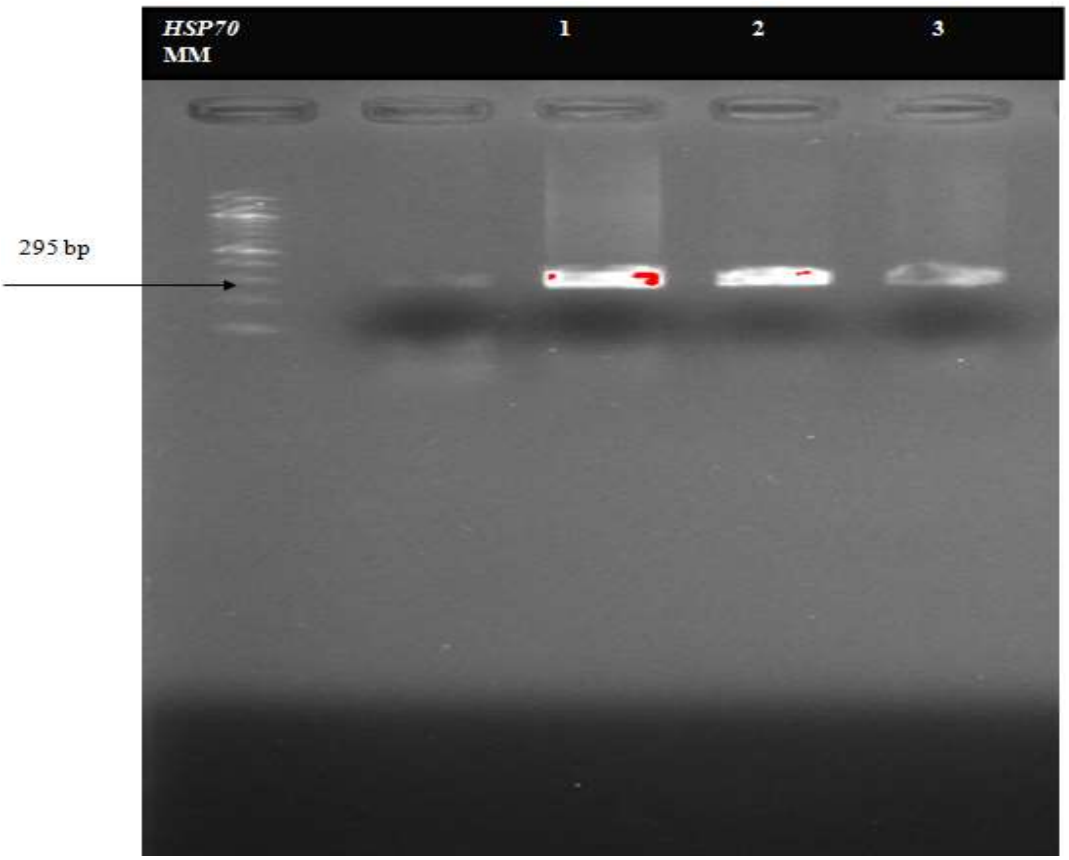
Identification of single nucleotide polymorphisms (SNPs) within exon 1coding region of HSP 70 gene in four Nigerian Zebu breeds of cattle and analyses of sequenced data

After sequencing of the samples, *HSP70* gene covering target region in exon 1 was aligned with NCBI-generated nucleotide sequence of unrelated *Bos taurus*. SNPs within the nucleotide sequences of four Nigerian bovine *HSP* 70 gene of were visualised, detected and bio-edited by chromatogram analyses using SeqMan Ngen Tool (DNASTAR®, Inc., Madison, Wisconsin, USA).

Results and Discussion

DNA extraction and isolation of HSP70 gene in four Nigerian Zebu cattle as revealed polymerase chain reaction and gel electrophoresis analyses

Plate 1 shows amplified region of *HSP70* gene of 295 bp at coding region of exon 1 in four Nigerian Zebu cattle breeds



Lane 1= MM= Molecular Marker; Lanes 1-3: Amplified fragment of *HSP70* gene 295 bp
Plate 1 Gel bands of amplified fragment of *HSP70* gene (295 bp)

Table 2: Occurrence and distribution SNP variants within the coding region of exon 1 of *HSP70* gene within four Nigerian zebu cattle breeds

Breed	Number of Animals examined	No. of animals with occurrence of SNP variants	Distribution of SNP variants across the four Nigerian Zebu cattle	No. of SNP Variants
White Fulani	25	19	A7Del, C145T, C154G, G220A, G220T	5
Ambala	23	16	C154T, G220T, C244T	3
Sokoto Gudali	21	10	C184T, T198A	2
Red Bororo	21	21	C154G, A78T, G157A, G106C, C154T, G196A, T198A, C224T, G199A, G220T, T245A	11
Total	90	66		21

