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NUCLEOTIDE POLYMORPHISMS OF HEAT SHOCK PROTEIN 70-2 GENE (HSP70-2) BETWEEN TROPICAL (THAI NATIVE) AND TEMPERATE (HOLSTEIN FRIESIAN) CATTLES

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ABSTRACT

Tropical breed cattle (*Bos indicus*) have more physiological tolerance to heat than temperate breed cattle (*Bos taurus*). This phenomenon involves cellular protein production related to various cellular proteins including heat shock protein. The study aimed to compare the nucleotide sequence of the heat shock protein 70-2 gene (HSP70-2) between Thai Native (NT) and Holstein Friesian (HF) cattle by using the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) method. DNA was extracted from 70 and 52 samples of NT and HF, respectively. Ten primers (HP1 to HP10), specifically designed for HSP70-2, were related to the data of *Bos taurus* in GenBank databases (accession number; BTU02892). The primers included three regions of the gene: (1) 5' flanking region, (2) coding sequence, and (3) 3' flanking region. The results of 10 primers showed 32 patterns in both NT and HF. Sequence analysis showed polymorphism in HSP70-2 and differentt nucleotide sequences at position 1262 (NT=A/C; HF=G) in HP5 primer, 2347 (NT=T; HF=C) in HP9 primer, and 2515 (NT=G/T; HF=C), 2516 (NT=C/T ; HF=G), and 2557 (NT=G; HF=T) in HP10 primer. The finding suggested that these primers were the best to separate NT from HF, and the different positions were found in HP5 and HP9 primers covering the coding sequence region, while HP10 primer covered the 3'flanking region. Thus, the coding sequence region and 3'flanking may affect the mechanism of HSP70-2 protein translation and HSP70-2 protein post-transcription, respectively.

Key words: *Tropical breed, Temperate breed, Nucleotide sequence, HSP70-2, Breed difference*.

INTRODUCTION

Tropical breed cattle (*Bos indicus*) exhibit greater physiological tolerance to heat compared to temperate breed cattle (*Bos taurus*) (Gaughan et al., 1999). Genetic divergence between these breeds occurred between 110,000 and 850,000 years ago, as evidenced by mitochondrial DNA and microsatellite analysis (Bradley et al., 1996; MacHugh et al., 1997). Heat shock proteins (HSPs) are molecular

chaperones induced by elevated temperatures and play crucial roles in cellular stress responses. Constituting 2-15% of total cellular protein, HSPs are essential for protein homeostasis under normal and stressful conditions (Morimoto et al., 1994). This study aims to characterize the HSP70-2 nucleotide sequence in *Bos indicus* and *Bos taurus* breeds to elucidate the genetic basis of their differing heat tolerances. By identifying nucleotide variations between breeds, we seek to develop selection markers for improving heat tolerance in cattle through breeding programs.

MATERIALS AND METHODS

Animals

Animals used in this study included Thai native cattle (NT), representing the tropical breed, and Holstein Friesian (HF), representing the temperate breed. The total number of NT and HF were 70 and 52 animals, respectively. The study was conducted at the Animal Biotechnology Laboratory, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, from June 2010 to May 2011.

Samples collection

Blood samples

Blood samples were collected from the jugular vein of each animal into 1 ml EDTA blood collection tubes. Nine milliliters of blood were collected per animal. All blood samples were kept at room temperature and DNA extraction was processed within 2 hours of collection.

Tissue samples

A 1 cm^3 tissue sample was collected from the sirloin muscle of each animal and stored in 95% ethanol until DNA extraction.

DNA extraction procedure

The whole blood or tissues from each sample was washed in 0.9% NaCl; Total genomic DNA was extracted following the protocols of Pure-Gene™ Blood/Tissue DNA Kit (GENTRA INC., MA). DNA concentration was determined by spectrophotometer at 260 nm and diluted with DNA hydration buffer to a concentration of 50 ng/µl. The genomic DNA prepared samples were stored at 4°C until the polymerase chain reaction (PCR) process.

Primers design

Design primer specific with HSP70-2 was reported in GenBank database (accession number; BTU02892) by GENEFISH2 and Primer3 program. NCBI BLAST was used to compare the nucleotide sequences, and Oligoanalyzer program was used for hairpin loop analysis. A total of ten primers (HP1 to HP10) were used in this study (Table 1).

