Heat shock protein 70 gene polymorphism in KU–Phuphan black–bone chicken

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ABSTRACT

The objective of this study was to investigate the polymorphism of heat shock protein 70 (*hsp*70) gene in the second generation of KU–Phuphan black-bone chicken populations comparing with crossbreed native chicken and commercial layer chicken populations. Thirty, twenty–five, twenty–three and twenty–three samples of the second generation of KU–Phuphan black–bone female chickens (FB) and male chickens (MB), crossbreed native chickens (BR), and the layer commercial line (LA) were genotyped *hsp*70 gene by the PCR–RFLP technique. The *hsp*70 gene showed two loci, locus *C* generated by *Eae*I and locus *M* created by *Mme*I. The locus *C* consisted of two alleles (C_1 and C_2) providing three genotypes of C_1C_1, C_1C_2 and C_2C_2. The allele and genotype frequencies of KU–Phuphan black-bone chicken populations were not different from other Thai indigenous breeds. The locus *M* consisted of two alleles (M_1 and M_2) whereas the two genotypes of M_1M_2 and M_2M_2 were observed. The allele M_1 showed low allele frequency and was only found in the MB population. The allele M_2 showed monomorphism in FB, BR and LA populations. The male KU–Phuphan black-bone chickens exhibited different M_1M_2 genotype from other detected populations but the allele frequency of MB was less than other Thai indigenous chickens. The genotype frequency of the candidate thermotolerance genotype C_1C_2/ M_2M_2 of both KU–Phuphan black-bone chicken populations was higher than other genotypes. According to the results, KU–Phuphan black-bone chickens showed variation in genotypes, especially for locus *C*, and contained high genotype frequency of the candidate thermotolerance genotype. Therefore, the locus *C* and the candidate genotype of *hsp*70 gene could be used as a genetic marker related with heat tolerance for selection and the breeding program of KU–Phuphan black-bone chickens to create heat tolerance chickens.

Keywords: heat shock protein 70 gene; heat stress; KU–Phuphan black–bone chicken; PCR–RFLP

INTRODUCTION

Tropical climate affects animal production. High ambient temperature is a major impact on performance of animals especially for poultry because they have no sweat glands, rapid metabolism, and high body temperature (Al-Aqil and Zulkifli, 2009). When high ambient temperatures are coupled with high humidity, the combination can become critical because the heat increment of animals is difficult to release from their bodies (Gaviol *et al*., 2008) and also can cause heat stress in chickens. Heat stress causes high mortality, low productivity, decline of feed intake and growth rate (Cooper and Washburn, 1998; Akaboot *et al*., 2010; Boonkum *et al*., 2014). Therefore, animal breeders attended to select heat tolerance poultry lines for solving these problems. At present, genetic markers are used for selection and breeding programs to detect the genes related with economic traits with marker assisted selection (MAS).

KU–Phuphan black–bone chicken is purebred black–bone chicken that was selected from native Mongolia black–bone chicken. KU–Phuphan black–bone chicken was selected under the project of the Faculty of Natural Resources and Agro Industry, Kasetsart University, Thailand. The selective breeding created the chicken to look different from other black–bone chickens such as the large body size, high growth rate, high egg production and disease resistance. Moreover, they were selected to have white color feathers and black color meat, skin, bones, tibias and combs. The black color is due to the melanin that is a polymer complex of the amino acid tyrosine. Natural melanin shows biochemical activities, including photoprotection, antioxidation, free radical sequestration and immunomodulatory effects (Hung *et al*., 2002). To improve the selective breeding program, the developed KU–Phuphan black–bone chickens should

be detected the variation of the functional genes related with the important commercial traits. The heat shock protein 70 (*hsp*70) gene is a candidate gene that is related with the heat tolerance trait. The *hsp*70 is the most widely studied member of the *hsp* family. This gene plays important roles for the survival and production performance of chickens, especially in high ambient temperature. Beckham *et al*. (2008) reported that lack of *hsp*70 gene led to a marked decrease in the heat resistance of cells. Tamzil *et al*. (2013) revealed about the polymorphisms of the *hsp*70 gene resulting in different *hsp*70 genotypes that are associated with differing heat tolerance levels in chickens. In addition, the association between polymorphisms of the *hsp*70 gene and heat tolerance has been regarded as a marker for selecting heat-resistant breeds of chickens (Mazzi *et al*., 2003; Gaviol *et al*., 2008). However, there is no evidence that polymorphisms of the *hsp*70 gene can be used effectively to select heat–tolerant KU–Phuphan black–bone chickens. Our study aims to observe the polymorphism of *hsp*70 of the second generation of KU–Phuphan black–bone chickens comparing with crossbreed native chickens and layer commercial chickens using PCR–RFLP.