Distribution of SNP variants detected within the coding region of exon 1 of HSP70 gene across the four Nigerian zebu breeds of cattle

The observed Seqman sequence alignment comparison between the bovine *HSP70* gene of consensus *Bos taurus* gene as obtained from NCBI and that of Nigerian zebu breeds of cattle revealed 21 SNP variants of *HSP70* gene within the four Nigerian zebu breeds of cattle as shown in Table 2. The distribution of the *HSP70* SNPs within the coding region of exon 1 within four Nigerian zebu breeds of cattle is presented as follows; Red Bororo: 11 > White Fulani: 5 > Ambala: 3>Sokoto Gudali: 2.

Table 3 Nature of SNP variants within the coding region of exon 1 of *HSP70* gene within four Nigerian zebu breeds of cattle

S/N	Breed	SNP variants	Nature of the SNP variants
1	White Fulani	A7Del	Indel
2	White Fulani	C145T	Transition
3	White Fulani	C154G	Transversion
4	White Fulani	G220A	Transition
5	White Fulani	G220T	Transversion
6	Ambala	C154T	Transition
7	Ambala	G220T	Transversion
8	Ambala	C244T	Transition
9	Sokoto Gudali	C184T	Transition
10	Sokoto Gudali	T198A	Transversion
11	Red Bororo	C154G	Transversion
12	Red Bororo	A78T	Transversion
13	Red Bororo	G157A	Transition
14	Red Bororo	G106C	Transversion
15	Red Bororo	C154T	Transition
17	Red Bororo	G196A	Transition
18	Red Bororo	T198A	Transversion
19	Red Bororo	C224T	Transition
20	Red Bororo	G220T	Transversion
21	Red Bororo	T245A	Transversion

Nature of SNP variants detected within the coding region of exon 1 of HSP70 gene within four Nigerian zebu breeds of cattle

Table 3 showed the nature of SNP variants detected within the coding region of exon 1 of *HSP70*, the nature of the detected SNPs are presented as follows; 1 Indel A7Del i.e deletion of A at base position 7 (White Fulani), 10 transversions (White Fulani: C154G and G220T, Ambala: G220T, Sokoto Gudali: T198A, Red Bororo: C154G, A78T, G106C, T198A, G220T and T254A) and 10 transitions (White Fulani: C145T and G220A, Ambala: C154T and C244T, Sokoto Gudali: C184T and Red Bororo: G157A, C157T, G196A, C244T and G199A).

Detection of HSP70 gene haplotype variants within the coding region of exon 1 of four Nigerian zebu breeds of cattle

The four (4) distinct haplotype variants were detected within the coding region of exon 1 of *HSP70*; haplotype TA at base positions 154 and 220 was detected in 5 White Fulani cattle, haplotype TC at base positions 78 and 106 were detected in four (4) Red Bororo cattle, haplotype AAAT at base positions 196, 198, 199 and 220 was detected, respectively in 5 Red Bororo cattle and haplotype TTA was detected at base positions 154, 244 and 245, respectively in five 5 Red Bororo cattle.

Seqman sequence alignment showing some of the detected SNPs of HSP 70 gene in four Nigerian cattle breeds

Figures 2 and 3 show some of the SNPs of *HSP* 70 gene detected at various base positions in four Nigerian cattle breeds

Test of selection

Darwinian selection pressure (w), as determined by codon-based *Z* test using the Suzuki and Gojobori method, revealed that at 5% level of significance, non-synonymous mutation (dN) is substantially greater than synonymous mutation (dS) as observed within the amino acid sequence of *HSP* 70 gene of four Nigerian cattle breeds (Suzuki and Gojobori, 1999).

Molecular screening of *HSP70* gene within the coding region of exon 1 of Nigerian cattle breeds detected 21 SNP variants thus depicting the variability of this gene at coding region of exon 1. This corroborated the reports of earlier findings of Singh *et al*. (2006) that *HSP* 70 was the most polymorphic and genetically diverse among the *HSPs* sub-family as their abundant nature confer on animal thermal tolerance advantage to wide range of stressful environmental and physio-thermal variations.

