RESEARCH ARTICLE

Genetic analysis of a novel polymorphism in coding region of *HSP70* gene and its association with some productive and reproductive traits in Mazandaran native breeder hens

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Najafi M, Rouhi MA, Mokhtari R, et al. Genetic analysis of a novel polymorphism in coding region of HSP70 gene and its association with some productive and reproductive traits in Mazandaran native breeder hens. J of Genet Disord and Genet Med September-30 2018;2(1):1-5.

Heat shock proteins (HSPs) are molecules that play an eminent role in protection of intercellular organs functions. These proteins could affect several cell activities such as regulation of cell signaling, protein misfolding, immune response regulation as well as apoptosis. This study aimed to evaluate the association between different allelic forms in coding region of *HSP70* gene and some productive and reproductive traits in Mazandaran native breeder hens. The blood samples were randomly collected from 305 native breeder hens. Then, DNA extraction carried out using modified salting out method and a 372 bp fragment was amplified in coding region of *HSP70* gene by PCR. In order to genotyping, PCR-RFLP technique and TaqI restriction enzyme was used. The genotyping results showed three genotypes of AA, AC and CC with following genotype frequencies of 0.32, 12.78 and 86.88, respectively. To confirm the genotyping results as well as detection of actual SNP, direct sequencing was applied. A novel SNP (A179C) was identified in the coding region of *HSP70* gene. Furthermore, association analysis between obtained genotypes and growth traits have shown that this novel polymorphism could significantly affect body weight, egg weight at 28 weeks of age, fertility and hatchability traits ($p<0.05$). According to our results, this polymorphism could further be considered as the molecular marker for the selection or breeding programs in the future.

Key Words: *Single nucleotide polymorphisms; Heat shock proteins; Growth traits; Mazandaran native chickens*

INTRODUCTION

Commercial poultry industry is suffered from various stresses associated with reduction of productive and reproductive performance [1]. High temperature is one of the most remarkable issues in both broilers and egg-laying chickens and produces stressful conditions for chickens, resulting in reduced nutrient utilization and performance, anorexia, heat stress, and consequently mortality [2-5]. It has been well documented that heat stress especially during the hot seasons leads to economic losses [6]. Broiler chickens are more sensitive to heat stress rather than other species of domestic breeds due to a rapid metabolism and high body temperature as well as having no sweat glands. During the heat stress, there is a mechanism for cell survival that provokes a rapid response at the transcription and translation level [7]. Exposure to extreme heat induces several genes associated with heat tolerance in chickens, including the naked-neck (Na), the frizzle (F), the dwarf (dw), as well as heat shock protein (HSPs) genes [8,9] and following the cells produce low molecular weight (LMW) proteins that are known as the heat shock proteins [10]. Despite thermal shock, these highly conserved proteins were shown to be activated due to other stressors such as ultraviolet radiation, oxidative stress, chemicals, changes in glucose levels, presence of glucose and amino acid analogues and different ions, ethanol, various metals, drugs, hormones as well as bacterial and viral infections [11]. The function of these proteins includes cell signalling control, immune response regulation, involvement in conditional infections, folding of proteins, and regulation of cell death [11]. The family of heat shock proteins includes HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP10 and small HSP [11]. Among these proteins, the HSP70 is highly sought after by researchers. According to the results of former studies, there is a significant correlation between heat stress tolerance and increased HSP70 protein levels, and HSP70-knockout organisms may be more vulnerable to the temperature changes [12]. Genetic diversity in the sequence of DNA or protein domains plays an eminent role in the survival and adaptability of a species. However, mutations may impact protein interactions which may influence the response of an individual to various environmental conditions [13]. In the chickens, polymorphisms of the HSP70 gene were correlated with the levels of mRNA expression as well as heat tolerance, which have been regarded as an effective marker for selecting heat-tolerant chickens [12,14] which is suitable for poultry farming in hot seasons especially in a high ambient temperature environment. Although the effect of HSP70 on heat stress has been well documented, fewer studies investigated the functional aspects of HSP70 and its association with production and reproduction system. It was reported that HSP70 expressed in the embryonic chicken lens constitutively, which was correlated with the early stages of fibre formation of cells, and increased mRNA abundance of this gene was part of the differentiation process [15]. Additionally, Binding of HSP70 to the sperm surface by interacting with VDAC2 and activating sperm motility is one of important roles of HSP70 in sperm migration within oviduct in quail [16]. Further association of HSP70 polymorphisms with the milk production traits including total milk yield, peak yield, yield at 300 days, protein% and fat% was observed in Frieswal cross bred cattle [17]. From one hand, the results of previous studies suggest that HSP70 gene is polymorphic and may be useful in selection of individual for relatively better thermo tolerance and higher productive performances and also some studies have shown a significant association between the HSP70 gene SNPs and some productive and reproductive traits. On the other hand, the genetic diversity of native chickens is undeniable for conserving valuable genetic resources which may be used in novel productive demands. Native chickens also are well in traditional management systems in Mazandaran province because of their ability to adapt to Mazandaran summer weather, during which the average temperature reaches more than 30°C and 74% relative humidity. Therefore, the purpose of this study was to identify the single-nucleotide polymorphism of the coding region of HSP70 gene as well as its association with some productive and reproductive traits in Mazandaran native hens.