MATERIALS AND METHODS

Chickens and sample collection

Thirty, twenty–five, twenty–three and twenty–three samples of the second generation of KU–Phuphan black–bone female chickens (FB) and male chickens (MB), crossbreed native chickens (BR), and the layer commercial line (LA) were studied. Blood samples (0.3 μL) were collected from the wing veins by syringes containing100 μL EDTA (0.5 M, pH 8.0) and then transferred to the laboratory to extract the genomic DNA. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University.

Genomic DNA extraction

Genomic DNA was obtained from 0.1 μL of whole blood using Genomic DNA isolation kit (Invitrogen®, California, USA). The quality of genomic DNA was determined using 1% agarose gel and the DNA concentration was calculated using the VC 100bp Plus DNA Ladder marker (Vivantis, Selengor Darul Ehsan, Malaysia).

PCR amplification of the chicken *hsp*70 gene

The reactions were assembled in a 25μl reaction volume containing: 100 ng genomic DNA, 0.2μM of each primer, 1x buffer (including 2mM $MgCl_2$), 100μM dNTPs (dATP, dTTP, dCTP and dGTP) and 1 unit of *Taq* DNA polymerase (Invitrogen®, California, USA). PCR amplification was performed using primers designed by Mazzi *et al*. (2003): F: 5′–AACCGCACCACACCCAGCTATG–3′ and R: 5′–CTGGGAGTCGTTGAAGTAAGCG–3′. The cycling protocol was 5 min at 94ºC, 34 cycles of denaturing at 94ºC for 30 sec, annealing at 62ºC for 30 sec and extending at 72ºC for 30 sec, with a final extension at 72ºC for 5 min. The amplification of *hsp*70 was displayed in 2% agarose gel.

Restriction with *Eae*I and *Mme*I

The pools contained 1 μg PCR product and were digested with the restriction enzyme *Eae*I and *Mme*I (NEB®, New England, USA) for 60 and 40 min, respectively, at 37°C and then followed by 20 min at 65°C. The samples were separated by electrophoresis in a 3%agarose gel for 40 min, at 100 V. Genotypes of *hsp* 70 were determined at locus *C* for *Eae*I and locus *M* for *Mme*I as following Duangduen *et al*. (2008) and Tunim *et al*. (2010).

Statistical analysis

Genotype frequencies, allele frequencies and Hardy–Weinberg equilibrium (HWE) based on the Chi–square (χ^2) were estimated using Genepop (v 1.2) (Raymond and Rousset, 1995). Observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}) were performed using FSTAT (v 2.9.3.2) (Goudet, 2002).

RESULTS AND DISCUSSION

Heat-shock proteins (HSP) play important roles in the folding, translocation, and refolding/degradation of proteins can affect the heat resistant in living organisms. According to Zhang *et al*. (2002), the polymorphism regulatory and coding regions of the *hsp*70 provided the different heat tolerance capability trait in chickens. In this study, the polymorphisms of *hsp*70 were detected in four chicken populations; KU–Phuphan black–bone female chickens (FB) and male chickens (MB), crossbreed native chickens (BR) and layer commercial chickens (LA).

Genotype and allele frequencies

At locus *C*, three populations (FB, MB, LA) were under Hardy–Weinberg equilibrium, except BR population (P<0.05). The departure from HWE might due to many reasons such as the population derived from various groups of native chickens leading to be

subpopulations, exceeding of homozygotes which explained by non-random mating and founder event (inbreeding) (Frankham *et al*., 2002). The BR population could strongly explained the heterozygote deficit because they showed low value of observed heterozygosity. A 360 bp amplified fragment of *hsp*70 was obtained for all breeds. Explained by Mazzi *et al*. (2003), this amplified fragment contains a point mutation of *hsp*70 and also the mutation created different patterns related with the tolerance of heat. In our study, the different restriction patterns of the *hsp70* were observed after digestion with the enzymes *Eae*I and *Mme*I. The *Eae*I digestion generated a polymorphic site called the locus *C* which contains two alleles: C_1 (229 bp) and C_2 (240 bp) (Figure 1).