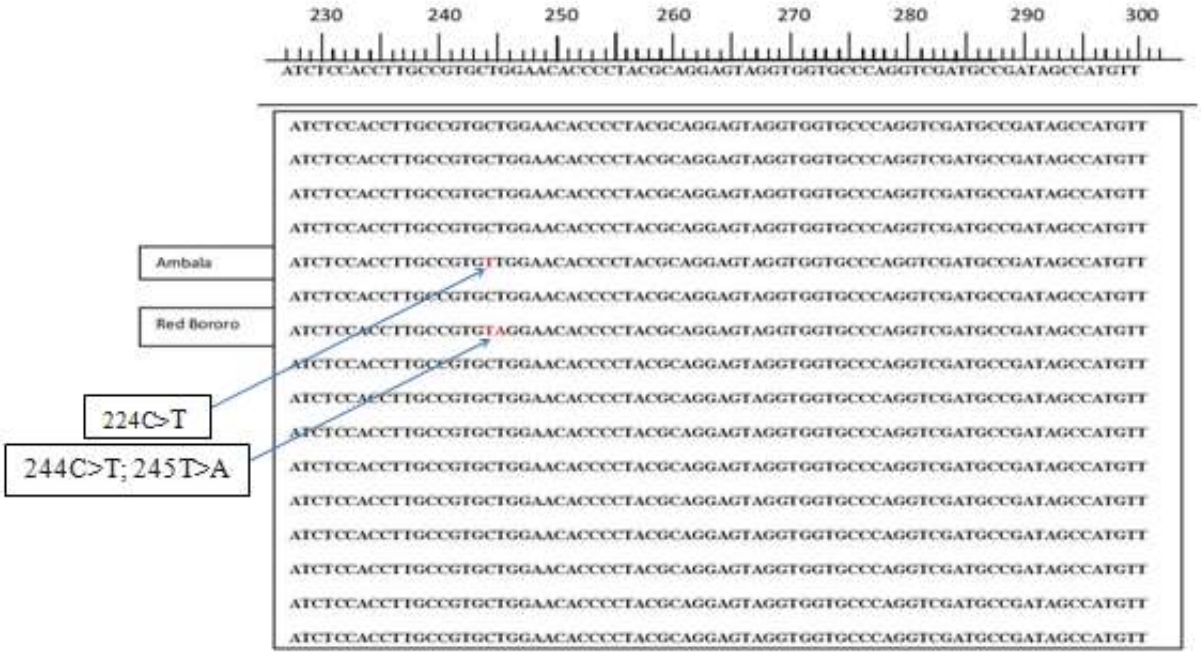


Fig. 2: SeqMan sequence alignment showing SNPs at base position: 224C>T (Ambala), 244C>T (Red Bororo) and 245T>A (Red Bororo) of HSP *70* gene in four Nigerian cattle breeds

Fig. 3: SeqMan sequence alignment showing; INDEL (Deletion of Adenine at base position 7 of *HSP70* gene in White Fulani cattle breed, SNP at position 78A>T of *HSP70* gene in Red Bororo cattle breed

In this study, 21 SNP variants of *HSP70* gene detected within coding region of exon 1demonstrated the following nature of polymorphisms: 1 Indel A7Del i.e. deletion of A at base position 7 (White Fulani), 10 transversions (White Fulani: C154G and G220T, Ambala: G220T, Sokoto Gudali: T198A, Red Bororo: C154G, A78T, G106C, T198A, G220T and T254A) and 10 transitions (White Fulani: C145T and G220A, Ambala: C154T and C244T, Sokoto Gudali: C184T and Red Bororo: G157A, C157T, G196A, C244T and G199A), thus indicating a very high genetic prevalence and variability in the *HSP70* gene of four Nigerian cattle breeds examined. The results agreed with the previous reports of Adamowicz *et al.* (2005) who detected a novel single-nucleotide polymorphism (SNP) in the 3' untranslated region of *HSP70* gene, Lamb *et al.* (2007b) who discovered 8 SNPs in *HSP70* gene of different cattle breeds and deduced that 5 of them were related to Brahman ancestry and Bhat *et al*. (2016) in Tharparker Indian cattle breed where two SNPs of *HSP* 70 gene (G > T and G > C) at 149th base positions were detected.

The detection of 21 *HSP* 70 SNPs in this study was similar to previous detection of 5 novel SNPs in the CDS and 3′-UTR region in the *HSP* 70 gene of Chinese Holstein cattle (Li *et al.*, 2011) and discovery of SNP variants of *HSP* 70gene in Frieswal crossbred cattle (Deb *et al*., 2013). The results of this work have equally confirmed the findings of Sodhi *et al.* (2013) who detected a total of 54 SNPs among three species in the *HSP* 70 gene of Indian zebu cattle and riverrine buffalo. Also, the observation of this study confirmed the discovery of SNPs of *HSP* 70 gene in Chinese Holstein cattle (Lamb *et al*., 2007a).

