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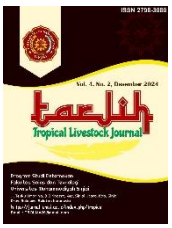

First Screening of Heat Shock 70kDa Protein 1A (HSPA1A) Gene Polymorphisms in Indonesian Local Sheep

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ABSTRACT

Heat shock proteins (HSPs) are a collection of proteins that promote thermotolerance in cells and protect against environmental stress. This study was a preliminary analysis to identify the nucleotide variant within the partial heat shock 70kDa protein 1A (HSPA1A) gene in several Indonesian sheep breeds. The blood samples were collected from five breeds of Indonesian sheep (Sapudi, Batur, Sakub, Wonosobo, and Garut) to be analyzed and amplified using a specific primer. The sequences were aligned using Clustal Omega to identify the single nucleotide polymorphism (SNP). An ExPasy program was used to detect the amino acid changes. The genotyping was done by direct examination of the peak of the electropherogram. As a result, based on the sequence alignment and direct observation of the electropherograms, three variants were found: SNP c.286A>G, c.503A>G, and c.733A>G. All SNPs were categorized as synonymous mutations since they did not alter the amino acid. The genotyping of samples showed two types of genotypes (AG and GG) from each SNP. In conclusion, our study suggests exploring other regions of the HSPA1A gene to find candidate mutations for further genetic analysis.

INTRODUCTION

In Indonesia, sheep are among the most widely raised livestock due to their important role in socio-cultural events, such as aqiqah and religious celebrations (Sujarwanta *et al*., 2024). Local sheep breeds, including Garut, Sapudi, Sakub, Batur, and Wonosobo, are central to the country's livestock economy. However, livestock productivity is heavily influenced by environmental factors and management practices. A growing concern in sheep farming is the decline in productivity caused by climate change or global warming. Rising temperatures significantly impact livestock performance by diverting energy from growth to coping with heat stress (Joy *et al*., 2020; Pérez-Barbería *et al*., 2020). Additionally, global warming affects the quantity and quality of available feed, further challenging livestock productivity (Gauly & Ammer, 2020).

Improving livestock's genetic quality through selection is a promising strategy to address these challenges (Hayes *et al*., 2013).

By selecting animals more resistant to heat stress, energy from feed can be better utilized for growth. This process can be accelerated through molecular selection, using genetic markers such as SNPs (single nucleotide polymorphisms) in genes linked to heat stress resistance. Unlike traditional selection methods, molecular selection could identify animals with the best genetic potential by early and accurate prediction of an animal's performance, even before phenotypic traits are expressed, thereby accelerating the breeding process (Ibtisham *et al*., 2017). Through the analysis of several SNPs throughout the genome, breeders can discern a diverse array of alleles associated with heat tolerance, encompassing both significant and subtle genetic influences. One such gene is HSPA1A, which encodes the heat shock protein 70kDa (HSP70) and is key in managing stress responses. In sheep, the HSPA1A gene is located on chromosome 20, spans 2454 base pairs, and has a single exon (positions 203-2128 bp).

Previous studies have identified polymorphisms in the HSP gene in several Indian sheep breeds, including Chokla, Magra, Marwari, and Madras Red (Singh *et al*., 2017). However, none of the reports identified the HSPA1A polymorphism in Indonesian sheep breeds. Exploring this gene in Indonesian sheep could reveal specific nucleotide variations that may serve as valuable selection markers for improving heat stress tolerance. Therefore, this study aims to identify nucleotide variations in the HSPA1A gene in several local Indonesian sheep breeds.

MATERIALS AND METHODS

A total of five blood samples consisting of Garut sheep from West Java Province, Sapudi sheep from East Java Province, and Batur, Wonosobo, and Sakub sheep from Central Java Province (one sample for each breed) were used for this preliminary study. The blood samples were taken through the jugular vein of approximately 3 milliliters using an EDTA vacutainer. Samples were then carried to the laboratory at 4 °C for DNA isolation using a gSYNC DNA Extraction Kit (Geneaid, New Taipei City, Taiwan).

DNA amplification using a pair of self-designed primers (F: 5'- TCC TCT CGA CCG TTT TCA GG-3' and R: 5'- GCT TGT TCT GGC TGA TGT CC -3') was done in Thermo Cycler to get the targeted PCR product (size 831 bp). The primer was designed using a template sequence from GenBank with accession number NC_056073.1. A 25 µL PCR reaction was formulated from 2 µL of genomic DNA, 12.5 µL of MyTaq HS Red Mix (Bioline, UK), 0.5 µL of each primer (forward and reverse), and 9.5 µL of double-distilled water (DDW). The amplification program was started from pre-denaturation at 95 °C for 5 min, pursued by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and then terminated with a final extension at 72 °C for 10 minutes. The PCR product was visualized in 2% standard agarose gel. After that, the PCR products were sent to Apical Scientific Sdn. Bhd. in Selangor, Malaysia, for the DNA sequencing process (Sanger DNA Sequencing by using Capillary Electrophoresis) for both directions (forward and reverse). The sequencing results were then used for alignment in Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) and for direct visual observation to check the peaks of the electropherograms in SnapGene Viewer software. Nucleotide Sequence alignment was done to detect the polymorphism and compare it with the genotypes of the HSPA1A gene in the GenBank at accession number NC_056073.1.

