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The genetic diversity of *heat shock protein 70* gene at promoter and 5' untranslated region in beef cattle

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ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi keragaman daerah promotor dan 5' UTR (*untranslated region*) gen *HSP70* pada bangsa sapi pedaging di Indonesia. Total sampel yang digunakan adalah sebanyak 86 sampel darah yang terdiri dari sapi Bali, Madura, PO (Peranakan Ongole), Limosin, dan Belgian Blue. DNA Sampel selanjutnya dianalisis menggunakan metode *direct sequencing*. Hasil *genotyping* menunjukkan adanya lima SNP (*single nucleotide polymorphysm*) di daerah promotor (g.-393T>C, g.-343C>A, g.-202T>C, g.-69T>G) dan 3 SNP di 5' UTR (g.19A>G, g.45C>T, g.100_101ins30). Frekuensi alel yang ditemukan pada sapi PO, Limosin dan Belgian Blue berada dalam keseimbangan Hardy-Weinberg, sedangkan pada populasi sapi Bali dan sapi Madura frekuensi alelnya disequilibrium. Secara umum keragaman alel yang diamati berkisar rendah hingga tinggi (0.26-1.00), SNP g.19A>G merupakan SNP yang memiliki keragaman tertinggi. Ditemukan bahwa SNP g.-69T>G berikatan dengan faktor transkripsi dari NF-Y dan CAAT box. Selain itu, ditemukan insersi 30 pb (pasang basa) (g.100_101ins30) pada sapi Bali dan sapi Madura belum pernah dilaporkan sebelumnya.

Kata Kunci: *5' UTR, gen HSP70*, *promotor, sapi potong, SNP*

ABSTRACT

This study was aimed to identify genetic diversity in the promoter area and 5' UTR (untranslated region) *HSP70* (heat shock protein 70) gene in several beef cattle in Indonesia. A total of 86 blood samples of Bali, Madura, PO (Peranakan Ongole), Limousine, and BB (Belgian Blue) cattle were used in this study. The extracted DNA of all blood samples was then analyzed using the direct sequencing method. The genotyping results showed the presence of five SNP (Single Nucleotide Polymorphism) in the promoter region, namely g.-393T>C, g.-343C>A, g.-202T>C and g.-69T>G and three SNPs at 5' UTR, i.e., g.19A>G, g.45C>T, and g.100_101ins30. The frequency of SNP alleles found in PO, Limousine, and BB cattle was in equilibrium, whereas in Bali and Madura cattle populations, the allele frequency was disequilibrium. In general, the level of diversity of observed alleles ranged from low to high (0.26-1.00), where SNP g.19A>G had the widest variety. It was successfully revealed in this analysis that the SNP g.-69T>G binds to both the NF-Y and CAAT box transcription factor. In addition, the 30 bp (base pair) insertions (g.100_101ins30) that were identified in Bali and Madura cattle have never been reported in previous research studies.

Keywords: 5' UTR, HSP70 gene, promoter, beef cattle, SNP

INTRODUCTION

Indonesia has a variety of cattle, both local, indigenous, and exotic cattle breeds, which have the potential to provide animal protein. Local and indigenous livestock are common cattle types that have long been developing and adapting to the local environment. Both local and indigenous cattle are suited to the local environment. They have the advantage of only require low input feeds, have the high reproducing capability, resistance to a parasite, and adapted to the humid tropical climates (Martojo, 2012). While exotic animals imported to Indonesia because of their superiority in production, both meat, milk, or eggs, but they cannot adapt to the harsh tropical environment (Pereira *et al.*, 2014). The various types of cattle breed have different responses in dealing with Indonesia's hot and humid tropical climate. Designing the breeding scheme by selecting the thermotolerant animals could be more productive and less costly than exploiting environmental conditions and management in the tropical region.

