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Analysis of genetic diversity of the heat shock protein 70 gene on the basis of abundant sequence polymorphisms in chicken breeds

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ABSTRACT. This study was designed to detect the sequence variation of the chicken heat shock protein 70 (*HSP70*) gene. A total of 102 individuals from 8 native Chinese breeds together with Dwarf White Chicken and Red Junglefowl were used to detect sequence variations. The coding regions of the chicken *HSP70* gene from 102 individuals were cloned and sequenced. Thirty-six variations were identified, which included 34 single nucleotide polymorphisms and 2 indel mutations. Fifty-seven haplotypes were observed, of which, 43 were breed-specific and 14 were shared. There were 7 Red Junglefowl-specific haplotypes, while Haidong and Silkie only had 2 specific haplotypes. Eleven and 3 haplotypes were shared between and within species, respectively. The variation in nucleotide diversity (Pi) and average number of nucleotide

differences (K) among species were consistent. The total Pi of *HSP70* was 0.0016, and the total K was 4.1998. The Pi value of Red Junglefowl was the highest (0.0018) and K was 4.8000, while the Pi of Silkie was the lowest (0.0010) and K was 2.5000. These results demonstrated that variation in chicken *HSP70* was abundant between and within species.

Key words: Chicken; *HSP70*; Single nucleotide polymorphism; Haplotype

INTRODUCTION

Heredity and variation are the cause of the species diversity, and heritable variation provides the raw material for biological evolution (Janecka et al., 2012). Remarkable differences in sequence evolution have been noted across lineages of mammals and other organisms (Welch and Bromham, 2005). Sequence mutations and the subsequent fixation of these variations yield the initial heritable variation that leads to adaptation and subsequently speciation (Bromham, 2009). Heat shock protein 70 (*HSP70*) is a member of the heat shock protein family (Kregel, 2002). As a molecular chaperone, it plays a critical role in the regulation of the heat shock response and the acquisition of thermotolerance (Mayer and Bukau, 2005; Guo et al., 2012). Local chicken varieties in China are diverse and have different levels of thermal resistance. We hypothesized that this could possibly be related to variations in the *HSP70* gene sequence. Furthermore, there could be differences in the *HSP70* sequence evolution among these local species and their ancestor Red Junglefowl. The *HSP70* gene families in *Stratiomys singularior* and *Oxycera pardalina* evolved quite differently from one another (Garbuz et al., 2011). In this study, we investigated the sequence variation in the *HSP70* gene in 10 populations, including 8 native Chinese chicken populations, Dwarf White chicken, and Red Junglefowl. Single nucleotide polymorphisms (SNPs) of the *HSP70* gene were detected by sequencing, haplotype and genetic diversity analyses of the *HSP70* gene were performed, and genetic differentiation of the *HSP70* gene was analyzed in the 10 chicken populations.

MATERIAL AND METHODS

Samples and DNA extraction

Blood samples of 102 chickens from 8 native Chinese populations, Dwarf White chicken (Xinguang Agriculture and Animal Husbandry Co., Ltd., China), and Red Junglefowl were collected. Information about the blood samples is listed in **Table S1**. Genomic DNA of the 102 individuals was extracted by a standard phenol-chloroform method.

Polymerase chain reaction (PCR) amplification

The following primer pair was designed based on the chicken *HSP70* gene (GenBank accession No. J02579): forward, 5'-CGATCTGGCTGCAATCTACG-3', and reverse, 5'-ATTTCCAGAAGCTGCACTTGG-3'. The amplification product was 2591 bp. The PCR mixture consisted of 1 µL chicken DNA, 0.25 µL 10 µM primers, 3.2 µL dNTP mixture, 0.2 µL LA Taq DNA polymerase (TaKaRa, Japan), 2.0 µL 10X LA PCR Buffer II, and ddH_2O (to

a final volume of 20 µL). After denaturation at 94°C for 3 min, samples were subjected to 32 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s, after which a final elongation step of 72°C for 6 min was included. PCR products were analyzed by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Cloning and sequencing

PCR products were purified using the E.Z.N.A.™ Gel Extraction Kit (Omega, USA). The purified products were ligated into the pGEM-T vector and cloned into *Escherichia coli* DH5α cells according to the standard protocol. Positive samples were sequenced by BGI Company (China).