RESULTS AND DISCUSSION

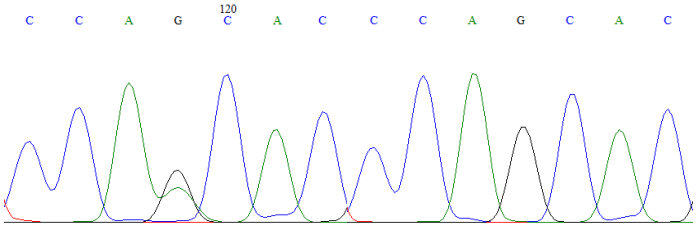
Heat-shock proteins (HSP) are well-known and highly conserved protein families responsible for cellular responses to various stresses (Rehman *et al*., 2020). HSPs are divided into several categories, including HSP40, HSP60, HSP70, HSP90, HSP100, and

small HSPs (Nagarajan *et al*., 2012). The HSP70 family is encoded by HSP70 gene and includes proteins of molecular masses ranging from 68 to 73 kDa (Hassan *et al*., 2019). Our study explored one of the gene (HSPA1A) belonging to the HSP70 family.

The DNA was successfully amplified using the designed primer. As a result, based on the sequence alignment and direct observation of the electropherograms, three variants were found: SNP c.268A>G, c.503A>G, and c.733A>G (named by its position on GenBank accession number NC_056073.1). Although only 3 SNPs were found in the electropherogram observation, based on the alignment results with the sequence from GenBank, it showed one additional SNP, namely SNP c.865T/C, as seen in Figure 4d. In this SNP, all samples analyzed had the CC genotype. For the SNP c.268A>G, c.503A>G, and c.733A>G, all the genotyping of samples showed two types of genotypes (one homozygous and one heterozygous), as shown in Figure 1-3. All SNPs were categorized as synonymous mutations since they did not alter the amino acid.

Exploration of HSP70 family polymorphisms in sheep has been extensively studied. In Pakistan, a SNP (222G>A) was identified in the HSP70 gene, with the wild-type GG genotype being dominant across several sheep breeds (Sheraz *et al*., 2023). Additionally, Singh *et al*. (2017, 2020) reported the discovery of 13 SNPs in the HSP90 and HSP70 genes of four local sheep breeds in India. Among these, four SNPs formed the TACCA haplotype, suggesting that selection operated directly (positively), with lower expression observed in less adapted animals. In Turkey, an indel polymorphism was found in the 5' flanking region of the HSP90AA1 gene, located at the −704 position (Öner *et al*., 2012).

Our study only found two genotypes for each SNP. This result was similar to Kumar *et al*. (2024), which reported an A to G mutation at the c.459 position of the HSP70 gene in Munjab sheep. Only AG and AA genotypes were identified. However, there was no association study revealed of identified genotypes with growth and thermotolerance traits.



AG GG

Figure 1. The electrophoregram of genotype AG and GG from SNP c.268A>G

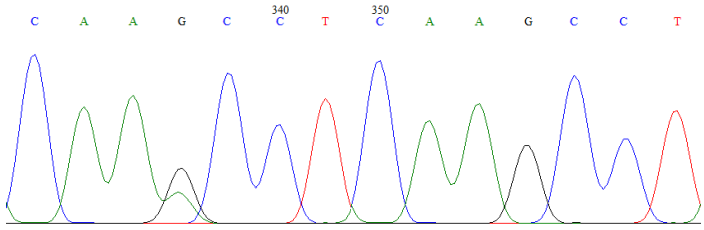


Figure 2. The electrophoregram of genotype AG and GG from SNP c.503A>G

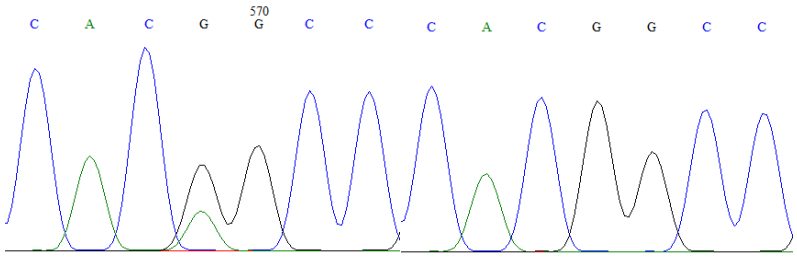


Figure 3. The electrophoregram of genotype AG and GG from SNP c.733A>G

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NC_056073.1     CGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCA
Sakub           CGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCA
Sapudi          CGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCA
Wonosobo        CGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCA
Batur           CGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCA
Garut           CGTGGGGGTGTTCCARCACGGCAAGGTGGAGATCA
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(a) SNP c.268A>G