Global warming has become a threat of livestock productivity by negatively affecting growth, reproduction, milk yield, and carcass traits of the livestock (Archana, 2017). The upper or lower critical temperature leads to heat stress conditions in most important for livestock, and it indicates the imbalance between heat production and heat loss (Kumar *et al.*, 2011). However, the animal has a regulation to maintain homeostasis by acclimation, acclimatization, and adaptation (Boyles *et al.*, 2011). Adaptation is a crucial movement for animals if the environmental stressor is present for an extended period. The regulation mechanisms of adaptation include biochemical, physiological, behavioural, and morphological changes (Archana, 2017).

Heat shock protein, include *HSP70 (Heat Shock Protein 70)*, is an essential biomarker as a biochemical mechanism against heat shock (Margel *et al.*, 2011; Abdelnour *et al.*, 2019). *HSP70*s have housekeeping roles and quality control functions because of their role in signalling the transduction pathways, proofreading the protein's structure, and repairing misfolded conformers on several stressors, especially heat stress (Mayer and Bukau, 2005). Bovine *HSP70* has been identified and mapped in the National Center for Biotechnology Information (NCBI). This gene is located on chromosome 23q22. The previous studies have reported that *HSP70* is associated with the reproductive properties of calves, the immune system (Dietz *et al.*, 1997), the quality of male semen (Gafer *et al.*, 2015), and heat resistance (Xiong *et al.*, 2013).

Several studies have identified the role of the *HSP70* gene on heat resistance in Chinese Holstein cattle (Xiong *et al.*, 2013), local Thai cattle (Charoensook *et al.*, 2012), Frieswal crossbred cattle (Deb *et al.*, 2013), Tharpakar and Karan fries (Singh *et al.,* 2014). However, studies of the *HSP70* gene in the CpG island (CGI) region of Indonesian beef cattle have not been carried out, so it is necessary to analyze the single nucleotide polymorphism (SNP) of *HSP70* gene structures in the CpG island of Indonesian beef cattle.

MATERIALS AND METHODS

Animal and Samples

This study utilized a total of 82 cattle blood samples, which were stored in the animal breeding and genetic laboratories, Faculty of Animal Science, IPB University (Table 1).

Table 1. Cattle breed, location, and year of blood samples collection

Breed	Collecting year	Total	Location
Bali	2019	28	BPTU-HPT Denpasar, Bali
Madura	2013	18	VBC Sapudi island
PO	2019	10	BET Cipelang, Bogor
Limousine	2019	12	BPTU-HPT Padang Mangatas
Belgian Blue	2019	14	BET Cipelang, Bogor
Total		82	

DNA Extraction, Amplification, and Sequencing

Total DNA was extracted by the *Phenol Chloroform* method (Sambrook and Russell, 2006) with some modification. The primers were designed based on the GenBank® from NCBI with access code AY149618.1. The primers were manual designed based on the CGI region in front of the *HSP70* first exon using Primer3 software and evaluated by Primer Stat. DNA amplification was performed using the polymerase chain reaction (PCR) with two pairs of forward and reverse primers. The CGI 1 has a product length of 308 bp with a forward primer 5'-GTTTGATACG GTTCGGATGG-3' of and reversal primer 5'-CTGAGGAGAA ACAGCAGCCT-3'. The CGI 2 was 503 bp from primer amplification by forward primer 5'-CATTACCCCT TTCCGAGACA-3' and reversal primer 5'-GAAGCTTATC TCGGAGCC-3'.

DNA amplification was performed using the polymerase chain reaction (PCR) method with a master cycler gradient machine (ESCO, Singapore). Each reaction was in a final volume of 25 µL, containing 2 µL DNA template, 9.4 µL nuclease-free water (NFW), 0.3 µL forward primer, 0.3 µL reverse primer, and 12.5 µL GoTaq® Green Master Mix (Promega, USA). An initial denaturation proceeded amplification of DNA at 95ºC for 5 min, followed by 35 cycles of 95ºC denaturation for 10 seconds, annealing 55ºC for 20 seconds, extension 72ºC for 30 seconds; and a final extension 72ºC for 5 min. The amplification products were visualized on a 1.5% agarose gel, stained with FlouroSafe DNA Stain (First Base, Singapore), and photographed using UV Transilluminator. The total volume 22 µL of each sample was sequenced by the commercial laboratory service at First Base (Malaysia), and direct sequencing was done using ABI Prism 96-capillary 3730 x 1 DNA analyzer (Applied Biosystems, USA).

