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In silico primer design and CpG island detection in HSP90 gene chicken (*Gallus gallus*)

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ABSTRACT. The HSP90 gene can be used as a marker of heat stress in chickens. The isoforms of the chicken HSP90 gene are HSP90 alpha (HSP90AA1 and HP90AB1) and HSP90 beta (HSP90B1). Each of these isoforms alpha and beta, possesses distinct features and gene polymorphism. This study aims to detect CpG islands using Primer3, Netprimer, and MethPrimer and primers designed *in silico*. About 24 primer candidates were identified in the HSP90AA1 (9), HSP90AB1 (10) and HSP90B1 (5) genes that can be used to detect polymorphisms that can be used as markers of heat stress in the HSP90 gene chicken. Detection of potential CpG islands was performed in the promoter region and the first exon. This was related to the methylation that affected gene expression in the promoter region. There was only one CpG island in the HSP90AA1 gene and two in the HSP90AB1 and HSP90B1 genes, as determined by the detection of CpG islands. The outcomes of this study can be applied to study on heat stress databases in Indonesian local chickens.

Keywords: CpG island; heat stress database; HSP90 chicken; *in silico*; primer design

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INTRODUCTION

Recently, climate change resulting from global warming has becoming increasingly significant, particularly for the poultry farming business. As temperatures continue to rise as a result of global warming, the detrimental effects of heat exhaustion (temperature exceeds thermo-neutral zone and animals are unable to control body temperature) in animal husbandry will become increasingly difficult to manage (Abd El-Hack *et al*., 2020; Ahmad *et al*., 2022). The chicken thermoneutral zone is typically between 18 and 25 °C. It is adequate to keep the normal body temperature of chicken at 41-42°C within this temperature range (Sohail *et al*., 2012).

In response to heat stress, the body tries to restore homeostasis. The body activates the Heat Shock Protein (HSP) gene to protect heat-sensitive proteins (Khalil *et al*., 2011; Calderwood *et al*., 2012). Heat Shock Protein 90 is a protein chaperone member with a size of 90 kilodaltons. Numerous isoforms of HSP90 exist, and HSP90 is divided into two classes: alpha isoform and beta isoform. There are structural differences that allow these isoforms to perform their functions effectively in different parts of the body, despite having the same function (Calderwood & Gong, 2016). At the molecular level, heat stress causes alterations in expression of genes involved in direct protection from high temperature stress. In high-temperature conditions, the modification of physiological and biochemical processes by changes in gene expression leads to the gradual development of heat tolerance in the form of acclimation or, preferably, adaptation. These processes could generate genetic polymorphisms. Genetic polymorphism is a variation in the DNA sequence (Ismail & Essawi, 2012; Hasanuzzaman *et al*., 2013).

A correlation between polymorphisms in the heat shock protein gene and heat tolerance has been observed. Research on Kampung Unggul Balitbangtan and Walik chickens with four distinct HSP70 gene haplotypes (H1', H1, H2, and H3), to determine the physiological response of acute heat stress. Based on rectal temperature measurements, the distribution of body surface temperature (head, neck, body, and leg area), hormone levels, and time of panting, the data was analyzed. Using infrared thermography, the body's surface temperature is measured. Based on rectal temperature, the results indicated that the haplotype H1 Walik chicken is more susceptible to heat stress. In contrast, the legs of H1' haplotype Walik chickens released more heat through their body surface (Aryani *et al*., 2021).

Research on Fowl Hyline Brown and Brown dominant chickens revealed that the polymorphism in the HSP90 alpha gene, specifically the single nucleotide polymorphism (SNP) detected from the HSP90AA1 gene, was associated with the observed traits, such as rectal temperature and respiratory rate (Irivboje *et al*., 2019). Local Chinese chickens, Lingshan and White Recessive Rock (WRR) chickens, also revealed that SNPs in the promoter region of the HSP90 beta gene affected heat tolerance (Chen *et al*., 2013). In Huainan chickens also originating from China, finding an SNP detected in HSP90B1 showed its relationship with heat tolerance traits (Wan *et al*., 2017). In addition to variations from single bases or SNPs, there is DNA methylation found on CpG island (Zhou *et al*., 2015; Shyamala *et al*., 2022). Previous research found that 3,133 CpG Islands were identified from 20 tissue data in one chicken sample that could affect gene expression. DNA methylation correlates with gene expression in normal adult tissues (Lim *et al*., 2019). No one has yet disclosed the chicken HSP90 gene's characterization. This study is conduct by designing numerous primers that can be used to amplify the HSP90 gene in silico, so that it can be used as a database of heat stress markers in local Indonesian chickens.