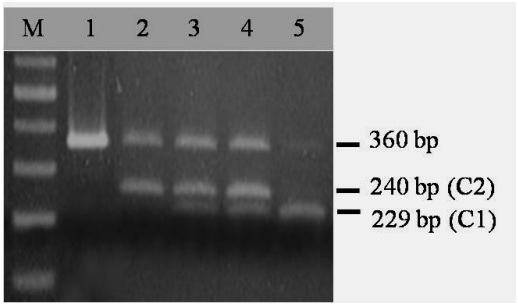

Figure 1 Alleles distribution of *hsp* 70 gene from PCR–RFLP cut by *Eae*I in 3% agarose gel. M is 100 bp ladder standard DNA marker, 1 is uncut 360 bp amplified *hsp* 70 gene, 2 is 240 bp of C2 allele, 3 and 4 are 229 bp and 240 bp of allele C1/C2, respectively, and 5 is 229 bp of C1 allele**.**

The allele frequencies of all populations were not different. Allele C_1 had frequencies in the range of 0.43 to 0.59 and allele C_2 had frequencies in the range of 0.41 to 0.57. The allele frequencies of our study were similar to the other Thai indigenous chickens (Pradu Hangdam C_1=0.45 and C_2=0.55, Lueng Hangkhao C_1=0.54 and C_2=0.46, and Dang C_1=0.55 and C_2=0.45) reported by Tunim *et al*. (2010). The locus *C* established three genotypes, C_1C_1, C_1C_2 and C_2C_2. The data was shown in Table 1. Genotype C_1C_2 was found for almost all individual chickens, except in BR population. The genotype frequency of C_1C_2 was 0.26, 0.39, 0.40 and 0.5 for BR, LA, MB, FB populations, respectively. The high genotype frequencies were found in both female and male KU–Phuphan black–bone chicken. This implied the KU–Phuphan black–bone chicken populations showed high polymorphism of *hsp*70. Mazzi *et al*. (2003) suggested that the polymorphism of *hsp*70 might be used by breeders to produce more heat tolerant chickens. In addition, Tamzil *et al*. (2013) found that the polymorphism of *hsp70* exhibited in native chickens had the highest heat resistance as compared to commercial breeds. According to our results, KU–Phuphan black–bone chickens might adapt in heat stress environment better than commercial chickens. Although, many researchers studied on the identification of *hsp*70 gene polymorphism and they found polymorphic sites that located upstream of the coding region (Mazzi *et al*., 2003; John *et al*., 2012; Abdolalizadeh *et al*., 2015; Gan *et al*., 2015). For our study, while the locus *C* was a polymorphic site, the locus *M* showed monomorphism for FB, BR and LA populations, containing one allele of M_2 (360 bp) (Figure 2). Only the MB chickens displayed both allele M_1 (229 bp) and allele M_2. The allele distributions

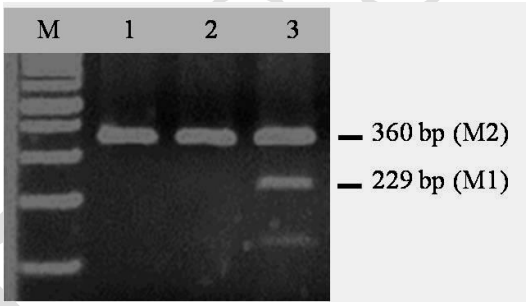

Figure 2 Alleles distribution of *hsp* 70 gene from PCR–RFLP cut by *Mme*I in 3% agarose gel. M is 100 bp ladder standard DNA marker, 1 is uncut 360 bp amplified *hsp* 70 gene, 2 is 360 bp of M2 allele, 3 is 229 bp and 360 bp of allele M1/M2, respectively.

of *EaeI* and *MmeI* cut of four chicken lines were displayed in Figure 3 and Figure 4, respectively. However in our study, allele M_1 was observed that only 4% of the population was not effective for the selection program whereas other Thai indigenous chickens were found 10–32% of populations (Tunim *et al*., 2010). This result might be caused from the selection process. Many alleles can be lost in the selection process due to random genetic drift, which is a consequence of finite populations and is proportional in the rate of inbreeding (Wolc *et al*., 2015). Therefore, the locus *C* seems effective to use as selection marker better than locus *M*.

Considering the genotype combination of locus *C* and locus *M*, our result found four possible genotypes of C_2C_2/ M_1M_2, C_1C_1/ M_2M_2, C_1C_2/ M_2M_2 and C_2C_2/ M_2M_2. Duangduen *et al*. (2008) detected effect of *hsp*70 genotype on thermotolerance in the native and broiler chickens. They reported that chickens containing C_1C_2/ M_2M_2 genotype showed better heat tolerance performance than other chickens consisting with other genotypes. In addition, chickens

Table 1 Allelic frequencies, genotypic frequencies and Hardy-Weinberg Equilibrium (HWE) test of *hsp*70 gene observed for each breed [n = 30 for female KU–Phuphan black bone chickens (FB), n = 25 for male KU–Phuphan black bone chickens (MB), n = 23 for crossbreed native chickens (BR) and n = 23 for commercial layer chickens (LA)] using Genepop (v 1.2) (Raymond and Rousset, 1995).

