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Research Article
Polymorphism of Duck HSP70 Gene and mRNA Expression under Heat Stress Conditions

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Abstract

Background and Objective: Tropical ambient temperature conditions that exceed comfortable temperatures for ducks and changes in maintenance systems from traditional to intensive conditions under low confined water cause ducks to have thermoregulation difficulties, causing the ducks to experience heat stress. The heat shock protein 70 (HSP70) gene, a known marker gene of heat resistance, can be a gene candidate for the selection of heat-resistant traits in local ducks to cope with heat stress. The objective of this study was to identify polymorphisms and the mRNA expression of the HSP70 gene under heat stress conditions. **Materials and Methods:** Genotyping was performed on 110 local duck breeds from West Sumatra (Pitalah, Bayang, Kamang and Payakumbuh). Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) and Sanger sequencing assays were used to identify polymorphisms. Polymorphism analysis was conducted with the MEGA 7 software. mRNA expression was identified in 12 liver tissue samples from 12 local ducks (3 birds of each local duck breed) under control conditions or heat stress treatment performed at 35°C for 1 or 2 h. **Results:** The amplification product was 466-bp. HSP70/HhaI was polymorphic in all the breeds and two SNPs (Single nucleotide Polymorphism), g.1696G >A and g. 1762C >T, were recognized in all the HSP70/HhaI loci. The g.1696g >A locus produced two alleles (A and G) with three genotypes (AA, AG and GG) and the g. 1762C >T locus also produced two alleles (T and C) with three genotypes (TT, TC and CC). The analysis showed that the G allele had a higher frequency than that of the A allele and the T allele had a higher frequency than that of the allele C in all breeds. χ^2 analysis showed that all the local duck breeds fit the Hardy-Weinberg equilibrium. The relative expression of the HSP70 gene mRNA in the liver and ovary tissues showed that heat stress had a significant effect ($p<0.05$) on the local ducks in the same breed. After 1 h of heat treatment, liver tissues from the Payakumbuh ducks showed the highest HSP70 mRNA expression, while after 2 h of treatment, the highest HSP70 mRNA expression was observed in the Bayang ducks. After the heat treatment of the ovarian tissues for 1 and 2 h, the Bayang ducks showed the highest HSP70 mRNA expression. **Conclusion:** HSP70 gene polymorphism and mRNA expression can be used as a candidate marker to increase heat resistance in ducks.

Key words: Heat resistance, heat stress, HSP70, local ducks, mRNA expressions, polymorphism

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The efforts of local duck businesses are focused on developing and improving efficiency to overcome current constraints, including lower optimal production and reproduction, which impact the quality and supply of local day-old duck; additionally, the current needs in terms of sufficient quantities have not been fulfilled. One of the factors that has been identified as the cause of less optimal local duck production and reproduction is their physiological conditions. The temperature conditions in tropical regions exceed comfortable temperatures for ducks and changes in maintenance systems from traditional to intensive conditions under minimal water confinement cause ducks to have thermoregulation difficulties, leading to ducks experiencing stress/heat stress.

As waterfowl, ducks have different physiologies from other birds and are more susceptible to heat stress[1]. According to Noor and Seminar[2], if stress continues and the body is unable to overcome it, genetic pathways will be pursued by activating the HSP gene that functions only under stressful conditions. The HSP70 gene, which has an important role in cell biology and biochemistry as a protective protein, is encoded by members of a *multigene* family that play a role in responding to heat stress[3].

Furthermore, Etches *et al.*[4] explained that the HSP70 gene aims to protect proteins that are sensitive to high temperatures from degradation and prevent permanent damage to proteins and cells that can further affect survival. Poultry exhibit HSP70 expression at a temperatures of 29.3°C and can reach a mRNA copy number of $86.54\pm0.15\times10^7$, although if the ambient temperature increases, HSP70 expression will continue to increase[5]. The HSP70 gene in ducks is 2543 bp and contains 15 *Single nucleotide polymorphisms* (SNPs), among which 12 are transitional and 3 transverse, leading to an SNP polymorphism frequency of 1 per 127 bp. All of the SNPs elicit *silent mutations* because they do not change the amino acids produced. The identified SNPs are polymorphic and can be used as markers for heat resistance in ducks. HSP70 mRNA expression is expressed in almost all tissues analyzed, though ovarian tissue and the liver display the highest expression after chest muscle and the hypothalamus[6].