MATERIALS AND METHODS

Data collection and analysis of polymorphism. HSP90 gene sequence data downloaded from the Ensembl online database (https://www.ensembl.org) in FASTA format by searching each isoform (HSP90AA, HSP90AB, HSP90B1) in the search toolbar, and then select restricting category and species to gene and chicken (*Gallus gallus*). Primer was designed using Primer3 automated method version 4.1.0 (http://bioinfo.ut.ee/primer3-0.4.0/). All primer pairs that were designed, then evaluated for their quality and secondary structure in order to identify prime candidates. Analysis was performed using the NetPrimer page (http://www.premierbiosoft.com/NetPrimer/Analyze Primer.jsp).

Identification of CpG island in the promoter region and exon of the HSP90 gene. MethPrimer was used to detect a potential CpG island in the chicken HSP90 gene (http://www.urogene.org/methprimer). MethPrimer operates by taking DNA sequences as input and searching for potential CpG island sequences. The CG sites obtained are then visualized and analyzed to determine if they are associated with transcription factors present in the promoter region (Li & Dahiya, 2002).

RESULTS AND DISCUSSION

HSP90 gene structure in chicken (*Gallus gallus*). The isoform of the HSP90 gene differ in length, quantity of exons, and quantity of promoter components from the isoform. The first isoform is HSP90AA1 gene, which is located on chromosome 5 and has eleven exons in chickens. Based on ENSGALT00000081765.3 on the Ensembl page (https://www.ensembl.org). The HSP90AA1 gene has a CDS length of 2187 base pairs and encodes 728 amino acids. The promoter region of the HSP90AA1 gene contains one TATA box, one CCAAT box, two Heat Shock Elements (HSE), and six putative SP1 binding sites. Similar to the unique intron of the Drosophila HSP83 gene, the first intron of the HSP90AA1 gene is located just before the ATG translation initiation codon and is -1.3 Kb in length (Vourc'h *et al*., 1989).

The second isoform of the HSP90 alpha class is HSP90AB1, located on chromosome 3. It has a CDS length of 2644 bp and codes for 725 amino acids. The HSP90AB1 gene sequence in chicken, has twelve exons and eleven introns. Similar to other HSP90 genes, the first intron is located just before the ATG initiation codon. The complete sequence of the chicken HSP90AB1 gene is available on the Ensembl page (ENSGALT00000016542.4). The promoter is the main distinction between the HSP90AA1 and HSP90AB1 genes. One CAAT box and one HSE element are located on the HSP90AB1 long before the TATA box, which is approximately -3 and -2 kb away. On HSP90AB1 there are also fewer SP1 sites than on HSP90AA1. Each of these features may explain why chicken

HSP90AB1 mRNA is generally lower than that of HSP90AA1, and cannot be induced by heat shock or serum/growth factor simulation (Meng *et al*., 1995).

The HSP90 beta or HSP90B1 gene in chickens is located on chromosome 1, has a CDS length of 3021 bp and codes for 795 amino acids. The HSP90B1 gene sequence has eighteen exons. The complete sequence of the chicken HSP90B1 gene is available on the Ensembl (ENSGALT00000081140.2). HSP90B1 has a length of 5' flanking region which is 600 bp. The HSP90B1 gene promoter consists of two CAAT boxes and one putative SP1 site.

Japanese quail (*Coturnix japonica* or Cj) and duck (*Anas platyrhynchos*) studied with regard to the structure and characteristics of the HSP90 gene and its isoforms (Nagahori *et al*., 2010; De-Qian *et al*., 2013). Quail have 94% similarity with chicken in the gene coding region CjHSP90AA1, CjHSP90AB1, and CjHSP90B1. In the 5′ flanking region of CjHSP90A, which contains the promoter and other elements, the putative TATA box sequence and eleven putative SP1/GC box binding sites are found. Similar to the HSE observed in HSP90AA1 in chickens, the CjHSP90AA1 promoter was also found two blocks of HSEs. The 5 flanking region of the CjHSP90AB1 gene contains a TATA box as well as three putative SP1/GC box sites. The 5′ flanking region of the CjHSP90B1 gene contains only one TATA box (Nagahori *et al*., 2010). Comparing the amino acid sequence of HSP90 from the liver of the Shaoxing duck (*Anas platyrhynchos*) to that of chicken revealed that the Shaoxing duck shared 92.6% to 99% homology with chicken (De-Qian *et al*., 2013).