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Received: September 06, 2018; Accepted: September 18, 2018; Published: September 30, 2018

MATERIALS AND METHODS

Experimental population, sampling and DNA extraction

In this study, records of the productive and reproductive traits (body weight, egg laying intensity, average egg weight at 28, 30 and 32 weeks of age, fertility and hatchability rates) of Native breeder fowls were individually measured and recorded by researchers using Excel software. The taking of a blood sample to provide material to identify an individual, or its genotype, would be regulated by the Act. Therefore, in the present study, the blood samples from birds were fully taken based on Animal Ethics Committees (AECs) which consider applications to conduct research that involves the collection of blood from animals and to assist animal researchers who collect blood from animals.

Fresh blood samples were collected in EDTA vacutainer tubes from 305 native breeder fowls from Mazandaran native fowls breeding station. Samples were immediately transferred to the laboratory by maintaining the cold chain conditions and stored at -20°C until DNA extraction process. DNA extraction was performed using modified salting out method [18], and the agarose gel electrophoresis was used to determine the quantitative and qualitative characteristics of DNA.

PCR reaction

In this study, a pair of specific primers [19] was used to amplify the coding region of the *HSP70* gene (Table 1).

TABLE 1
Primer sequences for amplify coding region of HSP70 gene

DNA Sequence	Acc. Number	Length	Region	Annealing
F: 5′-AGCGTAACACCACCATTC-3′				
R: 5′-TGGCTCCCACCCTATCTC-3′	J02579.1	372 bp	coding region	60°C

Polymerase chain reaction in 20 µl volumes including 10 µl PCR Master Mix (2x) (Aryatous company cat.no: lot#1454), 100 ng genomic DNA, 1 µl of each of the primers (10 pM) and 6.5 µl DNase-free water. Then the PCR thermal cycles were performed as follow: initial denaturation of the DNA at 95°C for 5 minutes and 35 cycles of denaturing at 95°C for 45 seconds, annealing at 60°C for connecting primers for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 5 minutes. To assess the quantity and quality of the PCR products, agarose gel 2% and ethidium bromide staining were used with the GeneRuler 100 bp DNA ladder (Thermo Scientific, SM0241).

Genotyping and sequence analysis

In order to detect different patterns of single nucleotide polymorphism in samples, PCR-RFLP method and TaqI restriction enzyme were used. The presence of the cutting site of this enzyme at position 1430 bp of this gene (according to the sequence recorded by JX827254.1) produces two fragments of 194 and 178 bp (C allele), while the absence of this position only produces a fragment of 372 bp (allele A). If there is a cutting site for the enzyme in both alleles, this leads to the presence of the CC genotype, and if there is no site in one or any of the alleles, then it leads to the presence of AC and AA genotypes, respectively. Additionally, to confirm the results of genotyping, from each genotype, two samples were selected for direct sequencing. After obtaining the raw sequences, bioinformatics analysis was performed using BioEdit software. Furthermore, to translate nucleotide acid sequence to corresponding peptide sequence and also for amino acid change analysis, Expasy server (https://www.expasy.org/) was applied.