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NC_056073.1     CGGAGACAAACCTAAAGTGCAGGTGAGCTACAAGC
Sakub           CGGAGACAARCCTAAAGTGCAGGTGAGCTACAAGC
Sapudi          CGGAGACAAGCCTAAAGTGCAGGTGAGCTACAAGC
Wonosobo        CGGAGACAAGCCTAAAGTGCAGGTGAGCTACAAGC
Batur           CGGAGACAAGCCTAAAGTGCAGGTGAGCTACAAGC
Garut           CGGAGACAAGCCTAAAGTGCAGGTGAGCTACAAGC
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(b)SNP c.503A>G

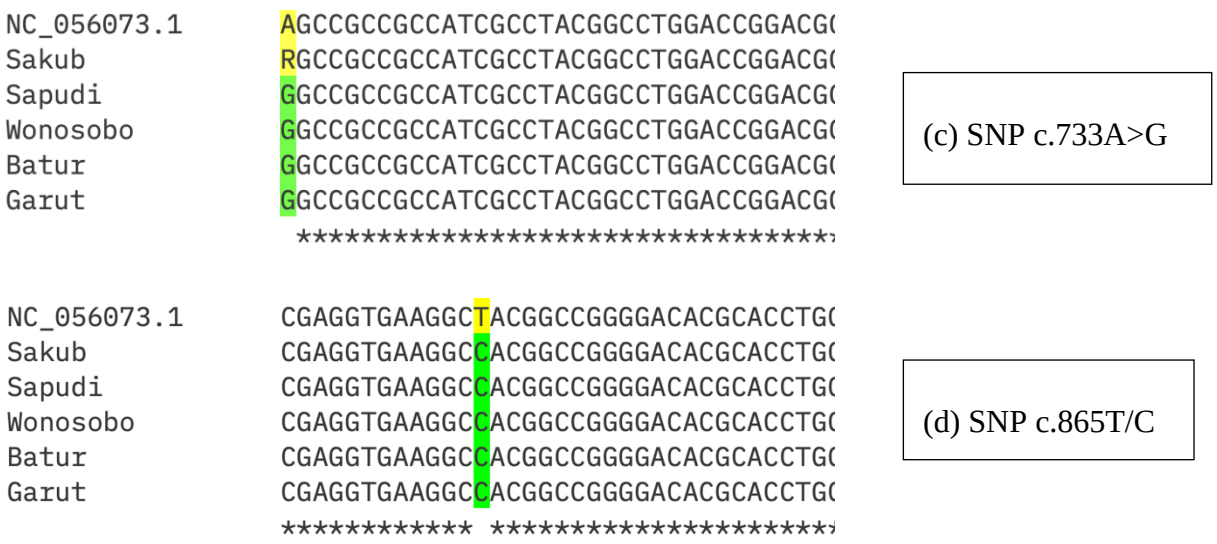

Figure 4. Sequence alignment for partial HSPA1A gene from Indonesian sheep breeds (the nucleotide for samples with heterozygous AG genotype was written as code R)

In the current data, the silent mutation that occurred in both genotypes did not result in any change in the amino acid structure because each amino acid has the same nucleotide form. The SNP c.268A>G, c.503A>G, and c.733A>G encode the amino acids glutamine, threonine, and lysine, respectively. Hunt *et al*. (2014) reported that synonymous mutation could still affect the role of translated protein through diverse cellular mechanisms. The statement strengthened by Bartoszewski *et al*. (2016) states that synonymous mutations can change the speed at which ribosomes translate mRNA, impacting the folding and function of the protein. The synonymous mutations could affect the level of protein expression and other events of the protein formation process (Baena *et al*., 2018). Thus, the SNP discovered in this work may be crucial for the development, function, and expression of HSP proteins, and future association studies. Employing the detected SNP may be valuable in creating techniques to implement marker-assisted selection for heat stress and tolerance in breeding programs.

CONCLUSION

In conclusion, this investigation discovered three synonymous SNPs (c.268A>G, c.503A>G, and c.733A>G) in the HSPA1A gene of the HSP70 family. Although these SNPs do not change the amino acid sequence, they may nevertheless impact protein function due to their impacts on translation speed and protein folding, implying a potential role in heat stress adaption. Future research should look into the possibilities for marker-assisted selection in breeding programs to improve thermotolerance in Indonesian sheep breeds.

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