Table 1. Primer design of the HSP90AA1 and SNP

Primer	Primer sequence	Target DNA (bp)	SNP
HSP90AA_1	F5'-CACACAGTTCTGAGAGAAGACG-3' R5'-CATACCGCCACCACACAGAG-3'	780	c.-59G>T dan c.-55A>G
HSP90AA_3	F5'-TTCGACTGTGCAGCAAGCTA-3' R5'-TGCTCGGGTCAGTCAAACTC-3'	353	c. 6G>A; c.93G>T; dan c.144C>T
HSP90AA_5	F5'-ATTGTCACGGCTTGGTTTGC-3' R5'-TGATGCTACTGGCTTGGCTC-3'	394	c.651T>C
HSP90AA_6	F5'-GCAAAAACTGGGCATTGTGC-3' R5'-GCTGCAGCCCAAGAATCTCT-3'	599	c.675T>C; c.708G>A; c.798G>A; c.723 gaa>gAaa; c.851A>G; dan c.861C>A
HSP90AA_7	F5'-AAAGGGGCCATGGGTAAGTG-3' R5'-TCCTCAGAGTCTACGACACCT-3'	591	c.1035T>C; c.1049 Aac>ac; c.1077C>G; dan c.1128A>G
HSP90AA_8	F5'-CTGCCTGGCAATTCAGAAGC-3' R5'-TGTGGGGTGCTCAGTATGTG-3'	367	c.1308G>A dan c.1320C>T
HSP90AA_10	F5'-ACAGTGCTGAGGCTCAAACA-3' R5'-GTCTGGTCAAGGGGCAACTT-3'	350	c.1569A>G; c.1608C>T; c.1653A>G; c.1704C>A; dan c.1736T>A
HSP90AA_11	F5'-AAGTTGCCCCTTGACCAGAC-3' R5'-ATCAACACACGCAGAGCTGA-3'	474	c.1797A>T; c.1807A>G; c.1833G>A; c.1857C>T; c.1876G>A; dan c.1916T>A
HSP90AA_12	F5'-TGGTCCTTT TTGCTTCTTGCG-3' R5'-TCC ACCTTTCAAGTGCTGGAG-3'	994	c.2127C>T; c.2135T>G; c.2153A>G; c.2169T>C; c.*80C>A; c.*193C>G; c.*297T>G; c.*303A>G; c.*336C>T; c.*350G>A; c.*645T>C; c.*656G>A; c.*671C>A; c.*710T>C; c.*719C>T; c.*723A>G; c.*731C>G, c.*732G>A; c.*762G>T; c.*765C>T; c.*81 caa>cAaa; c.*89 aac>aaAc; c.*758_*759dup

Notes: F = Forward, R= reverse

Primer candidate for HSP90 gene amplification in chicken (*Gallus gallus***).** The upstream 5' region and the exon of the HSP90 gene containing polymorphisms are used as a benchmark for primer design. Gene polymorphisms obtained from Ensembl data indicate the mutation's position, amino acid changes, and mutation type. When designing the primary HSP90 gene in chickens, this polymorphism is one of the characteristics considered.

Twelve primer pairs were designed (Table 1), but there were only nine good candidate's primers for HSP90AA gene, which is HSP90AA_1; HSP90AA_3; HSP90AA_5; HSP90AA_6;

HSP90AA_7; HSP90AA_8; HSP90AA_10; HSP90AA_11; and HSP90AA_12. This primer fits to a good primer standard, which has a hairpin value of more than -3 G, self complementary that is less than 8, self 3' complementary that is less than 3, GC clamp that range from 1 to 3, GC concentration that is between 47 and 60%, and a Tm difference of less than 5°C (Anika *et al*., 2019). In the exon 9 region, it is known that the primer pair HSP90AA_10 has the potential to amplify one of the SNPs, c.1608C>T. Previous research also demonstrated a link between these SNPs and heat stress.

Irivboje *et al*. (2019) discovered four SNPs, A7T, A160T, T223A, and C134T, in the HSP90AA1 gene of chickens. In the dbSNP online database, SNPs C134T (c.1608C>T link variation rs316136543) are recorded. Only SNP A7T has no known relationship with the observed traits, namely the rectal temperature and respiratory rate of chickens, among the four SNPs that are associated with these traits.

