Indian Journal of Animal Sciences **93** (7): 701–705, July 2023/Article
https://doi.org/10.56093/ijans.v93i7.132873

A novel SNP (c.1311C>T) on heat shock protein 70 (*HSP70*) gene of Kacang goat in Indonesia

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Received: 2 February 2023; Accepted: 6 June 2023

ABSTRACT

Heat shock proteins (HSPs) associated with stress reactions play an important role in cell survival by activating numerous regulatory proteins and inducing black apoptosis. This study aimed to identify the potential SNPs of *HSP70* gene in Kacang goats in Indonesia. Forty-three Kacang goats were selected from Sidoarjo and Tulungagung districts. The DNA isolated from blood samples was successfully amplified using the polymerase chain reaction (PCR) method with a pair of primers. The PCR products were sequenced in the coding region. The sequences were successfully aligned to determine the potential SNPs. A novel SNP (c.1311C>T) was found in this study. This SNP was categorized as a synonymous mutation. The insertion-deletion (in-del) mutation was also observed at the 1151st and 1161st nucleotide positions. Based on these mutations, four haplotypes were constructed where haplotype 1 had the highest frequency in Sidoarjo. Indonesian goats had no close relation with Iraqi goats according to Neighbor-Joing with Kimura's 2-parameter approach. Haplotypes 1 and 2 in Indonesian goats had three different bases with all haplotypes in Iraqi goats in Median Joining Network. The study concluded that a novel SNP of *HSP70* gene was identified in Kacang goats.

Keywords: DNA, Mutation, PCR, Polymorphism, Sequencing

Kacang goat is one of the original Indonesian goats (Nasich *et al*. 2018, Depison *et al*. 2020). Kacang goats are primarily raised to provide meat and as a side business to increase the income of farmers (Suyadi *et al*. 2020). Goat genetics have evolved due to changes in habitat and the adaptability of livestock since the domestication period (Zheng *et al*. 2020). The adaptability encouraged the formation of several goat breeds in various agroecosystems (Song *et al.* 2016, Hilmia *et al.* 2023). In a tropical country, goats are easily stressed due to nutritional and heat stress. This stress could decrease the goat's performance and production.

Heat stress could initiate the gene expression and the homeostasis system through biochemical and physiological mechanisms including endocrine and cellular heat stress responses (Suyadi *et al*. 2021). Based on the molecular weight, HSPs were classified into HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (Feder *et al*. 1999, Gade *et al*. 2010). One of them, HSP70 plays a key role in cell thermal tolerance and animal survival (Barbe *et al*. 1988, King *et al*. 2002). HSP70 has an important role in thermo-tolerance and immuno-modulation, so it is often associated with livestock performance (Hassan *et al*. 2019, Prasanna *et al*. 2021). Previous studies stated that *HSP70* gene was highly expressed in PBMC, liver, kidney, and heart during the summer in various goat breeds (Zulkifli *et al*. 2010, Dangi *et al.* 2012, Rout *et al*. 2016;). *HSP70* gene polymorphism was found in the exon region (C241T) as a non-synonymous mutation in Indian goats associated with the physiological vital parameters including rectal temperature, skin temperature, and respiration rate (Mohalik *et al*. 2021). Two synonymous mutations (A74C and C191G) were identified in Boer goats associated with sperm quality (Nibkin *et al.* 2014).

Recently, there has been no study on *HSP70* gene polymorphism in Kacang goats. This study will be important to support the breeding program for increasing the productivity of Kacang goats. This research aimed to identify *HSP70* gene polymorphism in Kacang goats with reference to the caprine genome database in National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Animals: Kacang goats (43 males) aged 1-1.5 years were selected for this study. The animals were from two populations in East Java Province; 29 goats from Sidoarjo

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District and 14 goats from Tulungagung District. In Sidoarjo, the goats were semi-extensively raised in the fishpond area while the animal management in Tulungagung was an intensive farming system. The populations are located in a tropical area. The coordinates of Sidoarjo and Tulungagung are 7.45303°S 112.71733°E and 8.0667°S 111.9°E, respectively.

