

Genetic Polymorphisms of the 3'-Untranslated Regions (3'-UTR) of the HSP 70 Gene in Moa Buffalo (*Bubalus bubalis*)

Maman Rumanta¹, Leonard Raden Hutasoit¹, Rony Marsyal Kunda^{2*}, Prasetyarti Utami³

¹ Biology Education, Study Program, Faculty of Education and Teacher Training, Universitas Terbuka, Banten, Indonesia.

² Biotechnology Study Program, Faculty of Sciences and Technology, Universitas Pattimura, Ambon, Indonesia.

³ Biology Study Program, Faculty of Sciences and Technology, Universitas Terbuka, South Tangerang, Banten, Indonesia.

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Corresponding Author:

Rony Marsyal Kunda

rony.kunda@lecturer.unpatti.ac.id

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Abstract: The Heat Shock Protein 70 (HSP70) gene, specifically the 3'-Untranslated Region (3'-UTR), is critical for regulating mRNA stability and translation under heat stress. This study aimed to identify genetic polymorphisms in the 3'-UTR of the HSP70 gene in Moa buffalo (*Bubalus bubalis*) and evaluate its potential role in heat adaptation. A total of 65 hair follicle samples (55 Moa buffalo and 10 Banten buffalo) were collected, followed by amplification by PCR and sequencing of the PCR product of the 3'-UTR regions. Alignment results from the 3'-UTR region showed the presence of 2 major polymorphic SNPs, i.e. g.1904C>A, and g.1910A>G. SNP (g.1904C>A) was found to be a cytosine (C) to adenine (A) substitution mutation type, and SNP g.1910A>G an adenine (A) to guanine (G) substitution. The discovery of 2 SNPs in the 3'-UTR of the HSP70 gene in Moa buffalo is very important for the development of genetic markers, helping to understand the mechanism of heat adaptation, to the conservation and improvement of livestock performance, especially Moa buffalo living in tropical environments.

Keywords: HSP70 gene; Moa buffalo; Mutation; Polymorphism; Thermal stress

Introduction

Moa buffalo (*Bubalus bubalis*), as one of the native Indonesian germplasms that grows on Moa Island, Southwest Maluku (Utami et al., 2023). This germplasm plays an important role in supporting local food security and the economic life of rural communities. Biological adaptation to the hot and humid tropical environment allows Moa buffalo to continue producing meat and labor compared to other livestock, such as Bali cattle. Suhendro et al. (2024) explained that in Bali cattle 15 polymorphic SNPs were found, leading to higher expression as an indication that the HSP70.1 gene is involved in reducing the damaging effects of stress. As a result, Balinese cattle experience a negative energy balance, resulting in reduced body size. Rumanta et al. (2023), successfully found a correlation between SNP

findings, and genetic drift with geographic isolation in Lakor goat species in Southwest Maluku.

Global climate change, especially increasing ambient temperatures and frequency of heat waves, puts significant physiological stress on livestock, resulting in decreased productivity, impaired reproduction, and increased mortality (Maulana et al., 2025; Mishra, 2021). In the context of thermal adaptation, the physiological and molecular responses of animals are the main focus to explain phenotypic variation in heat tolerance. One of the genes that plays an important role in this mechanism is Heat Shock Protein 70 (HSP70), a gene that encodes a chaperone protein involved in the refolding of denatured proteins, protection against cellular damage due to heat stress, and also plays a role in preventing protein aggregation and aiding protein transportation (B. Kumar et al., 2019; Suhendro et al., 2024).

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HSP70 gene expression has been shown to increase significantly when animals experience heat stress, making it a prime candidate for genetic studies on thermal adaptation in ruminants (A. Kumar et al., 2015). A study by Kumar et al. (2019) on Murrah buffaloes showed that HSP70 expression is closely related to the ability to maintain body temperature during summer. Meanwhile, Ismaeel et al. (2024) found polymorphisms in the 3'-UTR region of the HSP70 gene from endemic cattle from Sudan, which can affect mRNA stability and translational regulation through interactions with microRNA (miRNA), which ultimately impacts gene expression levels.