Since its discovery, scientists have been actively conducting research on the HSP70 gene, though most of the research has been focused on mammals and chickens. Research focused on using the HSP70 gene as a marker for heat resistance in ducks has not been widely performed. Therefore, this study aimed to determine the polymorphisms and mRNA expression of HSP70 genes in Indonesian local ducks.

MATERIALS AND METHODS

Experimental animals, blood samples and DNA isolation: This study used 110 ducks from West Sumatra (29 Pitalah birds, 24 Bayang birds, 29 Kamang birds and 31 Payakumbuh birds). All ducks are maintained in accordance with the principles of animal welfare and under the same management process conditions. As much as 1 mL of blood from each duck was taken through the *brachial vein* and placed into a tube vacuum containing 3 mL of EDTA.

DNA isolation was carried out based on a modified procedure according to Sambrook and Russel[7] whereby 20 µL of duck blood was placed in an Eppendorf tube (1.5 mL) with 1000 µL of 0.2% NaCl. Following incubation for 5 min, the sample was centrifuged at 8,000 rpm for 5 min and then the supernatant was removed. After the supernatant was removed, 10 µL of 5 mg mL^{-1} proteinase K, 40 µL of 10% sodium dodecyl sulfate (SDS) and 350 µL1×STE (sodium tris EDTA) were added and the mixture was gently shaken in an incubator at 55°C for 2 h. Next, 400 µL phenol, 400 µL CIAA (Chloroform: Isoamyl Alcohol = 24:1) and 40 µL 5 M NaCl were added to the mixture and it was slowly shaken for 1 h at room temperature. The mixture was then centrifuged at 12,000 rpm for 5 min. The, 400 µL of the clear portion (DNA) was transferred to a new tube (1.5 mL) using a pipette. Tubes that already contained DNA (clear part) were supplemented with 800 µL of absolute ethanol and 40 µL of 5 M NaCl and then stored in the freezer overnight. The next day, the solution was centrifuged at 12,000 rpm for 5 min, the supernatant was removed and then maintained in the open at room temperature until the ethanol evaporated. Next, 100 mL 80% TE was added to the tube. The obtained DNA was then stored in the *freezer* until its use.

HSP70 gene amplification and genotyping: A pair of primers (Table 1) was designed to identify the g.1696G>A and g.1762T>C polymorphisms in the HSP70 gene; these loci were previously reported by Xia *et al.*[6] and have the GenBank access code EU678246.2. The primers were designed using the primary designing tool[8]; the primers produce a 466 bp amplification product (Fig. 1). PCR was performed in a 15 µL volume (0.5 µL DNA, 8 µL *green master mix*, 0.2 µL HSP70 forward primer and 0.2 µL HSP70 *reverse* primer and 6.1 µL destruction water) and amplification was carried out using the GeneAmp® PCR 9700 System (GeneAmp PCR System 9700, AB Applied Biosystem, Singapore). The PCR cycling conditions

Fig. 1: The primers (forward and reverse) anneal to the bolded and underlined nucleotides
Red nucleotides show the SNP positions (*GeneBank* accession number EU678246.2)

Table 1: Primers for HSP70 DNA amplification.

Gene		Primer 5'-3'	Annealing temperature	Primer position	Product
HSP70	F	GGGAGACAAGTCCGAGAACG	60°C	1438-1903	466 bp
	R	CACCCGATCTCTGTTGGCTT			

F: Forward, R: Reverse, bp: Base pair

were as follows: (1) An initial denaturation at 95°C for five minutes, (2) 35 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec and (3) A final elongation at 72°C for five minutes. The results of DNA amplification were visualized by 1.5% agarose gel electrophoresis.

The PCR-RFLP technique was used to determine HSP70 gene SNPs and the HhaI restriction enzyme was used to identify the loci. PCR-RFLP products were visualized by 2% agarose gel electrophoresis using a UV Transilluminator (Alpha Imager, Alpha Innotech, Santa Clara, USA). Twelve PCR product results were sequenced using the Genetika Science services to verify the mutations.