Statistical analysis

Analysis of the relationship between genotypes and studied traits was performed using SAS 9.1 software based on the general linear model procedure (GLM). Hatching was carried out in 4 stages, and the breeder chickens were born in different hatchings (1 to 4), and the effect of the hatching stage was considered as the fixed effect. For all analyses, a significant level of 5% was considered.

RESULTS

DNA extraction and amplification of the specific DNA fragment

In this research, blood samples were collected from 305 Mazandaran native breeder hens and after DNA extraction, using a pair of specific primers, a 372 bp fragment of the coding region of the *HSP70* gene was amplified. All samples had a strong band, no extra band or dimmer.

Genotypic and allelic frequencies-PCR-RFLP

In this study, as shown in (Figure 1), three genotypes (AA, AC and CC) and two alleles of C and A with the frequency of 93.03 and 0.07 were observed in the studied population. The results of the Hardy-Weinberg equilibrium test showed that the studied population are in the Hardy-Weinberg equilibrium (Table 2).

Table 2
Gene and genotypic frequencies in the present study

Gene	Allele		Genotype			HWE*
HSP70	A	C	AA	AC	CC	
Number	-	-	1	39	265	0.74^{ns}
Frequency	0.07	0.93	0.003	0.127	0.868	

*Probability of Chi-square for Hardy-Weinberg equilibrium

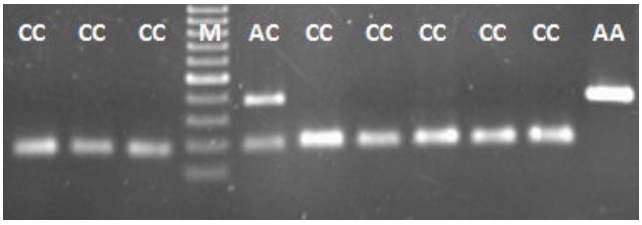

Figure 1) *Genotyping of Mazandaran native breeder hens using TaqI enzyme along with GeneRuler (M)*

Direct Sequencing

To confirm the results of PCR-RFLP technique and also identification of actual SNP, direct sequencing was used. Herein, we sequenced two samples for each genotype (except AA genotype that we had only one observation) and then the obtained sequences were aligned and SNPs were identified using bioinformatics software. Additionally, the obtained variants of DNA sequences for *HSP70* gene were deposited in NCBI database with accession number: MG575723, MG575724, and MG575725. Based on our results,

two SNPs were detected that after downstream analysis, the amino acid sequence is not altered in both positions (Figure 2). T224C SNP was transition and the C179A SNP was transversion which both were in the polypeptide combining region. Moreover, the identified SNPs in the present study were silent mutations (synonymous). Although this mutation leaves the amino acid unchanged, in some case could influenced phenotype by speeding up or slowing down protein synthesis or by affecting splicing through altering the composition, affinity and function of spliceosomes [20]. Occurrences of silent mutation vary among species as well as within genes. However, these changes without altering amino acid sequence have an impact on nucleotide stability, protein levels, structure and function [21]. Additionally, nowadays these silent mutations were applied for improving the safety and efficacy of the drugs [22].

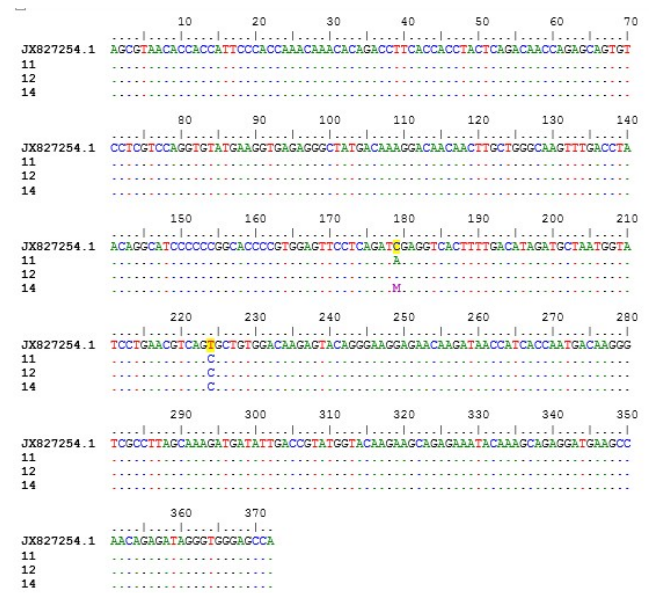

Figure 2) *Sequence alignment of three obtained genotypes using BioEdit software. Highlights shows SNPs positions, M represents heterozygote genotype (A/C SNP). Nucleotide position is based on our amplified fragments*