Frequency		Chicken lines			
		FB	**MB**	**BR**	**LA**
Allele frequency	C_1	0.48	0.56	0.43	0.59
	C_2	0.52	0.44	0.57	0.41
	M_1	0.00	0.02	0.00	0.00
	M_2	1.00	0.98	1.00	1.00
Genotype frequency	C_1C_1	0.23	0.36	0.30	0.39
	C_1C_2	0.50	0.40	0.26	0.39
	C_2C_2	0.27	0.24	0.44	0.22
	M_1M_2	0.00	0.04	0.00	0.00
	M_2M_2	1.00	0.96	1.00	1.00
Combination genotype frequency	C_2C_2/M_1M_2	0.00	0.04	0.00	0.00
	C_1C_1/M_2M_2	0.23	0.36	0.30	0.39
	C_1C_2/M_2M_2	0.50	0.40	0.26	0.39
	C_2C_2/M_2M_2	0.27	0.20	0.44	0.22
HWE	p-value	1.00	0.41	0.03*	0.40

*P<0.05

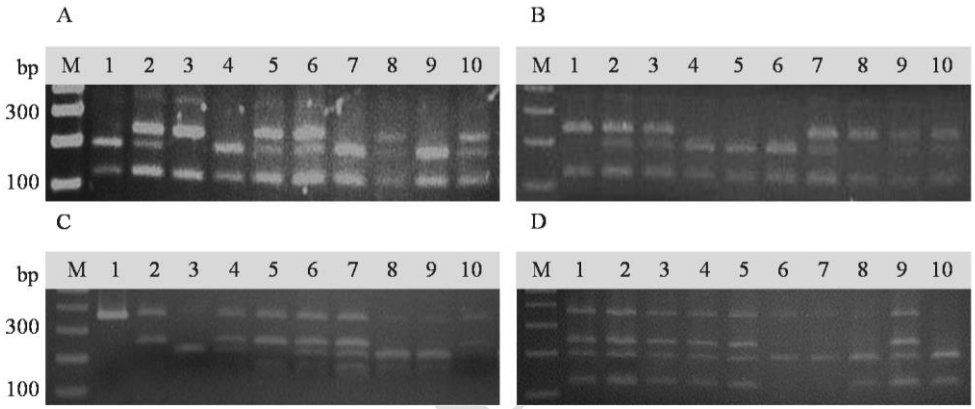

Figure 3 Some genotypes of *hsp* 70 gene of four chicken lines derived from PCR–RFLP cut by *Eae*I in 3% agarose gel. A is a male KU–Phuphan black bone chicken, B is a female KU–Phuphan black bone chicken, C is a crossbreed native chicken, and D is a commercial layer line. M is 100 bp ladder standard DNA marker. 1–10 are the samples of each chicken population.

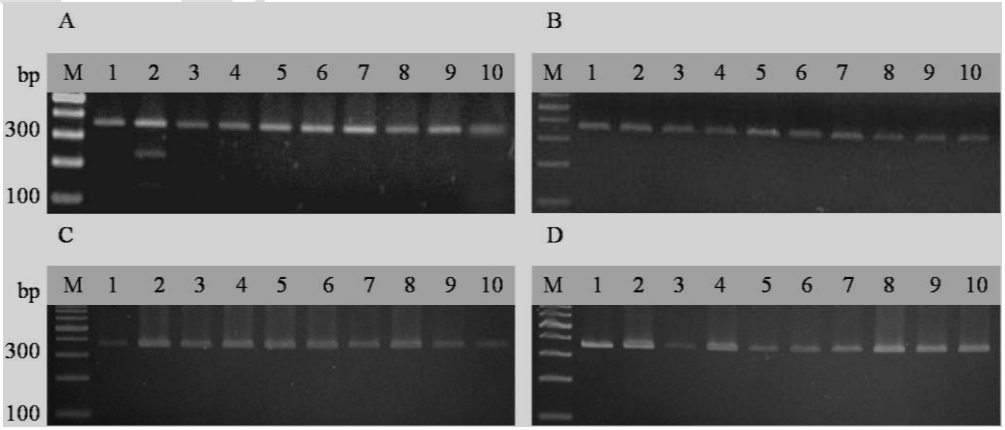

Figure 4 Some genotypes of *hsp* 70 gene of four chicken lines derived from PCR–RFLP cut by *Mme*I in 3% agarose gel. A is a male KU–Phuphan black bone chicken, B is a female KU–Phuphan black bone chicken, C is a crossbreed native chicken, and D is a commercial layer line. M is 100 bp ladder standard DNA marker. 1–10 are the samples of each chicken population.