Tissue samples, heat stress treatment and total RNA extraction: HSP70 gene expression analysis was performed using liver and ovary tissue samples from 12 selected local ducks including the Pitalah, Bayang, Kamang and Payakumbuh breeds with 3 birds from each breed. The treatments in this study were heat stress at 35°C for 0 h (control/without heat stress), 1 and 2 h. Ducks were placed in a simple modified environmental chamber with a wall made of mica and the front consisting of a 35×35×70 cm piece of glass. This simple chamber was equipped with a heater, blower, digital thermometer and thermostat. After the heat stress treatment, the duck was slaughtered to harvest liver and ovarian tissues using sterile equipment. The tissues were then placed in a tube containing RNA later and stored at -22°C, after which total RNA analysis was carried out.

RNA isolation from tissue (liver and ovary) was carried out using the RNeasy Mini Kit reagent (Qiagen, Germany). A total of 30 mg of sample tissue was placed into 300 µL of Lysis Buffer containing β-mercaptoethanol and then the sample was smoothed with a micro*pestle*. Next, 600 µL of Proteinase K was added (10 µL of Proteinase K diluted in 300 µL TE Buffer), vortexed, incubated for 10 min at room temperature and then centrifuged for 5 min at 12000 rpm. The sample supernatant was then transferred to a new tube that was supplemented with 450 µL ethanol and mixed by pipetting. The solution was then transferred to a column tube and centrifuged at 12000 rpm for 1 min. The solution was removed and 700 µL of wash buffer 1 (containing ethanol) was added to the column, which was then centrifuged at 12000 rpm for 1 min. Next, the solution was removed and 600 µL of washing buffer 2 (containing ethanol) was added to the column, which was then centrifuged again at 12000 rpm for 1 min. After the solution was removed, a second wash with 250 µL of washing buffer 2 was performed though with centrifugation for 2 min, after which the column was transferred to a 1.5 µL tube. After adding 100 µL of nuclease free water, the sample was centrifuged at 12000 rpm for 1 minute. The obtained RNA pellet (template) was stored at -20°C until use.

Reverse transcription, HSP70 gene primers and quantitative real time-PCR (qRT-PCR): RNA was transcribed into a complementary DNA (cDNA) using the Rever Tra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo Co., LTD, Japan). The reaction volume consisted of 2 µL of RNA template, 1 µL oligo (dT) and 9 µL of water. The solution was incubated at 65°C for 5 min. Next, 4 µLof 5×RB (buffer), 1 µL of riboblock, 2 µL dNTPs and 1 µL reverse transcriptase were added. The solution was then further incubated using PCR (GeneAmp PCR System 9700, AB Applied Biosystem, Singapore) at 42°C for 5 min and 78°C for 5 min. The complementary DNA was quantified (absorbance at 260:280 nm, i.e., 1.91-2.03) using a spectrophotometer (Agilent 8453, USA). The primers used to amplify the HSP70 mRNA in this study are presented in Table 2.

The complementary DNA was used to quantify the expression of the HSP70 gene using real-time PCR (qRT-PCR)

(Analytic Jena, AG q Tower 4 channels, Germany). The 10 µL real time PCR reaction containing SYBR qPCR Mix THUNDERBIRD (Toyobo Co., Ltd., Japan) consisted of 5 µL of master mix; 0.25 µL each of forward and reverse primer (10 pmol); 1 µLof sample cDNA and 3.5 µL of nuclease-free water. The PCR cycling conditions were as follows: 95°C for 5 min followed by 39 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec.

qRT-PCR uses a fluorescent reporter molecule to monitor the production of amplification products in the PCR cycle. The qRT-PCR reaction determines the cycle threshold (CT) value, which is the cycle when the fluorescent dye emission intensity exceeds the threshold value. The higher the initial copy number of the nucleic acid target is, the faster the increase in fluorescence, which yields a lower CT value[9].

Data analysis: The allele and genotype frequencies values and Hardy-Weinberg equilibrium were calculated per Nei and Kumar[10], while the observation heterozygosity (Ho) and expectation heterozygosity (He) were calculated per Weir[11]. The SNP and sequencing results were determined using Molecular Evolution Genetics Analysis (MEGA 7). HSP70 gene expression was calculated by comparing the relative target gene copy number (HSP70) with the control gene copy number (β-actin) using the CT (ΔCT) comparison method. Expression between target genes and control genes can be compared with the $2^{-\Delta CT}$ equation, with delta CT (ΔCT) = CT target gene-CT gene control (housekeeping gene)[12].