Association analysis

In the data analysis procedure, the AA genotype with a very low frequency (one observation) was eliminated from the analysis. Association analysis between identified allelic patterns and studied traits (body weight, egg laying intensity, average egg weight at 28, 30 and 32 weeks of age, fertility and hatchability rates) showed an remarkable association between genotypes and body weight, average egg weight at 28 weeks of age, fertility and hatchability rates in this population (p<0.05) (Table 3).

Table 3
Least Mean Square of observed genotypes in Mazandaran native breeder hens

Traits	Mean	Genotype	p-Value
	42.73	AC	
Body weight	39.86	CC	0.03*
	53.16	AC	
Egg weight at 28 weeks of age	51.49	CC	0.04*
	59.64	AC	
Fertility %	59.64	CC	0.02*
	45.92	AC	
Hatchability %	57.92	CC	0.03*

*Shows p ≤ 0.05

DISCUSSION

Nowadays, the use of modern molecular genetic technology to identify genetic markers is associated with economical traits to improve selection and breeding programs. Furthermore, the genetic changes by altering mRNA abundance of genes and their products can lead to the phenotypic difference among individuals and various species [23,24]. A growing body of evidence indicates heat stress is an important environmental challenge that affects poultry industry [25,26]. *HSP70* gene is one of the most important candidate genes for the heat stress and several studies have confirmed the importance of this genetic marker and its correlation with thermal stress. In *Drosophila melanogaster*, numerous mutations have been reported in the sequence of the *HSP70* gene that alter the gene expression and ultimately alter its morphology and physiology [27,28], which would apply to other organisms including birds. Similarly, SNPs in this gene may contribute to the binding of the peptide substrate to HSP70 or the activation of HSP70 [29]. In the chicken genome, the SNP density average was reported to be 5/1000 [30], where these SNPs exist in both coding and non-coding regions of the gene, and some of them alter the amino acid sequence and others are involved in splicing and eventually regulating gene expression [20]. Based on our best knowledge, so far 35 single-nucleotide polymorphisms and two deletions were identified in *HSP70* gene (Figure 3). Out of the 25 SNPs in the coding region, 17, 7 and 1 SNPs were located in the ATP enzyme active region, in the polypeptide combining region, and in C-terminal region, respectively. The average SNP density in the *HSP70* gene was reported 13/1000 [31].

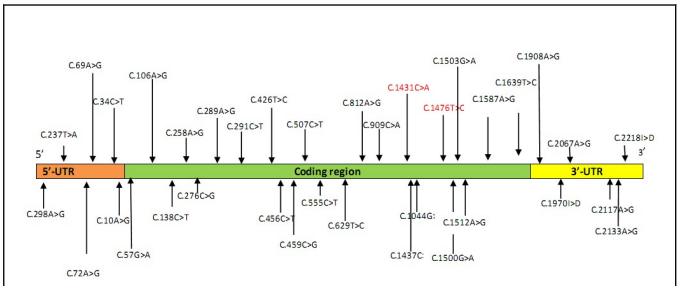

Figure 3) *Schematic representation of identified SNPs within HSP70 gene in chickens, Nucleotide position is based on sequence alignment with J02579.1. Highlighted SNPs were identified in the present study*

Association analysis

Considering the importance of this gene site, numerous association studies have shown that genetic polymorphisms of this gene have remarkable correlations with heat stress in chicken [6,32] as well as altering mRNA abundance of this gene [33]. It has also been reported that the changes in AT content in the promoter region of the *HSP70* gene can have a significant effect on the expression level of the *HSP70* gene as well as the regulatory evolution of this gene [34]. Moreover, genetic diversity in the non-coding region (C69A>G SNP) led to the formation of a MZF1 binding site that contributed to the transcriptional regulation of several genes, resulting in enhancing the expression level of *HSP70* gene as well as increasing the heat stress tolerance of chickens [35]. Further studies showed more single-nucleotide polymorphisms in the sequence of this candidate gene, encompassing identifying four SNPs in commercial broiler chickens [36], detection of 13 SNPs in the HSF3 gene, and the association of polymorphism C1388 A>G with heat stress [37] and identifying of three polymorphisms and their relationship with heat stress tolerance, growth traits, and egg production. In Liang's study, a significant correlation was observed between body weight gain and the genotypes (A258G) (p<0.001), and the AA genotype indicated the greatest weight gain at 0-16 weeks of age for both males and females, while no remarkable differences among