Table 2. Primer designs of the HSP90AB1 and SNP

Primer	Primer sequence	Target DNA (bp)	SNP
HSP90AB_1	F5'-CTGCCTTTGTTTGCTCTCCG-3' R5'-CTCCACCTCGTCCTCTCCAT-3'	689	c.-10A>C
HSP90AB_2	F5'-CTGTGTCCATCACTGTGGGG-3' R5'-GTCCTCTCCATGCTGCACTT-3'	529	c.-10A>C
HSP90AB_3	F5'-GCTGTAGCCTTGGTGCAAAC-3' R5'-GGCCAAAAGCAACTCACCTG-3'	567	c.119T>C; c.312C>T; c.345G>A
HSP90AB_4	F5'-AAGTGCAGCATGGAGAGGAC-3' R5'-AGCTCCCACAACAAGGAAGG-3'	555	c.119T>C; c.312C>T; c.345G>A
HSP90AB_5	F5'-CCTTACTCTCCCTCTGCCCT-3' R5'-GGTTTGGGCGGTGTAGAAGA-3'	324	c.373A>C; c.416T>G ; c.458A>G; dan c.477G>A
HSP90AB_6	F5'-TCTTCTACACCGCCCAAACC-3' R5'-GCTTTCCCAGTACAGTCCCC-3'	214	c.588G>A
HSP90AB_7	F5'-GTAGAGGTGGCCTGGGACTA-3' R5'-AAGTCAGTACAGCCCACAGC-3'	503	c.673A>G; c.693G>A; c.704C>T; c.720A>G; c.741A>G; c.767A>C; c.782_784 gAGGag>gag; c.810A>C, c.819C>T; dan c.857C>T
HSP90AB_9	F5'-GTGGGCTGACAGCAATGTCT-3' R5'-TGCAGTATGTCCAAGCTCTCA3'	298	c.1195A>C
HSP90AB_ 10	F5'-GGGCAGCTGGACTCTTATGG-3' R5'-TATTCTTCCCCCAGCACCCA-3'	383	c.1338G>A; c.1341G>A; dan c.1460T>C
HSP90AB_ 13	F5'-AGGCTTCATCTCTTCTTTCCC-3' R5'-GCATGAAACACACAGAACCTT-3'	600	c.2084A>G; c.2088G>C; c.2130A>C; c.2162T>G; c.2171T>G; c.*50C>T; c.*180G>A; c.*289C>T; c.*323C>T; c.*330A>C; c.*334T>G; c.*361A>T; c.*382C>T; c.*415A>G; c.*424A>C; c.*199_*200insGT; c.*369_*370insG

Notes: F = Forward, R= reverse

Thirteen primer pairs were designed for the HSP90AB1 gene, but only ten pairs matched good primer standards and contained SNPs. The selected primer pairs HSP90AB_1; HSP90AB_2; HSP90AB_3; HSP90AB_4; HSP90AB_5; HSP90AB_6; HSP90AB_7; HSP90AB_9; HSP90AB_10; and HSP90AB_13 was showed in Table 2. Chen *et al*. (2013) identified five SNPs and one indel variation in a total of 498 bp of the 5' flanking region HSP90AB1 gene of chickens. Analysis of the 5' flanking region of HSP90AB1 revealed the presence of multiple putative transcription factor binding sites. Three mutations in the 5' flanking region were found to be associated with mutations of the transcription factor binding site, but the detected SNP information could not be located on the corresponding Ensembl page. Single nucleotide polymorphisms C.-141G>A, C.-94A>C, and C.-39C>T were identified, indicating that the three mutations were serum response components binding factor (SRF), transcription factor MYB in cells and viruses, and factor GATA binding. It is known that SRF contributes to the development and occurrence of cancer (Shan *et al*., 2010). Given the presence of SRF binding sites in the promoter regions of a number of genes regulating mitochondrial

biogenesis, it is suspected that SRF was also involved in the mRNA expression of the HSP90AB1 gene (Chen *et al*., 2013).