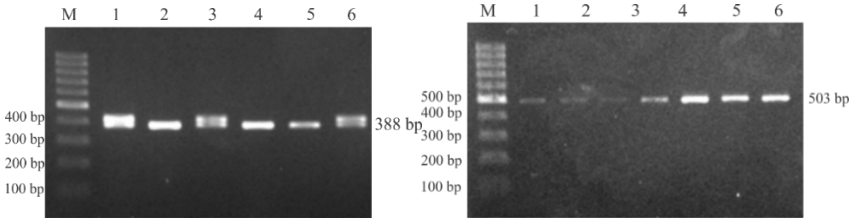
Data Analysis

The result of product target amplification after sequenced was combined to make the consensus sequence by removed the overlap sequence. The total product was 606 bp, consisting of 188 bp 5' *untranslated region* (5' UTR) and 418 bp promoter regions. The results of sequencing in the form of nucleotide chromatograms were then identified. The double-peak bands were considered as a heterozygous using FinchTV 1.4.0 (Geospiza, Inc.; Seattle). The sample sequences aligned with reference sequences through the accession numbers AY149618.1 using the MUSCULAR alignment technique in MEGA X (Kumar *et al.*, 2018). Genotype reconstruction and SNP diversity were done using the PopGene32 (Yeh *et al.*, 2000) and Bioedit programs (Hall, 1999).

RESULT AND DISCUSSION

Amplification of Bovine *HSP70* Gene

Partial amplification of the *HSP70* gene of Bali, Madura, Peranakan Ongole, Limousine, and Belgian Blue cattle produces DNA fragments that are categorically divided into two regions of CpG island (CGI), which are 388 bp CGI 1 and 503 bp CGI 2 (Fig. 1). This two amplification, when combined they will form overlapping sequences, so the sequences were combined to produce 606 bp consensus fragments. This sequence is located in the promoter region and 5' UTR (Fig. 2).



(a) (b)

Figure 1. Electrophoresis PCR product of HSP70 gene (a) promoter and (b) 5' UTR.

The Structure and Diversity of *HSP70* Gene on Promoter and 5' UTR

HSP70 is intronless genes that only have one exon along 2103 bp consisting of 177 bp 5' UTR