As obtained in this study, previous studies reported five SNPs in the 5-flanking region of *HSP* 70 gene (Chen *et al*., 2000; Huang *et al*., 2002; Hess and Duncan, 1996). As per the nature of SNPs detected in this study where purine base changed to pyrimidine base, this was similar to earlier works of Nikbin *et al*. (2014) in Boer goats and Boer crosses who detected two transversions in *HSP* 70 gene at base positions 74A>C (ss836187517) and 191C>G (ss836187518). The changes observed in the amino acid sequences in our study showed that these amino acid changes can bring about subsequent changes in protein product and function (Gade *et al*., 2010) which will consequently improve the ability of the animals to better cope perform and survive under the assault of thermal condition

Regarding the SNPs detected in this study and its possible effect of performance of animals under thermal pressure, Nikbin *et al*. (2014) reported the detection of two SNPs of *HSP* 70 gene in Boer goats and Boer crosses at base positions 74A>C (ss836187517) and 191C>G (ss836187518) and these were reported to have had remarkable significance on most of the fresh and post-thaw semen quality traits examined especially velocity traits. Similarly, SNPs of *HSP* 70 gene affected motility traits of fresh semen, frozen semen and semen quality traits after ejaculation and during storage (Elliott *et al*., 2009; Lloyd *et al*., 2009). Also the detected SNP variants at different loci within the nucleotide sequence of *HSP* 70 gene in these dairy cattle had evident differences in thermo-tolerance traits of animals examined (lamb *et al.*, 2007a)

Previous works of earlier researchers showed differences of body parameters of livestock animals due to effect of SNP variants, for example, SNP variants in *HSP* 70 gene showed association with sperm quality of boars (Huang *et al*., 2002), sperm characteristics in bull (Shrum *et al*., 2010) and calving traits (Rosenkrans *et al*., 2010). It was also reported that semen quality may be influenced by *HSP* 70 gene in boars (Huang *et al*., 2000). Elliott *et al*. (2009) found that *HSP* 70 gene, as sperm-binding oviductal proteins, increases longevity and viability of sperm in bulls and boars. The lack or knockout of the *HSP* 70 gene caused a significant increase in apoptosis (Dix *et al*., 1996). Govin *et al*. (2006) found an evidently remarkable association between thermo-regulatory functions of *HSP* 70 gene and spermatid DNA-packaging proteins during spermatogenesis. Similarly, authors have reported the significant effect of SNPs on various dairy traits of cattle for instance, SNPs of *HSP* 70 gene was reported to have a remarkable influence on milk yield and milk content of Chinese Holstein cattle (Lamb *et al*., 2007a).

Knockout of *HSP* 70 gene in mice showed structural abnormalities in spermatocytes, arrested evolution of primary spermatocytes, and increased apoptosis of these cells (Christians *et al*., 2003). *HSP* 70 gene plays a protective role in reaction to hyperthermia as well as other stress conditions by providing a balance between synthesis and degradation of cellular proteins (Shi *et al*., 1998). It also acts as a molecular chaperone, which assists in the process of folding, transporting and assembling proteins in the cytoplasm, mitochondria and endoplasmic reticulum (Georgopoulos and Welch, 1993). It was found that adding recombinant *HSP* 70 gene to semen increased longevity and viability of spermatozoa during cooling and after freezing (Lloyd *et al*., 2009) as presence of *HSP* 70 gene is required to protect the proteins involved in DNA repair or recombination (Jeong *et al*., 2009).

Regarding test of selection of *HSP* 70 gene in Nigerian cattle population, Darwinian selection pressure (w), as determined by codon-based *Z* test using the Suzuki and Gojobori method, revealed that at 5% level of significance, non-synonymous mutation (dN) is substantially greater than synonymous mutation (dS) within the amino acid sequence evaluated. Thus, *HSP70* gene might have evolved through positive selection (dN > dS) within the population of four Nigerian breed of cattle and this is suggestive of change in protein product which is occasioned by SNPs resulting in possibly positive biological effect of the fitness of the animals in response to thermo-tolerance at *HSP* 70 gene loci (Gade *et al*., 2010)

Conclusion

Molecular screening of four Nigerian breeds of cattle for SNPs in the coding region of exon 1 of the *HSP70* gene indicated that there is high variability in this region of *HSP* 70 gene examined. Also, higher dN and dS ratio estimated in four Nigerian breeds of cattle suggested that *HSP* 70 gene could cause positive changes in the fitness of the animals for better thermo-tolerance under assault of thermal stress and as such the gene could be used as a candidate gene / bio-markers for thermo-tolerance selection of animals under thermal stress. Further studies should be conducted to interrogate the association between SNPs of *HSP70* gene and thermo-tolerance traits and other physiologically important traits of animals under thermal assault of the tropics.

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Conflict of Interest

Authors declare that there is no conflict of interest in this study.

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