RESULTS AND DISCUSSION

Polymorphism of HSP70 gene: Based on the HSP70 gene PCR results from 110 samples separated on a 1.5% agarose gel with a 100 bp ladder, the amplified 466-bp DNA band was obtained (Fig. 2). SNP determination via the PCR-RFLP method used the HhaI restriction enzyme (GCG |C); the results showed the presence of 2 SNPs g.1696G>A and g.1762T>C. At the g.1696G>A locus, there are two alleles (A and G) with 3 genotypes (AA, AG and GG), while the g.1762T>C locus also has 2 alleles (T and C) with 3 genotypes (TT, TC and CC). The following is the combination of genotypes with their respective lengths: AA/TT genotype (466 bp), GG/TT (259 and 207 bp), AG/TT (466, 259 and 207 bp), AA/CC (325 and 141 bp), GG/CC (259, 141 and 66 bp), AG/CC (325, 259, 141 and 66 bp), AA/TC (466, 325 and 141 bp), GG/TC (259, 207, 141 and 66 bp) and AG/TC (466, 325, 259, 207, 141 and 66 bp) (Fig. 3). The two SNPs obtained are the same as those obtained in the study by Xia *et al.*[6], which identified HSP70 gene SNPs in *Shansui white* commercial duck (Fig. 6).

The G locus allele g.1696G>A HSP70/HhaI gene in the four local ducks from West Sumatra dominate the allele frequency. The value of the G allele frequency from the four duck breeds ranged from 0.76-0.90, while the allele A frequency value ranged from 0.10-0.24. The GG genotype dominates the genotype frequency at this locus. The GG genotype frequency values for the four local duck breeds ranged from 0.59-0.81. The frequency of the AG genotype is in the lower range from 0.19-0.41. The lowest frequency genotype is AA, which had a genotype frequency of 0.10. Bayang, Kamang and Payakumbuh ducks did not show the AA genotype. The χ^2 value indicated that the genotype frequency and locus allele are in Hardy-Weinberg equilibrium (Table 3).

For the HSP70/HhaI gene g.1762T>C locus, the T allele dominated the allele frequency in all four local duck breeds. The T allele frequency in the four duck breeds ranged from

Table 2: Primers for HSP70 mRNA amplification.

Gene		Primer 5'-3'	Annealing temperature	Product
HSP70	F	CAATGCCGACCTCTTCCGT	60°C	158 bp
	R	CTTTGCCATTGAAGAAGTCCTGTAG		
β-actin	F	TGGACTCTGGTGATGGTGTTA	60°C	174 bp
	R	CACGCACAATTTCTCTCTCGG		

F: Forward, R: Reverse, bp: Base pair

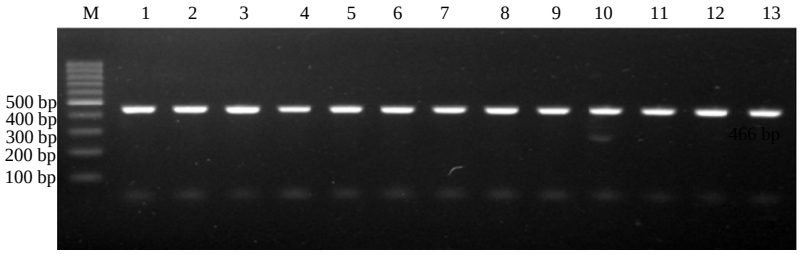

Fig. 2: Amplification of HSP70 gene in a 1.5% Agarose Gel. Lines 1-13: Samples, M: 100 bp DNA ladder

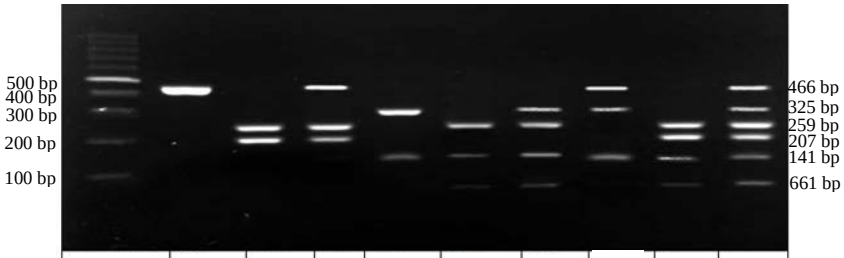

Fig. 3: Identification of the g. 1696G >A and G. 1762T >C polymorphism loci in HSP70/HhaI via PCR-RFLP
AA, AG, GG, TT, CT, CC: Genotypes

Table 3: The genotype and allele frequencies for the g.1696G>A HSP70/HhaI locus in local duck were in Hardy-Weinberg equilibrium