Analysis of polymorphism by PCR-SSCP and sequencing HSP70-2 gene

For PCR procedure

PCR mixture: Genomic DNA from NT and HF was used as template DNA. The PCR reaction mixture consisted of 1 µl DNA template, 1 µl 10× PCR buffer, 0.8 µl 50 mM $MgCl_2$, 1 µl dNTPs (1 mM each), 1 µl of each 1 µM forward and reverse primer (Table 1), 0.1 µl 5 U/µl Taq polymerase, and 4.1 µl sterile water, for a total volume of 10 µl.

PCR amplification

Initial denaturation was performed at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at [temperature]°C for 45 seconds (primer annealing temperatures are listed in Table 1), and extension at 72°C for 30 seconds. A final extension step was carried out at 72°C for 5 minutes. PCR products were visualized on a 2% agarose gel and stored at 4°C until subsequent SSCP analysis.

SSCP procedure

PCR products were denatured and converted to single strands by mixing with SSCP dye in a 1:1 ratio. Three microliters of the resulting mixture were subjected to Single-Strand Conformation Polymorphism (SSCP) analysis on a 5% non-denaturing polyacrylamide gel under conditions specified in Table 1. Gels were stained with GelStar (GelStar Inc., NY) to visualize SSCP patterns. One representative sample from each unique SSCP pattern in NT and HF groups was sent to Marcogen (South Korea) for nucleotide sequencing. The obtained sequences were compared to GenBank reference sequences using DNAMAN version 5.2.2, BioEdit, and ClustalW2.

Table 1. Primers of HSP70-2 gene and condition of PCR-SSCP.

Primer	nucleic acid Sequence (F, Forward; R, Reverse)	PCR product size (bp)	Annealing temp (îC)	Position	Electrophoresis protocol For SSCP (volt/time)
HP1	F: CTCCTGTTTCCTCCAGCGAA R: GTCGTTGGCGATGATCTCCA	517	63	Position 6-523 in 5'flanking region and coding sequence	200 V/360 min
HP2	F: GCACCACCTACTCCTGCGTA R: CTTCATGTCCGACTGCACCA	230	66	Position 461-691 in coding sequence	200 V/180 min
HP3	F: CGCAGAACACGGTGTTCGA R: GTGATCACCGCGTTGGTCA	271	62	Position 614-862 in coding sequence	200 V/180 min
HP4	F: GCTGACCAAGATGAAGGAGA R: GTCGATCGTCAGGATGGACA	253	61	Position 795-1066 in coding sequence	200 V/180 min
HP5	F: GTTCGACGTGTCCATCCTGA R: GAACAGGGAGTCGATCTCCA	271	64	Position 1038-1291 in coding sequence	200 V/180 min
HP6	F: GAGAACCTTGTCGTCCAGCA R: CAGGATGCCATTGGCATCGA	646	66	Position 1239-1885 in coding sequence	200 V/390 min
HP7	F: AGATCGAGGTGACCTTCGACA R: CTTCAGCCCCTCATCCTCCA	260	66	Position 1844-2304 in coding sequence	200 V/240 min
HP8	F: GTCGTACGCCTTCAACATGA R: AGACCCAGAGCCCCCTTTA	271	66	Position 2055-2325 in coding sequence	200 V/240 min
HP9	F: GTGTAACCCCATCATCAGCAGA R: TCGAAACATTCTGGTGAACACA	290	64	Position 2232-2522 in coding sequence and 3'flanking region	200 V/240 min
HP10	F: GTTATAGTGAGTGTGTTCACCAGA R: AAGCCATTATCCCTCCCCAA	238	64	Position 2489-2709 in 3'flanking region	200 V/180 min

RESULTS AND DISCUSSION

PCR-SSCP patterns were generated for each breed using primers HP1 to HP10 (Table 2). A total of 32 unique patterns were identified across both breeds. The HSP70-2 gene was 2,720 bp in length. Seven out of the ten primers exhibited breed-specific PCR-SSCP patterns, but only three primers (HP5, HP9, and HP10) clearly differentiated NT from HF breeds. As shown in Table 2 and Figure 1, primer HP5 produced pattern 1 in 100% of NT samples and pattern 2 in 100% of HF samples. Similarly, primer HP9 generated patterns 1-7 in 100% of NT samples and pattern 8 in 100% of HF samples. Primer HP10 yielded pattern 1 in 100% of NT samples and pattern 2 in 100% of HF samples.