Polymorphism in the 3'-UTR region is very important because this section not only affects mRNA stability but also the effectiveness of protein translation, especially under environmental stress conditions (Abbas et al., 2020). Recent studies have shown that variations in this region can contribute to phenotypic differences in response to thermal stress in local cattle and buffalo (Prihandini et al., 2022). Rehman et al. (2020) study in Buffalo found that variations in the HSP70 regulatory region were associated with physiological performance and heat stress responses.

Until now, there have been few reports on the characterization of polymorphisms in the 3'-UTR region of the HSP70 gene in local Indonesian buffaloes, especially the Moa buffalo. In fact, analysis of genetic diversity in this region can provide important information regarding natural selection and local adaptation to environmental pressures (Kim et al., 2025). Recently, the several genomic study by also revealed that the HSP70 gene is under strong selection pressure in species living in tropical regions, including buffaloes (Domple et al., 2021; Rehman et al., 2020).

Identifying and understanding polymorphisms in the 3'-UTR region of the HSP70 gene in Moa buffalo will not only provide insight into the molecular adaptation mechanism to thermal stress, but also provide important contributions to the development of marker-based breeding strategies to improve heat resistance. Thus, this study is important to strengthen the scientific basis for conservation and development of local buffalo genetics in Indonesia.

Method

Ethical Approval

All procedures of this study were approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia. Animal handling followed international guidelines for animal welfare used in scientific research. Sampling was performed non-invasively using hair follicles to

minimize stress on the animals. Figure 1 is the overall stage of the research which is shown in the following flowchart.

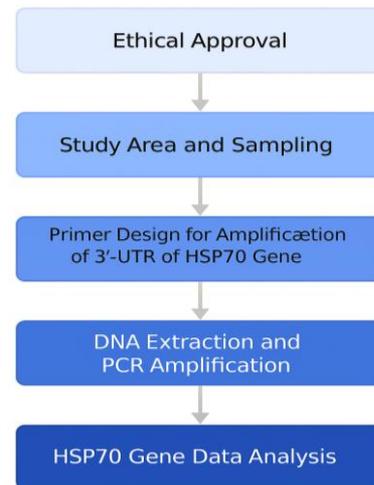


Figure 1. Research flow chart

Study Area and Sampling

This study was conducted from February to September 2024. The samples collected included Moa buffalo and Banten buffalo as a comparison to other local buffalo species in Indonesia. The Moa buffalo specimens collected included non-invasive samples (hair follicles) from 4 villages, i.e Werwaru, Tounwawan, Klis, and Moain (Figure 2), while Banten buffalo were collected from Majau village, Pandeglang, Banten. The total number of samples was 65 (30 males and 35 females), including Moa buffalo (25 males and 30 females), and Banten buffalo (5 males and 5 females). Molecular analysis was carried out at the Genetic Engineering Laboratory, Veterinary Research Center, National Research and Innovation Agency (BRIN), Indonesia.



Figure 2. Buffalo sampling location map

Primer Design for Amplification of 3'-UTR of HSP70 Gene

Primers were designed to target the 3'-untranslated region (3'-UTR) of the HSP70 gene, based on the *Bubalus*

bubalis reference sequence from GenBank (Accession Number: MH814759.1). Primers were designed using Primer3Plus software and verified by BLAST against the NCBI database to ensure specificity to the target gene. Primer sequences: Forward-HSP70-3UTR-F: (5'-ATCCCCAAGGTGCAGAAGC-3'), and Reverse-HSP70-3UTR-R: (5'-ACACAGAACCAAACATCACTCG-3'). These primers were designed to amplify a fragment of ± 1213 bp from the 3'-UTR region (Figure 3).