		Allele frequency		Genotype frequency			
Duck breed	N	G	A	GG	AG	AA	χ^2
Pitalah	29	0.76	0.24	0.62	0.28	0.10	1.77^{ns}
Bayang	24	0.90	0.10	0.81	0.19	-	0.23^{ns}
Kamang	26	0.80	0.20	0.59	0.41	-	1.45^{ns}
Payakumbuh	31	0.82	0.18	0.65	0.35	-	1.44^{ns}

N: No. of samples, ns: Non significant, $\chi^2_{(0.05, 1)}$: 3.84

Table 4: The genotype and allele frequency for the g.1762T>C HSP70/HhaI locus in local duck were in Hardy-Weinberg equilibrium

		Allele frequency		Genotype frequency			
Duck breed	N	T	C	TT	CT	CC	χ^2
Pitalah	29	0.62	0.38	0.41	0.41	0.17	0.43^{ns}
Bayang	24	0.69	0.31	0.43	0.52	0.05	1.07^{ns}
Kamang	26	0.57	0.43	0.32	0.50	0.18	0.01^{ns}
Payakumbuh	31	0.53	0.47	0.26	0.55	0.10	0.32^{ns}

N: No. of samples, ns: Non significant, $\chi^2_{(0.05, 1)}$: 3.84

0.53-0.69, while the allele C frequency value ranged from 0.31-0.47. The CT genotype dominated the genotype frequency, as the CT genotype frequency in the four local duck breeds ranged from 0.41-0.55. The frequency of the TT genotype is in the lower range from 0.26-0.43. The lowest frequency among the genotypes was for the CC genotype, which ranged from 0.05-0.19. The χ^2 value indicates that the genotype frequency and allele locus are in Hardy-Weinberg equilibrium (Table 4).

The allele frequencies of the two HSP70 SNP loci are polymorphic; this outcome is in accordance with Nei and Kumar[10]. If there are two or more alleles in breeds with relative frequency values that are greater than 0.01 (1%), they are called polymorphic. The allele frequencies and SNP genotypes in this study were higher for the polymorphisms when compared with the results obtained by Xia *et al.*[6]. The χ^2 genotype frequency and allele values of all the loci in this gene showed that they are in Hardy-Weinberg equilibrium. Vasconcellos *et al.*[13] stated that a breed is in Hardy-Weinberg equilibrium if the genotype and allele frequencies are constant from generation to generation. Genotype equilibrium in a fairly large breed occurs if there is no selection, mutation, migration or genetic drift. Conversely, if there is an accumulation of genotypes, divided breeds, mutations, selection, migration and mating in the same group, this equilibrium can cause an imbalance in the genotype or allele frequencies in the breed[14,15].

Table 5: Heterozygosity of the HSP70/HhaI locus in local duck

	g.1696G>A		g.1762C>T	
Duck breed	Ho	He	Ho	He
Pitalah	0.28	0.37	0.41	0.47
Bayang	0.19	0.17	0.52	0.43
Kamang	0.41	0.33	0.50	0.49
Payakumbuh	0.35	0.29	0.55	0.50

Ho: Observed heterozygosity, He: Expected heterozygosity

Table 5 shows that the observed heterozygosity for the HSP70/HhaI gene at position g.1696G>A in each local duck breed ranged from 0.19-0.41, while the value of the expected heterozygosity ranged from 0.17-0.37. The observation heterozygosity value of the HSP70/HhaI gene at g.1762T>C in

each of the local duck breeds ranged from 0.41-0.55, while the value of the expected heterozygosity ranged from 0.43-0.50. Tambasco *et al.*[16] stated that if the observed heterozygosity value (Ho) is smaller than the expected heterozygosity (He), intensive endogamy/inbreeding has occurred in the cluster intended for selection purposes.