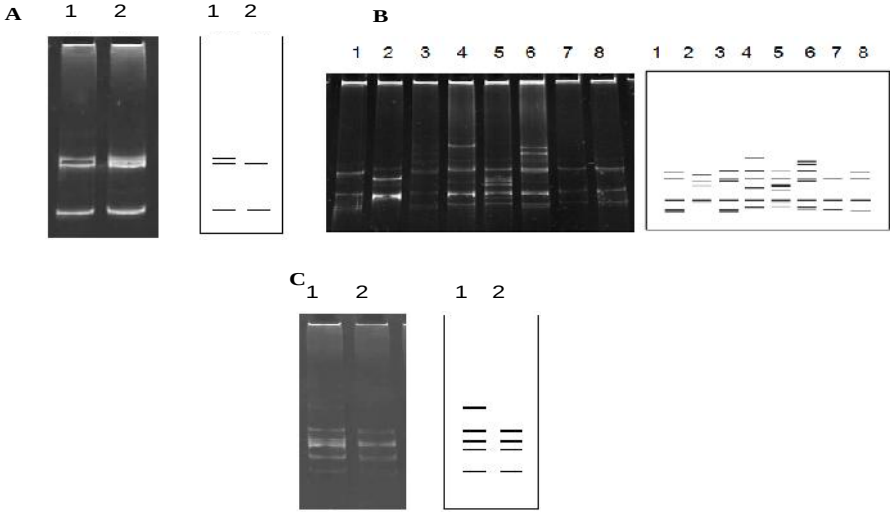

Figure 1. PCR-SSCP pattern of 2 breeds. NT (Thai Native cattle); HF (Holstein Friesian cattle).

A) HP5 primer: 1 (NT), 2 (HF). B) HP9 prime: 1-7(NT), 8(HF).

C) HP10 primer:1 (NT), 2 (HF).

Table 2. PCR-SSCP patterns frequency results of each breed from 10 primers and different nucleotides.

Primer	Breed	Percent of SSCP pattern frequency (head of animals)								P value	Position in *HSP 70-2* (nucleotide seq. different)
		Pattern 1	Pattern 2	Pattern 3	Pattern 4	Pattern 5	Pattern 6	Pattern 7	Pattern 8		
HP1	NT	41.43 (29)	35.71 (25)	20.00 (14)	2.86 (2)	0.00 (0)	0.00 (0)			0.001*	6-523 in 5'flanking region and coding sequence (14 nucleotide different)
	HF	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	63.46 (33)	36.54 (19)				
HP2	NT	68.57 (48)	20.00 (14)	8.57 (6)	2.86(2)					0.001*	461-691 in coding sequence
	HF	0.00 (0)	0.00 (0)	0.00 (0)	100 (52)						
HP3	NT	51.43 (36)	48.57 (34)							0.001*	614-862 in coding sequence
	HF	100.00 (52)	0.00 (0)								
HP4	NT	100.00 (70)								-	795-1066 in coding sequence
	HF	100.00 (52)									
HP5	NT	100.00 (70)	0.00 (0)							0.001*	1038-1291 in coding sequence (1262 NT = M (A ; Thr. aâ/ C; Pro.aâ), HF= G ; Ala. aâ)
	HF	0.00 (0)	100.00 (52)								
HP6	NT	100.00 (70)								-	1239-1885 in coding sequence
	HF	100.00 (52)									
HP7	NT	90.00 (63)	10.00 (7)							0.370	1844-2304 in coding sequence
	HF	84.62 (44)	15.38 (8)								
HP8	NT	17.14 (12)	82.86 (58)	0.00 (0)	0.00 (0)					0.018*	2055-2325 in coding sequence
	HF	0.00 (0)	0.00 (0)	7.69 (4)	92.31 (48)						
HP9	NT	47.14 (33)	12.86 (9)	11.43 (8)	8.57 (6)	2.86 (2)	14.29 (10)	2.86(2)	0.00 (0)	0.001*	2232-2522 in coding sequence and 3'flanking region (2347NT = T, Asp. aâ, HF= C; Asp. aâ (silent substitution)
	HF	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	100.00 (52)		
HP10	NT	100.00 (70)	0.00 (0)							0.001*	2489-2709 in 3'flanking region (position 2515, 2516, 2556)
	HF	0.00 (0)	100.00 (52)								