Sample collection and DNA extraction: A total of 43 blood samples (3 mL) were collected through jugular vein using a tube containing EDTA. The blood samples were immediately stored in a cooler box (4°C) for the next analysis. A total of 300 µL of fresh blood samples were used to extract the DNA. The DNA extraction was conducted according to the manufacturer's protocol for Genomic DNA Mini Kit Blood/Cultured Cell (Geneaid, Taiwan). The protocol consisted of sample preparation and 4 steps of extraction. The steps were cell lysis, DNA binding, washing, and DNA elution. The final volume of DNA yield was 100 µL. The DNA purity and concentration were quantified using NanoDrop™ One^{C} Spectrophotometer (Thermo Scientific).

DNA amplification and sequencing: A pair of primers were designed according to the caprine genome sequence in NCBI with the GenBank accession number NM_001285703. The forward primer was 5-CTCAACAAGAGCATCAACCC-3. The reverse primer was 5-CCTCTGCCTTGTACTTCTCC-3. The total volume of PCR solution was 30 µL consisting of 1 µL of 50 ng/µL DNA sample, 0.3 µL of each primer (forward and reverse), 15 µL of 1× Go Taq Green Master Mix (Promega, USA), and 13.4 µL of nuclease free water. PCR was performed in a T100 Thermal Cycler machine (Bio-Rad, Singapore) with following conditions; one cycle of pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 45 sec, and then extension at 72°C for 1 min and one cycle of final extension at 72°C for 5 min. The PCR product was qualitatively evaluated in 1.5% electrophoresed agarose gel and visualized on blue light Gel Doc (Glite 965 GW, Taiwan). Furthermore, the PCR products were sequenced at 1st Base, Selangor, Malaysia using the Sanger method.

Data analysis: Sequence alignment was performed in Bioedit compared to caprine reference sequences of GenBank accession numbers (LC616785.1, LC616786.1, LC616787.1, LC616788.1, LC616789.1, LC616790.1, and, LC616791.1). A novel SNP was determined based on the mutation site in aligned sequences. The number of Haplotypes was determined using the DNAsp package. The phylogenetic tree was reconstructed using MEGA11 based on Neighbor-Joining with Kimura 2-parameter. Median-Joining Network was performed by popart (Leigh and Bryant 2015).

RESULTS AND DISCUSSION

Kacang Goats have an important role in the economy of small farmers because they are easier to raise in large numbers than cattle. In Indonesia, most farmers raise the goat using semi-intensive farming to get meat and milk products. In addition, Kacang goat has good adaptability to the tropical climate and can survive with poor feed, especially during the dry season. As a homeothermic animal, the goat must maintain its body temperature at the same level despite the change in temperature around their environment. However, cellular homeostasis can be affected by heat stress (Hasan *et al.* 2019). Regarding the adaptability to the tropical climate, HSP70 has a crucial role in thermal tolerance against heat stress (Shende *et al*. 2019).

Several studies analyzed the *HSP70* gene polymorphism in goats (Fatima *et al*. 2019), buffaloes (Habib 2020), and poultry (Habib *et al*. 2020). Caprine *HSP70* is located on chromosome 23 with a length of 2476 nucleotide bases with 641 amino acids. The gene consisted of two exons and one intron. In this study, the partial exonic DNA of *HSP70* gene was successfully amplified using the PCR method at the annealing temperature of 60°C. The length of the PCR product was 511 bp and could be visualized in 1.5% electrophoresed agarose gel. This sequence was located in the coding region of *HSP70* starting from 1075th to 1585th nucleotides. A novel SNP was detected in this study and categorized as a synonymous mutation with no amino acid change. The SNP occurred in the 1311th nucleotide in the coding region of *HSP70* gene with nucleotide change of Cytosine to Thymine (Fig. 1). This result was different from the previous study on some Indian goats. Mohalik *et al.* (2021) found a non-synonymous mutation (C241T) that significantly affected rectal temperature, skin temperature, and respiration rate. The synonymous mutation did not change the amino acid. Nevertheless, the synonymous mutation was able to change the gene expression, conformation, and function (Sauna and Kimchi-Sarfaty 2011). The synonymous mutation was also implicated in several classes of human disease and exerts a physiological effect (Stark *et al*. 2010).