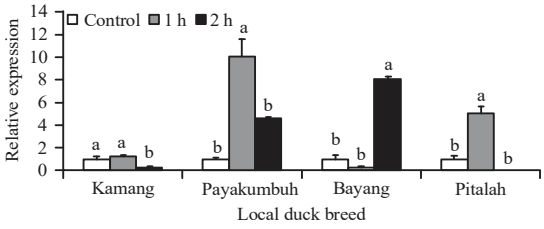

Fig. 4: HSP70 mRNA expression in liver tissues from local ducks

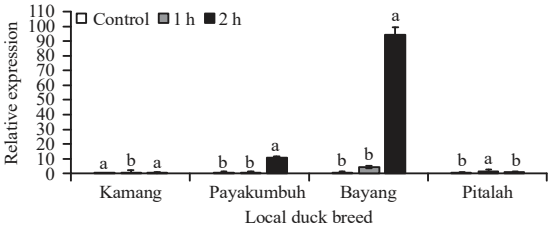

Fig. 5: HSP70 mRNA expression in liver tissues from local ducks

Relative mRNA expression of HSP70 gene: Analysis of the HSP70 gene mRNA expression experiments showed that the HSP70 gene was expressed in the liver and ovary tissue and that heat stress treatment had a significant effect ($p<0.05$) on the relative mRNA expression in the same duck breed. Payakumbuh duck liver tissue showed the highest HSP70 expression after heat stress treatment for 1 h, whereas Bayang duck liver tissue showed the highest expression after heat stress treatment for 2 h (Fig. 4). According to King *et al.*[17], liver tissue is one of the most sensitive tissues expressing HSP70 in response to heat stress.

For ovarian tissue, Bayang ducks showed the highest expression after heat stress treatment for either 1 or 2 h. This proved that the relative HSP70 mRNA expression was higher in the ovary than in liver tissue in laying-type ducks (Fig. 5). Based on research by Xia *et al.*[6], HSP70 gene mRNA is

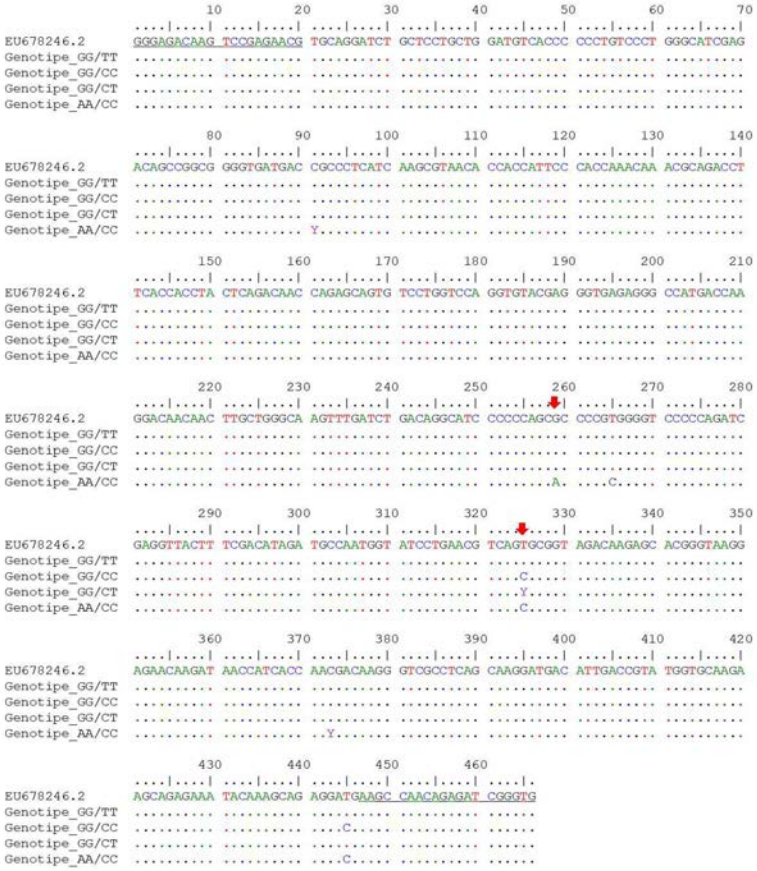

Fig. 6: Partial sequence of the duck HSP70 gene

The underlined sequence shows where the forward and reverse primers anneal. The arrows show HSP70/HhaI SNP target (GenBank accession No.: EU678246.2)

expressed in almost all tissues, with ovarian and liver tissues showing the highest expression after chest muscle and the hypothalamus. Heat stress of ducks affects HSP70 gene expression[18]. A greater increase in gene expression (*upregulated*) indicates more individual resistance to heat and other stresses[19,20].

CONCLUSION

The SNPs of HSP70/HhaI gene were polymorphic in West Sumatera, Indonesian local ducks. The high mRNA expression of HSP70 gene in Bayang ducks showed that these ducks have good heat resistance under heat stress conditions.

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