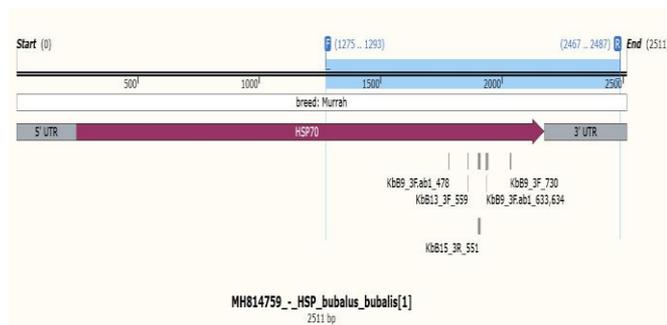


Figure 3. Region fragment (3'-UTR) of HSP70 gene targeted for amplification

DNA Extraction and PCR Amplification

DNA extraction was performed using the GeneJET Whole Blood Genomic DNA Purification Kit (Thermo Scientific®) according to the manufacturer's protocol. DNA quality and quantity were evaluated using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific) at a ratio of 260/280 and 1% agarose gel electrophoresis to confirm DNA integrity. PCR reactions were performed in a total volume of 25 μ L consisting of: 12.5 μ L 2X PCR Master Mix (GoTaq® Green, Promega), 1 μ L forward primer (10 μ M), 1 μ L reverse primer (10 μ M), 2 μ L template DNA (± 100 ng), 8.5 μ L nuclease-free water. PCR conditions: Pre-denaturation: 95°C for 5 min, Denaturation: 95°C for 30 s, Annealing: 60°C for 30 s, Elongation: 72°C for 45 s, and Final extension: 72°C for 7 min, with a total of 35 cycles. PCR products were confirmed by 1.5% agarose gel electrophoresis and visualized using a UV transilluminator (Applied Biosystems, Foster City, California, USA).

HSP70 Gene Data Analysis

The amplified PCR fragments were then purified using the QIAquick PCR Purification Kit (Qiagen). The purified products were sent to PT. Genetika Science Indonesia for bidirectional sequencing using the Sanger method. The DNA sequence data obtained were analyzed using the following software: DnaSP v6 for calculating genetic variations, Chromas v2.6.6 for chromatogram visualization and editing, BioEdit v7.2.5, and MEGA X for multiple alignment, Single Nucleotide Polymorphisms (SNPs) detection, and haplotypes. The

3'-UTR sequence of the HSP70 gene was compared with the GenBank reference sequence with Accession Number (MH814759.1).

Results and Discussion

Genetic Polymorphism and Haplotype Analysis in the 3'-UTR Region of the HSP70 Gene

Sequence analysis of PCR products revealed 2 major Single Nucleotide Polymorphism (SNP) positions in the 3'-UTR regions of the HSP70 gene, i.e in the sequence g.1904C>A, and g.1910A>G compared to the reference (MH814759) of the HSP70 gene from Genbank (Figure 5). Haplotype analysis showed that Haplotype AA had the highest frequency (0.45), followed by AG (0.27), CA (0.18) and CG (0.09) (Figure 4). The dominance of haplotype AA (45.5%) suggests that the combination of allele A at both SNP positions is very common in the Moa buffalo population. This could reflect a form of ancestral haplotype or a haplotype that provides an adaptive advantage to extreme tropical environments such as the high temperatures in Southwest Maluku.

The high frequency of AG (27.3%) suggests a diversification of haplotypes that remains stable in the population. This is important because haplotypes such as AG may be associated with different thermal responses in HSP70 gene expression (Xu et al., 2025). Haplotypes CG and CA, although lower in frequency (9.1% and 18.2%), are still important as they indicate the presence of minor variants that contribute to haplotype diversity. This provides a basis for the possibility of natural selection against changing environments.

SNPs in the 3'-UTR regions have significant potential to alter gene expression regulation because this region contains microRNA (miRNA) binding sites and other factors that regulate mRNA stability (Haas et al., 2012). Several SNPs found in this study overlapped with predicted miRNA binding sites using RegRNA 2.0, indicating a possible biological impact on HSP70 gene regulation (Koopae et al., 2014).