Nucleotide sequence analysis results

HSP70-2 genes have 3 regions. The 5' flanking region with a size of 423 bp (starting from 1-423 bp), nucleotide sequence analysis was found for Invert CCAAT box (70 to 74 bp, and 153 to 157 bp), Promoter (100 to 136 bp), SP1 binding site or GC box (172 to 177 bp), HSE (185 to 190 bp), and TATA box (192 to 197 bp) at the same position in both breeds. Mutation points found 13 positions as shown in Figure 2. The mutation point revealed that these may affect the heat response differently in the two breeds. The regulated structure controls the transcription factors (TF) and enzymes that activate the transcription process. Mutation of nucleotide base can affect the heat shock factor that will bind with the heat shock element at the starting point of the transcription process. Therefore, different sensitivities and volumes of HSP70-2 mRNA synthesis were derived (Cai *et al*., 2005).

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HF  CTCCTGTTTC CTCCAGCGAA TCCTAGAAGA GTCTGGAGAG TTCTGGGAGG KGAGGCATWC   60
NT                                                          A       C

                                                                 promoter
          Invert CCAAT
HF  AGGGGGRTGA TTGGTTCCCG AAAACCAGGG GGCAGGACTT GAGGCGAAAC CCCTGGAATA  120
NT  ----C-C--- --------A- ---G--T--- ---------- ---------- ----------

          promoter                    Invert  CCAAT                 GC box
HF  TTCCCGACCT GGCAGCCCCA CTGAGCTCGG TCATTGGCTG ACGA-GGGAA AAGGCGGGGC  180
NT  ---------- ---------- ---------- ---------- ----A----- ----------

          HSE      TATA box
HF  TTGATGAAGA ATTATAAACA CAGAGCCGCC TGAGGAGAAA CAGCAGCCTG GAGAGAGCTG  240
NT  ---------- ---------- ---------- ---------- ---------- ----------


HF  ATAAAACTTA CGGCTTAGTC CGTGAGAGCA GCTTCCGCAG ACCCGCTATC TCCAAGGACC  300
NT  ---------- ---A------ ---------- ---------- ---------- ----------


HF  GCCCCGAGGG GCACCAGAGC GTTCAGTTTT CGGGTTCCGA AAAGCCCGAG CTTCTCGTCG  360
NT  ----------A -------C-- T--------- ---------- ---------- ----------

HF  CAGATCCTCT TCACCGATTT CAGGTTTGAA GCTTATTTCG GAGCCGGAAA AGCAGGGCAC  420
NT  ---------- ---------- --- ------ ------C--- ---------- ----------

HF  CGG
NT  ---
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Figure 2. The nucleotide sequence of 5'flanking region of Heat shock protein 70-2 gene. Invert CCAAT, promoter, SP1 binding site/GC box, HSE, TATA box (under line) with sequence positions given above, are shown. The first line shows the HF(Holstein Friesian cattle) sequence, and the second line shows the NT(Thai Native cattle) sequence. Identity with the first line sequence is denoted by a dash, substitution by a different base letter.

The Coding region with a size of 1929 bp starts from 424 to 2353 bp. This region used 8 primers (HP2 to HP9) to amplify the fragment of the gene. The nucleotide sequence is shown to be highly conserved in PCR-SSCP product from HP4 and HP6, which is then transformed to conserve protein (no difference is found in the two breeds with SSCP pattern, though 1 pattern was not found in the mutation point during nucleotide analysis (Silent substitution). On the other hand,

PCR_SSCP products from HP5, HP9 showed significant difference between breeds, but only 2 SNPs clearly separated NT from HF; significant difference of nucleotide was observed at positions 1262 bp in HP 5 primer (NT=A/C; HF=G), and 2347 bp in HP9 primer (NT=T; HF=C) as shown in Figure 3. These two positions affect the translation of protein. It is only one position at 1262 bp (missense mutation) that NT can be translated to Threonine (A) or Proline (C), and HF to Alanine (G). While at position 2347 bp is a silent substitution, the two breeds are transformed to Asparagine. Amino acid performs different protein functions, such as: refolding protein, membrane translocation, degradation of misfolded proteins, and other regulatory processes. Therefore, it has different heat responsibilities for different breeds.