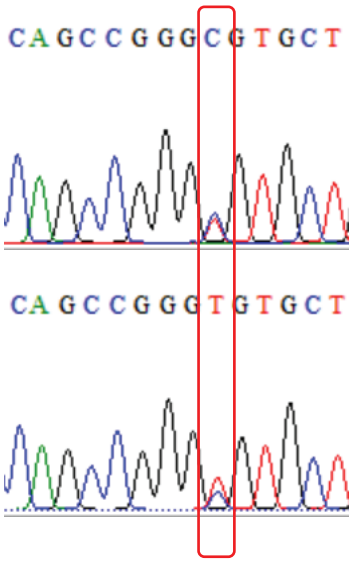

Fig. 1. A novel SNP (c.1311C>T) of *HSP70* gene in Kacang goats.

The use of flavonoids in feed such as phloretin can improve growth performance in livestock with heat stress and high HSP70 expression (Hu *et al*. 2021). In addition, the use of amino acids such as glutamine can improve the quality of meat in chicken that is given heat stress (Hu *et al*. 2020). HSP synthesis is observed not only when exposed to high temperatures, but also when exposed to toxic animal and plant products, infectious diseases, inflammation, and fever (Zugel and Kaufmann 1999, Danzer *et al*. 2011, Bianchi *et al.* 2014, Hu *et al*. 2022). Therefore, it is important to study HSP70 to mitigate heat stress in livestock and increase productivity in hot areas with high humidity.

According to the mutation, four haplotypes were observed in this research. Haplotypes 1 and 2 were determined by the difference of nucleotide bases at the 1311th position (c.1311 C>T) whereas haplotypes 3 and 4 were formed by two deletion sites at 1151st and 1160th, respectively. In Sidoarjo, the frequencies of Haplotype 1, Haplotype 2, Haplotype 3, and haplotype 4 were 0,68; 0.63; 1.00; and 1.00, respectively. Haplotype 1 and Haplotype 2 were only observed in Tulungagung with frequencies of 0.31 and 0.37, respectively (Table 1). Haplotype diversity in Indonesia was 0.55 with 4 haplotypes while haplotype diversity in Iraq was 1.00 with 7 haplotypes (Table 2). In this study, haplotype 1 had the highest frequency in Indonesian goats. Iraqi goats had the highest haplotype diversity because seven samples had their own haplotype. It was different in Kacang goat in Indonesia which had a large sample size but fewer haplotypes were found. In Indonesian goats, haplotype 1 had a similar sequence with the GenBank access number NM_001285703.1.

Table 1. Haplotype frequency of *HSP70* gene in Indonesian goat

Haplotype	Population (Freq)		Total (Freq)
	Sidoarjo	Tulungagung	
Haplotype 1	15 (0.68)	7 (0.31)	22 (0.51)
Haplotype 2	12 (0.63)	7 (0.37)	19 (0.45)
Haplotype 3	1 (1.00)	0 (0.00)	1 (0.02)
Haplotype 4	1 (1.00)	0 (0.00)	1 (0.02)

Table 2. Haplotype diversity of *HSP70* gene in Indonesian goat and Iraqi goat

Country	Number of Haplotype	Haplotype Diversity
Indonesia (n = 43)	4	0.55
Iraq (n =7)	7	1

The phylogenetic tree was constructed from a partial exonic sequence of HSP70 that had a synonymous mutation inside. The result of the phylogenetic tree analyzed with Neighbor-Joing with Kimura 2-parameter showed that Kacang goat had no close relationship with the Iraqi goat. It was indicated by a separate cluster in the phylogenetic tree (Fig. 2). According to neighbor-joining results, two clades of *HSP70* diversity were observed in this study, namely