genotypes regarding egg weight at first lying or the number of eggs laid until 40 weeks of age were found [38]. To conclude, the results from the aforementioned studies consistently demonstrate the polymorphic nature of the different regions of HSP70 and their relationships with important economic traits. Additionally, our results have shown that there is a significant associations between C179A SNP and body weight, average egg weight at 28 weeks, fertility and hatchability rates, which can provide strong evidence for importance of this candidate gene. Therefore, it can be concluded that these polymorphisms can be used as an effective marker in breeding programs and some of them have no negative effect on productive performances.

Phylogeny analysis

HSP70 is expressed in other avian species and is also shown to be well conserved among various species. Alignment sequence analysis for coding region of HSP70 indicated homology of 97%, 96%, 94%, 93%, 92%, 91%, 90% and 83% with *Numida meleagris*, *Coturnix coturnix*, *Anser cygnoides*, *Columba livia*, *Corvus cornix*, *Falco peregrinus*, *Haliaeetus albicilla*, *Gavia stellata*, and *Acanthisitta chloris* respectively. This finding shows the high similarity of this candidate gene among birds and also our obtained sequences were grouped in *Gallus Gallus* clade. Based on phylogenetic analysis of this region following neighbour-joining algorithm, chickens is located in distinct cluster, and helmeted Guinea fowl and quail were grouped close to chickens (Figure 4).

Notably, HSP70 protein in *Gallus Gallus* showed 99% homology with *Numida meleagris* and 98% homology with quail HSP70 [9]. These findings support the notion that the HSP70 protein is more conserved compared to DNA sequence of this gene probably because of silent mutations. Considering the fact that there was a genetic differentiation in the *HSP70* gene between birds due to evolution, artificial selection may also have a prominent role in this process.

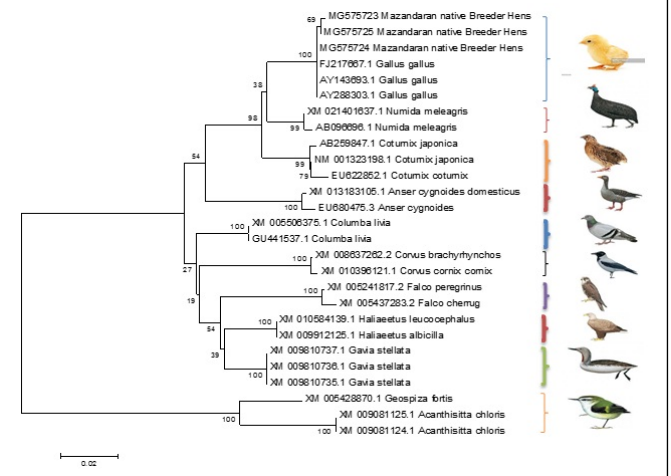

Figure 4) *Phylogeny analysis of HSP70 gene across different species based on coding region sequence using Mega6.0 software following neighbour-joining algorithm*

CONCLUSION

Optimizing the production and reproduction function and hence reducing the overall economic losses are desirable proposition for producers. Maintaining bird's performance especially in unfavourable conditions requires improved management strategies and genetic advancement. The results of the present study indicate the importance of this candidate gene, and given the fact that the number of SNPs detected in this gene is increasing, so further studies on this breed and other breeds is recommended. Regarding the results of the present research work and finding a SNP associated with body weight, average egg weight at 28 weeks, fertility and hatchability rates, this SNP could be considered as a candidate marker in breeding programs and can be used in studies related to the identification of QTLs associated with the production traits.

CONFLICTS OF INTEREST

All Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. The authors confirm that there are no known conflicts of interest.

ACKNOWLEDGEMENT

The authors of this study have thanked the staff and management of the Mazandaran native fowls breeding station for collaborating on the trait recording. Also, we sincerely grateful of Dr. Hassan Baneh for his contribution to the statistical analysis of this study.

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