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HF  GGCCAGCCTG GAGATCGACT CCCTGTTCGA GGGCATCGAC TTCTACACGT CCATCACCAG GGCGCGGTTC 1330
HF  ---------- ---------- ---------- ---------- ---------- ---------- ----------
NT  -M-------- ---------- ---------- ---------- ---------- ---------- ----------
NT  -M-------- ---------- ---------- ---------- ---------- ---------- ----------

HF  GGTGGACTAG GGGCCTTACT TTTTGTCTGT CTGTAGTAGA CCTATGGACT TTGGTCTTGC CCTATATTTA 2410
HF  ---------- ---------- ---------- ---------- ---------- ---------- ----------
NT  ------T--- ---------- ---------- ---------- ---------- ---------- ----------
NT  ------T--- ---------- ---------- ---------- ---------- ---------- ----------
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Figure 3. Multiple sequence alignment in positions 1261 to 1330, and positions 2341 to 2410 of *HSP70-2* of HF 2 and NT 2 samples. Breed abbreviations are as follows, HF (Holstein Friesian cattle), NT (Thai Native cattle). Identity with the first sequence is denoted by a dash, substitution by a different base letter at 1262 bp (NT=M (A/C, HF=G;), and 2347 bp (NT=T, HF=C).

The 3' flanking region has a size of 416 bp starting from 2354 to 2770 bp. This region used two primers (HP9 and HP10) to amplify the fragment of the gene. The different points between the two breeds were seen at three positions: at 2515 bp (NT=G/T; HF=C), 2516 bp (NT=C/T; HF=G), and 2557 bp (NT=G; HF=T) in HP10 primers as shown in Figure 4. The different positions may affect the post-transcription of HSP70-2, and the complete translocation process of mRNA. This can be found in the report of Adamowicz et al. (2005) who reported new SNPs in 3'-UTR regions of HSP70-1 gene of *B. taurus* and *B. indicus* found to be inserted in G and C nucleotide or substituted for U/A in some positions that affected the complete translocation process of mRNA but did not affect the quantity of HSP70 mRNA synthesis.

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HF  TCTCTAACTA GCTCGATTTT TGTTATTTCT GTATGTTATA GTGAGTGTGT TCACCAGAAT GTTTCGATTT 2520
HF  ---------- ---------- ---------- ---------- ---------- ---------- ----------
NT  ---------- ---------- ---------- ---------- ---------- ---------- ----KY----
NT  ---------- ---------- ---------- ---------- ---------- ---------- ----KY----

HF  TCATGCAAGT TGGTAATAAG GATGGCTTTC CGTGGGTTTT TTTTTGTTTG TTTCAAGTGA GTCAACACTG 2590
HF  ---------- ---------- ---------- ---------- ---------- ---------- ----------
NT  ---------- ---------- ---------- ------G--- ---------- ---------- ----------
NT  ---------- ---------- ---------- ------G--- ---------- ---------- ----------
```

Figure 4. Multiple sequence alignment in positions 2451 to 2590 of HSP70-2 gene for HF 2 and NT 2 samples. Breed abbreviations are as follows, HF (Holstein Friesian cattle); NT (Thai Native cattle). Identity with the first sequence is denoted by a dash, substitution by a different base letter at 2515, 2516 bp (HF=CG; NT=K(G/T) Y(C/T), and 2557 bp (HF=T; NT=G).

In order to confirm the homology of HSP 70-2 gene between NT and HF in this study (122 animals), the nucleotide sequence of template *B. taurus* HSP70-2 gene (GenBank accession number BTU02892) was determined by comparison of the similarity analysis. The results showed that there was 96 to 98% similarity. In addition, HF nucleotide sequence at positions 1262, 2347, 2515, 2516 and 2557 bp were not different from the template. These results indicated that these positions can be used in separating HF from NT.

CONCLUSION

The results of this study indicate that HSP70-2 gene between tropical and temperate cattle have nucleotide sequence polymorphisms in 3 regions of the gene. However, different nucleotide sequences between tropical and temperate breed cattle have clear mutation points that can separate tropical breeds from temperate breeds. Their positions are at 1262 (NT=A/C; HF=G), 2347 (NT=T; HF=C), 2515 (NT=G/T; HF=C), 2516 (NT=C/T; HF=G), and 2557 bp (NT=G; HF=T) with a total length of 2770 base pairs. The differences observed in their positions may be related to the gene that makes the tropical breed cattle to have more physiological tolerance to heat than the temperate breed cattle.

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