Only five pairs of primers (Table 3), namely primer HSP90B_1, HSP90B_4, HSP90B_11, HSP90B_13, and HSP90B_15, were detected in the target region, despite the fact that eighteen primer pairs were designed. According to Ensembl, primer HSP90B_15 had the potential to amplify c.1974G>A, one of the SNPs Wan *et al*. (2017) discovered an SNP (g6798G>A or c.1974G>A) in exon 14 of HSP90B1 that does not result in an amino acid change (glutamic:GAG>GAA). At the g6798G>A (NC 006088.3) site, a single nucleotide polymorphism results in three genotypes: AA, GA, and GG. The longer survival time of the GG genotype indicates that the G allele is advantageous for heat tolerance. Despite the fact that these are silent mutations that not alter the amino acid sequence or function of the resulting protein, these SNPs can be associated with target properties by influencing mRNA stability and translation efficiency. The c.1974G>A polymorphism has the potential to be used in culture to increase heat tolerance after conducting further study of various chicken populations (Wan *et al*., 2017). Table 1-3 shows that the primary pair of chicken HSP90 genes that meet the criteria for good primers may also be capable of SNP amplification.

Table 3. Primer design of the HSP90B1 and SNP

Primer	Primer sequence	Target DNA (bp)	SNP
HSP90B_1	F5'-GCCTGCCACTTGCGCTTAG-3' R5'-CCAGAAAGGCTACAGCACTCA-3'	693	c.-84T>G; c.-59T>C; dan c.-23C>G
HSP90B_4	F5'-TGCTCCTGCCAGGTCATAAA-3' R5'-GTGAGCTCCTCATTACCAGCA-3'	399	c.204C>A
HSP90B_11	F5'-AATTCTGCTCCACGTGGCTT-3' R5'-TGCTGCTGAAGTGTTTCACG-3'	353	c.1215T>G
HSP90B_13	F5'-TTGACACCTGTGCTTTCCCT-3' R5'-ACTCTTCTTCCTTCCCTCCTCA-3'	319	c.1497C>T; c.1557C>T; c.1600C>A; dan c.1626T>G
HSP90B_15	F5'-GGGACGTAGAGCTGACAGTG-3' R5'-TCATCCCCCGTTACTGGCTA-3'	588	c.1915C>T; c.1935A>G; c.1938C>T; c.1974G>A; dan c.1995A>T

Notes: F = Forward, R= reverse

Detection of CpG island in the chicken HSP90 gene. Epigenetic changes is the mechanism for altering gene expression without altering the DNA sequence. DNA methylation, histone modification, and non-coding RNA are all examples of epigenetic processes. DNA methylation is the most studied mechanism out of the three. DNA methylation has considerable biomarker potential (Handy *et al*., 2011). CpG is the site of methylation, and approximately 80% of CpG sites methylated. CpG sites dispersed throughout genes. DNA methylation is the addition of a methyl group (-CH3) at position 5 of the cytosine ring in the gene sequence to form 5-methylcytosine (5mC) (Wang *et al*., 2020). Methylation of the CpG site in the gene promoter can inhibit gene expression (Jang *et al*., 2017). The existence of CpG island can be identified using MethPrimer. CpG island are found in the promoter and the first exon of several genes. Table 4 displayed the base composition of the promoter gene sequences and exon of the HSP90 gene in chickens.

Table 4. Bases composition in the sequence of the promoter and exon gene HSP90 Chicken

HSP90 gene	Promoter and exon sequence (bp)	Bases composition (%)					
		A	T	G	C	A+T	G+C
HSP90AA1	681	16.01	13.36	33.48	37.15	29.37	70.63
HSP90AB1	637	15.07	16.8	33.59	34.54	31.87	68.13
HSP90B1	750	16.27	17.47	32.13	34.13	33.74	66.26

CpG island will indicate differences in mean G+C content and gene relationships among vertebrate classes. According to Table 4, the G+C content of the CpG island chicken HSP90 gene range from 66.27 to 70.63%. CpG island in chickens contains more G+C than in mammals, whereas

CpG island in humans and mice contains between 67% and 64% G+C (Deaton *et al*., 2011; Haberman *et al*., 2012; Mantsoki *et al.*, 2015). CpG island in *Xenopus* smaller and contains less G+C than in mammals due to its diminutive size and lower G+C content (Stancheva *et al*., 2002).