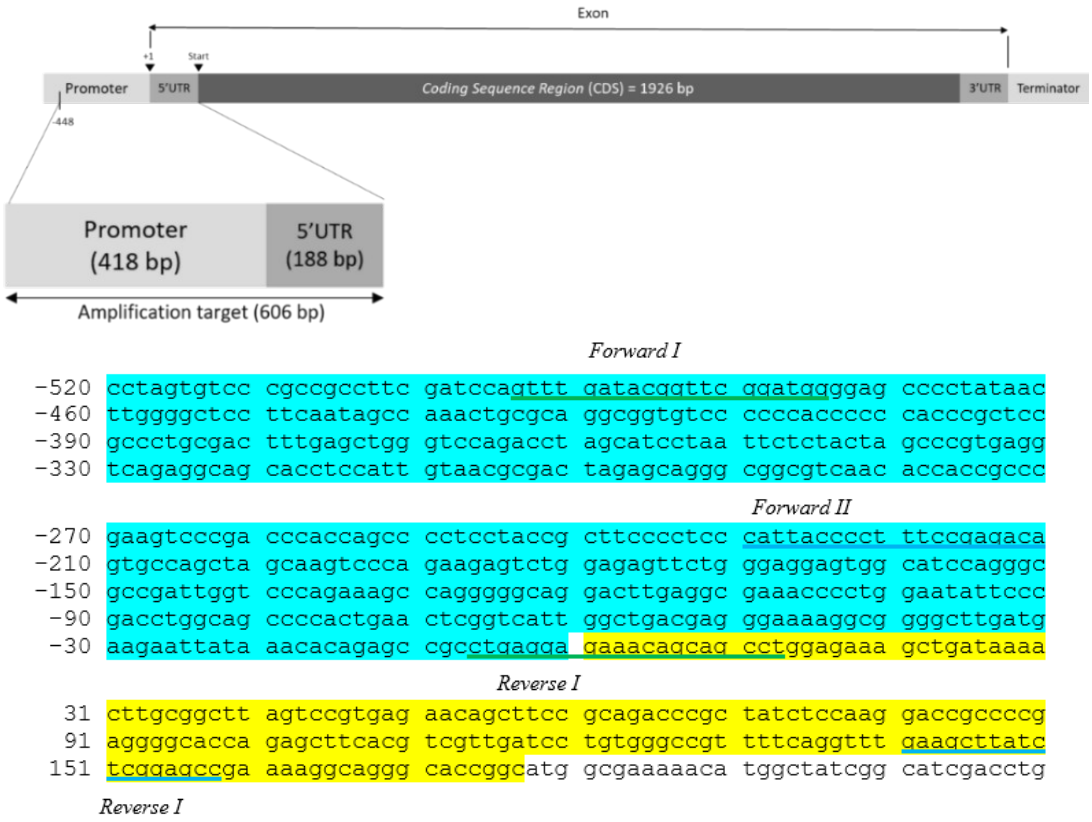
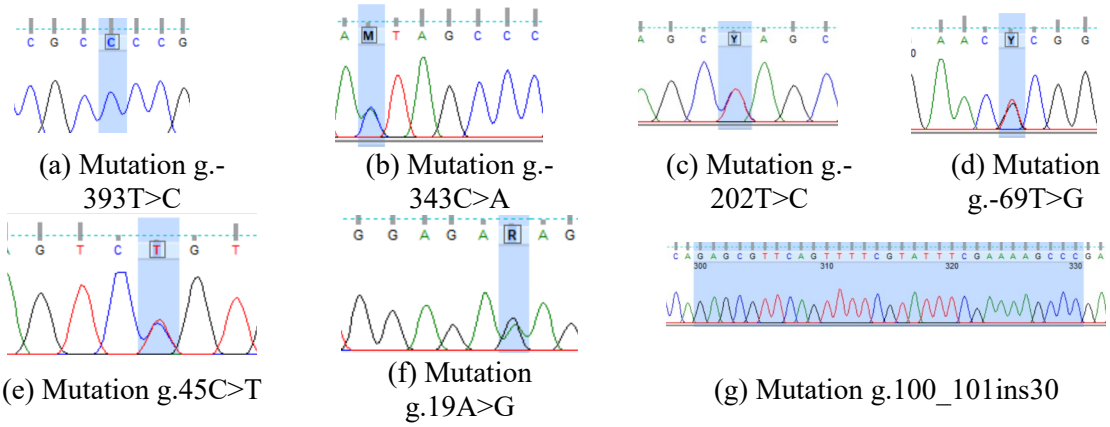


Figure 2. Reconstruction of the HSP70 gene in cattle based on the GenBank® sequence where (...) is a promoter and ( ) is 5' UTR

and 1926 bp coding sequence (CDS). The structure of this gene begins with a 1290 bp promoter. This study obtained sequence with 418 bp of the promoter region and 118 of 5' UTR (Fig. 2). Nicholas (2010) explained that the nucleotides of the gene structure numbered positively from the beginning, where transcription began. While the precede nucleotide bases numbered negatively, namely -1, -2, and so on, so +1 numbering will start from the beginning of 5' UTR (downstream) and -1 will begin from the end towards the beginning of the promoter (upstream), there is no 0 number (Fig. 2). SNP nomenclature in the recent study uses guidance from Ogino *et al.* (2007) whose mapped the SNP accessions by numbering based on gene structures. The SNPs obtained from the chromatogram analysis can be seen in Figure 3.