presented the genotype of C_2C_2/ M_1M_2 was sensitive to heat stress (Duangduen *et al*., 2008) which should be considered for the breeding program. In our study, female and male KU–Phuphan black–bone chicken populations showed the high C_1C_2/ M_2M_2 genotype frequency (FB was 0.5 and MB was 0.4). This combination genotype showed the higher genotype frequency than other three genotypes (Table 1). The female and male KU–Phuphan black–bone chickens exhibited the higher genotype frequency of C_1C_2/ M_2M_2 than various strains of Thai native chickens (Pradu Hangdam=0.48, Lueng Hangkhao=0.34, Dang=0.30 and Chee=0.28) reported by Tunim *et al*. (2010). These results indicated that KU–Phuphan black-bone chicken populations showed good performance at high ambient temperatures to tolerate from heat stress. Moreover, the polymorphic locus *C* and C_1C_2/ M_2M_2 genotype of *hsp*70 could be used as candidate markers to select the heat tolerant chickens.

Genetic diversity of *hsp*70

Locus *M* showed monomorphism for most of the populations. A reduction of heterozygosity could occur during the selection and inbreeding process (Akaboot *et al*., 2012), and resulted in a loss of alleles of *hsp*70 that could affect to the adaptation to heat stress of chickens. Therefore, the utilization from locus *M* was carefully considered for our chicken populations, especially for KU–Phuphan black–bone chickens. The observed (H_o) and the expected (H_e) heterozygosities were detected from all populations for locus *C* but locus *M* was determined only the MB population. At locus *M*, the H_o and the H_e of the MB population were 0.04. At locus *C*, the H_o varied from 0.21 to 0.50 and H_e ranged from 0.48 to 0.51 (Table 2). The H_o of BR was lower than other breeds (H_o=0.26). The highest value of H_o and H_e were found in female KU–Phuphan black–bone chicken population which were 0.50 and 0.51, respectively. The expected heterozygosity at locus *C* of all chicken populations was not different from the H_e of the other Thai native breeds (Pradu Hangdam=0.500, Lueng Hangkhao=0.502 and Dang=0.501) reported by Tunim *et al*. (2010) (H_e = 0.398 to 0.502) and also was not different from the H_e of Pradu Hangdam (H_e = 0.49) reported by Akaboot *et al*. (2012). However, although FB and MB showed high value of the H_e but the inbreeding coefficient within population (F_{IS}) was detected. All populations remaining F_{IS} values were positive, suggesting that inbreeding was common in almost all chicken populations. The F_{IS} was 0.016, 0.208, 0.486 and 0.214 for FB, MB, BR and LA populations, respectively. Although the F_{IS} of both KU–Phuphan black–bone chicken populations was detected the low level of inbreeding, a need for genetic intervention, for examples, exchange of breeding cocks among flocks to avoid the possible negative impact of inbreeding depression on survival, reproduction and productivity, should be considered in the KU–Phuphan black–bone chickens.

Table 2 Observed and expected heterozygosity, and Inbreeding coefficient (F_{IS}) of *hsp*70 gene for each breed [n = 30 for female KU-Phuphan black bone chickens (FB), n = 25 for male KU-Phuphan black bone chickens (MB), n = 23 for crossbreed native chickens (BR) and n = 23 for commercial layer chickens (LA)] using FSTAT (v 2.9.3.2) (Goudet, 2002).

Loci	Heterozygosity	Chicken lines			
		FB	MB	BR	LA
C	Observed	0.50	0.40	0.26	0.39
	Expected	0.51	0.50	0.48	0.48
M	Observed	0.00	0.04	0.00	0.00
	Expected	NA	0.04	NA	NA
F_{IS}		0.016	0.208	0.486	0.214

NA is Not Available

In conclusion, Chen *et al*. (2016) found that the use of molecular breeding method to identify genetic markers related to thermotolerance could allow the possibility of direct gene selection. According to our study, both the KU–Phuphan black–bone chickens exhibited genotype related with heat tolerance. The polymorphisms in the *hsp70* gene might be used by the breeders to produce chickens that are more tolerant to high temperature.

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