This finding is in line with the results of studies by Habib et al. (2017) and Cwiklinska et al. (2020), which reported that SNPs in the 3'-UTR can contribute to heat stress responses and cell protection mechanisms through modification of miRNA binding and mRNA degradation. In addition, studies in cattle and buffalo also showed that variants in the 3'-UTR of the HSP70 gene can be associated with production performance and resistance to environmental stress (Badri et al., 2021; B. Kumar et al., 2019).

In context genetic diversity in the region 3'-UTR of the HSP70 gene, haplotype identification is important because it can represent patterns of genetic variation related to the regulation of gene expression through

post-transcriptional mechanisms, such as mRNA stability, and microRNA binding. Haplotype analysis using DnaSP v6 showed that there were two main haplotype combinations. The two SNPs formed several unique haplotypes, each of which showed a distinct genetic profile of Moa buffalo. The haplotype diversity value (Hd) of 0.683 indicates a moderate level of genetic variation within the population. This reflects that Moa buffalo populations harbor significant genetic variation in the 3'-UTR region of the HSP70 gene, which is important for thermal adaptation and tropical environments. This indicates the potential for genetic selection based on molecular characters (Abbaya et al., 2024).

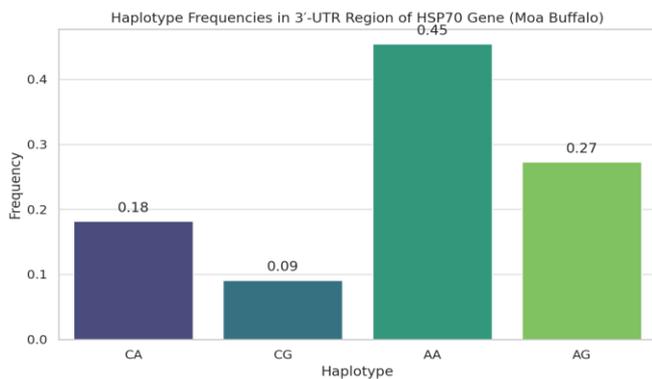


Figure 4. SNP frequency in the 3'-UTR of the HSP70 gene in moa buffalo

The alignment results showed that although it does not encode a protein domain, the 3'-UTR region contains post-transcriptional regulatory elements such as microRNA binding sites, AU-rich elements (AREs) sequences, and mRNA stability determinants. SNPs in this region can affect HSP70 expression, not its structure, but its adaptive function at the transcript level (Prasanna et al., 2021). The results of this study strengthen the findings of research by Pires et al. (2019), which stated that the diversity of the 3'-UTR regions of the HSP70 gene has an important role in the thermal stress response in tropical ruminants.

SNP Nucleotide Alignment in the 3'-UTR of the Moa Buffalo HSP70 Gene

Nucleotide alignment analysis is an important step in identifying genetic variations, especially Single Nucleotide Polymorphisms (SNPs) in non-coding regions such as the 3-UTR Region. In study here, alignment was performed to compile and compare DNA sequences from the amplification and sequencing of the 3-UTR region of the HSP70 gene in the Moa buffalo population. The alignment process aims to identify specific positions of nucleotides that have undergone substitution among the individuals analyzed (Figure 5).

The main findings from this alignment process indicate that the SNP (g.1904C>A) is located at nucleotide position 1904 in the 3'-UTR regions, with the finding of a Cytosine (C) to Adenine (A) nucleotide substitution indicating a change that has the potential to affect mRNA stability (Mayr, 2017). The C>A substitution could potentially disrupt or create cis-regulatory elements, including AU-rich elements (AREs) or microRNA (miRNA) binding sites, thereby altering how long an mRNA transcript remains intact within the cytoplasm or how efficiently it is translated into protein. Such modifications may affect HSP70 protein levels, which are crucial for cellular protection against heat-induced protein damage (Hu et al., 2024).

The altered expression could lead to either enhanced or reduced ability to respond to thermal or oxidative stress, especially under the environmental conditions common in tropical regions like Southwest Maluku. Therefore, the g.1904C>A SNP may serve not only as a candidate functional mutation but also as a molecular marker in genetic selection programs aimed at improving stress resilience in local buffalo populations.