The 5' UTR is the regions that are part of the exon but do not directly contribute to the protein sequence because they are only transcribed but not translated (Leppek *et al.*, 2018). Some mutations in 5' UTR have affected the expression of the genes, such as those that arise in AR gene expression causing cancer of the prostate gland (Crocitto *et al.*, 1997) and mutations in 5' UTR in the *HSP70* gene are also associated with service per conception in Pasundan cattle (Said and Putra, 2018).

Based on the diversity analysis of *HSP70* gene, we successfully indicated four polymorphic SNPs in the promoter region (g.-393T>C, g.-343C>A, g.-202T>C, g.-69T>G) and three polymorphic SNPs in 5' UTR (g.19A>G, g.45C>T, g.100_101ins30) (Fig. 3). The analysis of alignment sequence based on the reference sequence reveals four transition mutations, two transversion mutations, and one insertion mutation. Transition mutations occur at point -393 from thymine (T) to cytosine (C), -202 thymine to cytosine (T>C), +19 C (cytosine) to T (thymine), and +45 A (adenine) to G (guanine). The transversion mutation occurred at position -343 is a change of base cytosine to adenine (C>A) and -69 thymine to guanine (T>G). Meanwhile, insertion of 30 bases (GAGCGTTCAG TTTTCGTATT TCGAAAAGCCC) occur in position +100 to +101.



(a) Mutation g.-393T>C (b) Mutation g.-343C>A (c) Mutation g.-202T>C (d) Mutation g.-69T>G

(e) Mutation g.45C>T (f) Mutation g.19A>G (g) Mutation g.100_101ins30

Figure 3. HSP70 partial chromatogram with SNP in the promoter {(a), (b), (c), (d)} and 5' UTR region {(e), (f), (g)}

Transcription Factor in Promoter Region

Nicholas (2010) stated that the upstream region of genes (promoter region) is an important location for the sites of RNA polymerase transcription factors to attach; in this way, they control the transcription of a gene. The recent study reveals six putative transcription factor binding domains, namely CACC box, AP-2, CAAT box, CAAT box that bind to the NF-Y site, TATA box, GC box or specificity protein 1 (SP 1), and Heat Shock Elements 2 (HSE 2) (Fig. 4). The

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-510 CCTAGTGTCC CGCCGCCTTC GATCCAGTTT GATACGGTTC GGATGGGGAG CCCCTATAAC
-450 TTGGGGCTCC TTCAATAGCC AAACTGCGCA GGCGGTGTCC CCCCACCCCC CACCCGCTCC
                                          CACC box                (T/C)
-390 GCCCTGCGAC TTTGAGCTGG GTCCAGACCT AGCATCCTAA TTCTCTACTA GCCCGTGAGG
                                                    (C/A)
-330 TCAGAGGCAG CACCTCCATT GTAACGCGAC TAGAGCAGGG CGGCGTCAAC ACCACCGCCC
                                          GC box/SP-1
-270 GAAGTCCCGA CCCACCAGCC CCTCCTACCG CTTCCCCTCC CATTACCCCT TTCCGAGACA
                          AP-2
-210 GTGCCAGCTA GCAAGTCCCA GAAGAGTCTG GAGAGTTCTG GGAGGAGTGG CATCCAGGGC
           (T>C)                                                AP-2
-150 GCCGATTGGT CCCAGAAAGC CAGGGGGCAG GACTTGAGGC GAAACCCCTG GAATATTCCC
          CAAT box          AP-2          CAAT box              HSF2
 -90 GACCTGGCAG CCCCACTGAA CTCGGTCATT GGCTGACGAG GGAAAAGGCG GGGCTTGATG
                           (T>G)    NF-Y
 -30 AAGAATTATA AACACAGAGC CGCCTGAGGA GAAACAGCAG CCTGGAGAAA GCTGATAAAA
       TATA box                                        (A>G)
  31 CTTGCGGCTT AGTCCGTGAG AACAGCTTCC GCAGACCCGC TATCTCCAAG GACCGCCCCG
                    (C>T)
  91 AGGGGCACCA GAGCTTCACG TCGTTGATCC TGTGGGCCGT TTTCAGGTTT GAAGCTTATC
     (-/GAGCGTTCAGTTTTCGTATTTCGAAAAGCCC)
 151 TCGGAGCCGA AAAGGCAGGG CACCGGCATG GCGAAAAACA TGGCTATCGG CATCGACCTG
 211 GGCACCACCT ACTCCTGCGT AGGGGTGTTC CAGCACGGCA AGGTGGAGAT CATCGCCAAC
 271 GACCAGGGCA ACCGCACCAC CCCCAGCTAC GTGGCCTTCA CCGATACCGA GCGGCTCATC
 331 GGCGATGCGG CCAAGAACCA GGTGGCGCTG AACCCGCAGA ACACGGTGTT CGACGCGAAG
 391 CGGCTGATCG GCCGCAAGTT CGGAGACCCG GTGGTGCAGT CGGACATGAA GCACTGGCCT
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Figure 4. *HSP70* gene sequences and SNP, description with colour: *coding sequence*, 5' UTR, promoter, *binding site*, substitution mutations, and insertion mutation