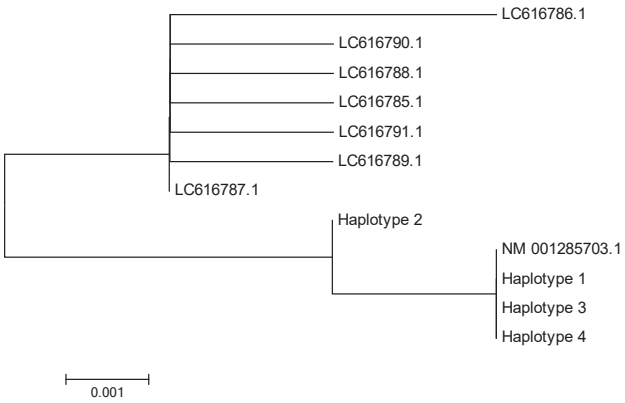

Fig. 2. Neighbor-joining tree with kimura 2-parameter.

the Indonesian and Iraqi clades. Iraqi clades consisted of LC616785.1, LC616786.1, LC616787.1, LC616788.1, LC616789.1, LC616790.1, and LC616791.1. The Iraqi clades were located in one cluster whereas the Indonesian clade had two clusters. One cluster was haplotype 2 and the others were haplotypes 1, 3, and 4. It indicated the difference between haplotype 2 and the others.

A similar result was found in Median Joining Network that Indonesian goats had no close relationship with Iraqi goats based on haplotype 1 and haplotype 2 (Fig. 3). The difference between haplotype 2 and all haplotypes in Iraqi goats was only three nucleotides. Because haplotypes 3 and 4 were in-del mutation, they could not be analyzed using the median-joining network. Median-Joining (MJ) has been proposed as a phylogeographic analysis method showing evolutionary relationships and probable ancestral connections between haplotypes (Kong *et al*. 2015). The heat shock protein gene is an established phenotype model for studying the evolutionary importance of regulatory mutations in response to environmental changes (Chen *et al*. 2011). Therefore, a lot of *HSP70* sequence data is needed to determine the genetic changes of the *HSP70* gene in goats. Furthermore, Nagayach *et al*. (2017) stated that HSP70 plays a key role to provide the heat stress

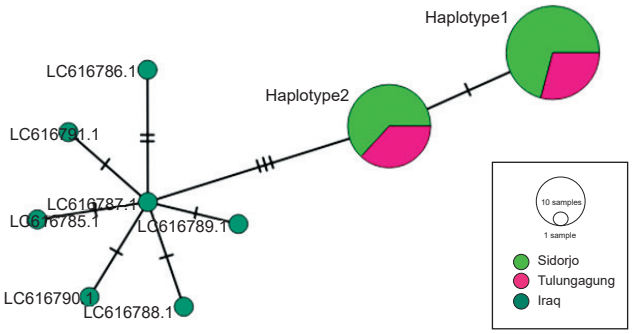

Fig. 3. Median Joining Network of *HSP70* gene in Indonesian goat and Iraqi goat.

tolerance mechanism during different seasons in goats.

In conclusion, a novel Single Nucleotide Polymorphism (c.1311C>T) in the coding region of *HSP70* gene was found in this study. Indonesia Kacang goats in this research had

no close relationship with Iraqi goats. The Kacang goats with the T allele (haplotype 2) was unique haplotype that showed different clusters with haplotypes 1, 3, and 4. In the future, an association study of a novel Single Nucleotide Polymorphism (c.1311C>T) could be performed to understand the effect of a synonymous mutation in heat stress mechanisms in Kacang goats. For the breeding program, haplotype 2 could be used as a specific marker to determine the unique cluster of Kacang Goats in two different populations

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the member of the Genomic and Proteomic Research Group Laboratory, Faculty for providing data. This research was supported by the Ministry of Education and Culture, Republic of Indonesia through the Hibah Peneliti Muda 2019 project.

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