In chicken, the HSP90AA1 gene contain one CpG island with 66 CG sites on the gene promoter and the HSP90AA1 exon. The CpG island is 537 base pairs long and contains 70.63 % G+C. The HSP90AB1 gene contains two CpG islands with respective lengths of 140 bp and 392 bp. CpG Island 1 contains 11 CG sites, whereas CpG island 2 contains 49 CG sites. Both were identified in the promoter and exon of the HSP90AB1 gene in chicken. The HSP90AB1 gene has a 61.7% G+C base composition. The HSP90B1 gene contains two CpG islands. CpG island 1 contains 15 CG sites, whereas CpG island 2 contains 55 CG sites. Both were identified in the promoter and exon of the HSP90B1 gene in chicken. CpG island 1 is 156 bp in length, whereas CpG island 2 is 472 bp. CG site. The HSP90B1 gene has 66.26% G+C. Figure 1 shows the detection of a CpG island on the promoter and the first portion of the exon of the chicken HSP90 gene.

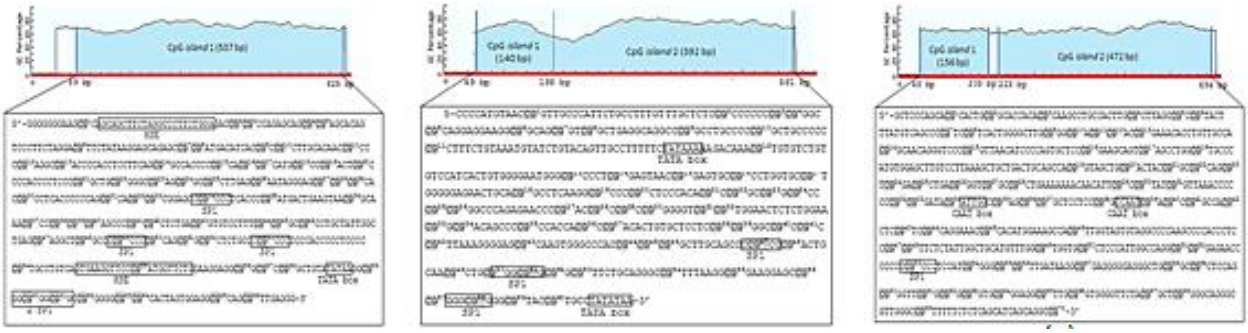

Fig. 1. CpG island from promoter and exon: a. HSP90AA1 gene (ENSGALT00000081765.3); b. HSP90AB1 gene (ENSGALT00000016542.4); c. HSP90B1 gene (ENSGALT00000081140.2).

The CG site discovered in the promoter region of the chicken HSP90 gene, specifically in the SP 1 and HSE regions. Vinoth *et al*. (2018) found the emergence of CpG regions within transcription factor binding sites in the HSP90 promoter region, one of which is SP 1, UCE, PEBP-2, and GCF. Methylation at specific CpG positions can affect the DNA-binding affinity of specific transcription factors (Deaton & Bird, 2011). However, due to the presence of multiple CG sites and transcription factors within the promoter region of the HSP gene, the study conducted by Vinoth *et al*. (2018) failed to distinguish which CG sites and transcription-binding factors are responsible for epigenetic adaptation.

The methylation level of the CG sites in the HSP70 core promoter in chickens was also analyzed. CpG sites at positions -426, -419, and -413 in the chicken HSP70 gene were identified as SP 1 transcription sites, whereas CpG sites at position -172 were found in HSE. The result showed that there was no correlation between DNA methylation of transcription factor CpGs and HSP70 expression (Gan *et al.*, 2013).

CONCLUSION

Twenty-four primary candidates for the chicken HSP90 gene were successfully designed in silico, including: HSP90AA 1; HSP90AA 3; HSP90AA 5; HSP90AA 6; HSP90AA 7; HSP90AA 8; HSP90AA 10; HSP90AA 11, HSP90AA 12; HSP90AB 1; HSP90AB 2; HSP90AB 3; HSP90AB 4; HSP90AB 5; HSP90AB 6; HSP90AB 7; HSP90AB 9; HSP90AB 10; HSP90AB 13; HSP90B 1; HSP90B 4; HSP90B 11; HSP90B 13; and HSP90B 15. In addition, the potential of the CpG Island in the HSP90 gene has been identified in silico. In the promoter region and exon of the HSP90 gene in chickens, the HSP90AA1 gene contains a 537-bp CpG Island with 66 CG sites. The HSP90AB1 gene has two CpG islands; CpG island 1 is 140 bp in length and contains 11 CG sites, while CpG island 2 is 392 bp in length and contains 49 CG sites. Likewise, the HSP90B1 gene possesses two

CpG islands. CpG island 1 is 156 bp in length and contains 15 CG sites, while CpG island 2 is 472 bp in length and contains 55 CG sites.

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