sequence at the -19 to -24 position contains 5'-TATAAA-3' (Fig. 4), which is a TATA box; this is based on Nicholas's (2010) finding, who stated that the position of the TATA box is around 25 upstream bases. Specificity Protein 1 (SP1) in the form of GC box with sequence 5 '-GGGCGG-3' located at -88 to -93. CACCC box located at -403 to -407.

Genes with CGI-rich sequences generally have SP1 as an option for binding transcription factors such as in *Dag1* gene expression (Rettino *et al.*, 2009) and *p35* genes in mice (Ross *et al.*, 2002). The area of transcription factors in this study was very conservative, and no mutations occurred, except the NF-Y (nuclear transcription factor Y) binding site bound to the CAAT box, which had a mutation g.-69T>G. NF-Y is one of the transcriptional factors that bind to the CAAT box in the promoter region of various eukaryote genes (Li *et al.*, 1992). NF-Y is emerging as a regulatory factor for many genes overexpressed in multiple biological pathways (Mantovani, 1999) and several different kinds of cancer (Dolfini and Mantovani, 2013). This mutation has strong potential that indicates methylation because there are transcription factors that interact with the methyl-CpG binding domain (MBD) (Zhu *et al.*, 2016).

Allele Frequency of *HSP70* Gene

Allendorf (2013) stated that the SNP is considered polymorphic if it has a major allele frequency smaller than 0.99 for large populations (n = 50) and lower than 0.95 for smaller

Table 2. Allele frequencies of HSP70 gene and Chi^2 test results

SNPs	Allele	Population						
		Bali	Madura	PO	Limousine	BB	mean	sd
Promoter								
g.-393T>C	T	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	C	1.00	1.00	1.00	1.00	1.00	1.00	n/a
	Chi^2 test	n/a	n/a	n/a	n/a	n/a	n/a	n/a
g.-343C>A	C	0.93	1.00	0.45	0.75	1.00	0.83	0.21
	A	0.07	n/a	0.55	0.25	n/a	0.17	0.21
	Chi^2 test	**	n/a	ns	ns	n/a		
g.-202T>C	T	0.75	0.78	0.9	0.87	0.93	0.85	0.07
	C	0.25	0.22	0.1	0.13	0.07	0.15	0.07
	Chi^2 test	ns	ns	ns	ns	ns		
g.-69T>G	T	0.79	0.81	1.00	0.87	0.93	0.88	0.08
	G	0.21	0.19	n/a	0.13	0.07	0.12	0.08
	Chi^2 test	ns	*	n/a	ns	ns		
5' UTR								
g.19A>G	A	0.52	0.31	0.05	0.08	0.36	0.26	0.18
	G	0.48	0.69	0.95	0.92	0.64	0.74	0.18
	Chi^2 test	ns	ns	n/a	ns	ns		
g.45C>T	C	0.91	0.75	0.7	0.71	0.57	0.73	0.11
	T	0.09	0.25	0.3	0.29	0.43	0.27	0.11
	Chi^2 test	*	ns	ns	ns	ns		
g.100_101ins30	-	0.93	0.89	1.00	1.00	1.00	0.96	0.05
	Insertion	0.07	0.11	n/a	n/a	n/a	0.04	0.05
	Chi^2 test	**	**	n/a	n/a	n/a		