SNP g.1910A>G is only 6 bases different after g.1904C>A. The alignment results showed that the substitution of Adenine (A) to Guanine (G) nucleotides was found. The position of the SNP at both nucleotides is located in the microRNA binding site fragment that regulates the post-transcriptional mechanism. Bartel (2018), Song et al. (2020) found that mutations in DNA fragments from the 3'-UTR regions have the potential to alter the regulation of HSP70 expression.

Other studies have also reported that SNPs in the 3'-UTR can interfere with the binding of miRNAs such as miR-21, miR-106a, or miR-34, which are known to be involved in the regulation of stress response genes (Sharma et al., 2019). Therefore, the g.1910A>G SNP may have important functional consequences in regulating HSP70 expression through post-transcriptional mechanisms, especially in animals facing heat stress in their natural habitat.

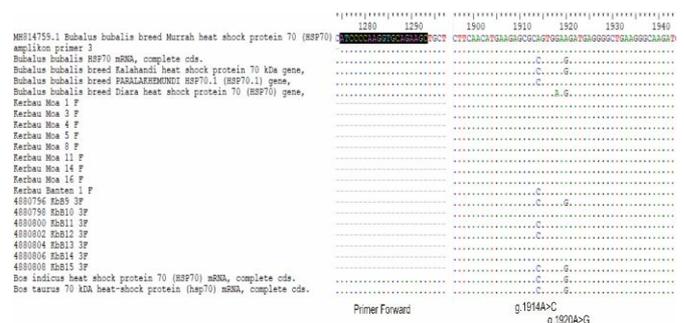


Figure 5. Moa buffalo (*Bubalus bubalis*) SNP variation and position compared with GeneBank database

The 3-UTR region is known to contain many regulatory elements, including binding sites for miRNAs, RNA-binding proteins, as well as other elements that affect mRNA stability and translational efficiency (Bartel, 2018; Skeeles et al., 2013). The discovery of SNP g.1910A>G, in this region has the potential to alter the binding affinity of miRNAs, which in turn can inhibit or increase HSP70 gene expression. Research by Raza et al. (2021) also confirmed that mutations in the 3-UTR region can disrupt normal regulatory pathways, and can cause dysregulation of the HSP70 gene, especially under heat stress conditions. This effect is particularly relevant in species such as Moa buffalo, which are adapted to tropical environments with exposure to high temperatures. Overall, several studies have reported that SNPs in the 3'-UTR regions of the HSP70 gene are associated with resistance to heat stress and other environmental stresses in various livestock species including ruminants (Hariyono et al., 2022; Romero-Ramírez et al., 2024).

Conclusion

This study successfully identified the presence of single nucleotide polymorphisms (SNPs) in the 3'-UTR regions of the HSP70 gene in Moa buffalo (*Bubalus bubalis*) that live on Moa Island, Southwest Maluku. Two major SNPs detected, i.e g.1904C>A, and g.1910A>G. SNP (g.1904C>A) was found to be a cytosine (C) to adenine (A) substitution mutation type, and SNP g.1910A>G an adenine (A) to guanine (G) substitution. These two SNPs each have varying allele frequencies among the individuals studied. The presence of SNPs in the 3'-UTR of the HSP70 gene has the potential to affect gene expression regulation through disruption of interactions with miRNA or mRNA stability. This variation also has implications for the Moa buffalo thermal stress response capacity and environmental adaptation, making it an important target in local livestock conservation and genetic improvement programs. Further studies linking these SNPs to HSP70 gene expression and Moa buffalo physiological performance are highly recommended.

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Author Contributions

Conceptualization, MR and RMK; methodology, RMK, SDV and IMM; validation, PU and RMK; formal analysis, MR, and PU; investigation, SDV, IMM and RMK; resources, RMK and PU; data curation, MR and RMK; writing-original draft preparation, RMK, IMM and PU; writing-review and editing, IMM and PU; visualization, RMK and PU. All authors have read and approved the published version of the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest in this article. The funder (LPPM UT) had no role in the research design, collection, analysis, interpretation of data, or writing of the publication from the beginning to the completion of this study.

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