Note: Chi^2 test df = 1, χ^2 table = 3.84. **: highly significant (P<0.01), *: significant (P<0.05), ns: not-significant (P>0.05); n/a: not available

populations. Table 2 presents the *HSP70* gene allele frequencies in the observed population. Alleles are variant forms of genes located in the same locus. In a population, allele frequencies are a reflection of genetic diversity (Noor, 2008).

This study revealed seven polymorphic loci with 4 transition substitution (g.-393T>C, g.-202T>C, g.19A>G, and g.45C>T), 2 transversion substitution (g.-343C>A dan g.-69T>G), and 1 insertion mutation (g.100_101ins30). The number of SNPs found in this study was less than the number of SNPs found by Öner *et al.* (2017) who obtained 43 SNPs in local Turkish cattle in the 5' UTR and also fewer than the study of Sodhi *et al.* (2013) whose discovered 5 SNPs in the 5' UTR and 11 SNPs in the promoter region in the *Bos taurus*. A large minor allele frequency (MAF) value indicates that the locus is highly polymorphic, while (for small populations) a MAF value of smaller than 0.05 is considered monomorphic (Allendorf *et al.*, 2013). Monomorphic loci showed by zero MAF of the g.-343C>A locus in Madura and BB cattle, g.-69T>G locus in PO cattle, and g.100_101ins30 locus in PO, BB, and Limousin cattle (Table 2). MAF variations range from 0.05 to 0.68, with the highest MAF at the g.19A>G locus and the lowest MAF at the g.100_101ins30 locus.

Population in Hardy-Weinberg Equilibrium

Genetic diversity is an essential component of population selection. Populations with high genetic diversity vary in response to environmental stresses, so selection to produce heat tolerance cattle is possible. Genetic diversity in populations can be described by the number of polymorphic loci in the population, heterozygosity in Hardy-Weinberg equilibrium (HWe), and gene frequency or haplotype frequency (Nei and Kumar, 2000). The population will be in HWe if the population is large, so there have been random mating, nor any mutations, no selection, and no migration have occurred. (Noor, 2008). Some loci in Bali and Madura cattle populations are not in the HW equilibrium (Table 2), i.e., g.-343C>A, g.45C>T, and g.100_101ins30 in Bali cattle; g.-69T>G and g.100_101ins30 in Madura cattle.

The potential SNP loci in this study are insertions g.100_101ins30, mutation g.-69T>G, and mutation g.19A>G. The locus of insertion 100_101ins30 only found in Bali and Madura cattle that have banteng (*Bibos banteng*) blood is encouraging considering this type of cattle has better adaptability in a hot and humid climate that other type of beef cattle studied. This study revealed that the g.-69T>G locus was the only SNP found to mutated in the MBD-transcription factor zone. The g.19A>G locus was the most polymorphic because it has the highest MAF. So the subsequent research has the potential to be carried out in Bali cattle to reveal the role these loci in adapting harsh environments

CONCLUSION

The study found seven SNPs *HSP70* gene in the promoter region and 5' UTR of Bali, Madura, PO, BB, and Limousine cattle located in the bases g.-393T>C, g.-343C>A, g.-202T>C, g.-69T>G, g.19A>G, g.45C>T, and g.100_101ins30. The potential SNPs for future research are in the loci of insertions g.100_101ins30, mutation g.-69T>G, and mutation g.19A>G. The majority of SNP loci studied were in polymorphic form and were in the Hardy-Weinberg equilibrium, except for the Bali, Madura, and BB cattle population.

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