Statistical analysis

Sequence alignment and polymorphism detection were performed using DNASTAR. Haplotypes were determined by Clustal X 1.81 and MEGA 4.0 (Tamura et al., 2007). Haplotype diversity, nucleotide diversity, and neutral tests were determined using DnaSP4.9 (Rozas et al., 2003). Analysis of molecular variance (AMOVA) of the *HSP70* gene was carried out using Areliquin3.11, and genetic variation was calculated by the fixation index.

RESULTS

Polymorphisms of the chicken *HSP70* gene

A product of 2591 bp was obtained by PCR amplification, which included a 322-bp 5'-flanking region, a 1905-bp open reading frame, and a 364-bp 3'-noncoding region. Sequence alignment revealed 34 SNPs and 2 indel mutations (Table 1), among which 6 variations were located in the 5'-flanking region, 24 were in the coding region, and 6 were in the 3'-noncoding region. Thirty SNPs were transitions and the other 4 were transversions. Of the 24 SNPs in the coding region, 16 were in the ATP enzyme active region, 7 were in the polypeptide combining region, and 1 was in C-terminal region; additionally, 7 SNPs caused amino acid changes **Table S2**.

Table 1. Distribution of single nucleotide polymorphism locations in the chicken *HSP70* gene.

Number	Position	Variation type	Region	Number	Position	Variation type	Region
1	C.298A>G	Transition	5'-flanking region	19	C.629T>C	Transition	Coding region
2	C.237T>A	Transversion	5'-flanking region	20	C.812A>G	Transition	Coding region
3	C.72A>G	Transition	5'-flanking region	21	C.909C>A	Transversion	Coding region
4	C.69A>G	Transition	5'-flanking region	22	C.1044G>A	Transition	Coding region
5	C.34C>T	Transition	5'-flanking region	23	C.1431C>A	Transversion	Coding region
6	C.10A>G	Transition	5'-flanking region	24	C.1437C>T	Transition	Coding region
7	C.57G>A	Transition	Coding region	25	C.1476T>C	Transition	Coding region
8	C.106A>G	Transition	Coding region	26	C.1500G>A	Transition	Coding region
9	C.138C>T	Transition	Coding region	27	C.1503G>A	Transition	Coding region
10	C.258A>G	Transition	Coding region	28	C.1512A>G	Transition	Coding region
11	C.276C>G	Transversion	Coding region	29	C.1587A>G	Transition	Coding region
12	C.289A>G	Transition	Coding region	30	C.1639T>C	Transition	Coding region
13	C.291C>T	Transition	Coding region	31	C.1908A>G	Transition	3'-noncoding region
14	C.426T>C	Transition	Coding region	32	C.1970I>D	T indel	3'-noncoding region
15	C.456C>T	Transition	Coding region	33	C.2067A>G	Transition	3'-noncoding region
16	C.507C>T	Transition	Coding region	34	C.2117A>G	Transition	3'-noncoding region
17	C.550T>C	Transition	Coding region	35	C.2133A>G	Transition	3'-noncoding region
18	C.555C>T	Transition	Coding region	36	C.2218I>D	A indel	3'-noncoding region

Haplotype analysis

Fifty-seven haplotypes were found (Table 2).

Table 2. *HSP70* haplotypes in 10 chicken breeds.

Haplotype number	1 1111111111 2222 22233445 5667788891 2377788899 2344 2855817257 9114727753 3655922306 2835 5614928799 7027781603 0528714380 9884
H1	ATAACAGACG CACTCCTCTA CGCCCGGAAT AAAA
H2	.....G.... ....
H3	.... G...
H4	.... .GG.
H5	......A... G.T....... ....
H6	...G...... ....
H7	.... A...T..... ....
H8	....C. A...T..... ....
H9	.... A...T.A... ....
H10	.... A......... ....
H11	..G....... ....T..... ....
H12	....T..... ....
H13	.... .A..T..... ....
H14	.... .A.TT..... ....
H15	.......G.. .....T...G ....TA.... ....
H16	....G ....TA.... ....
H17	....C...... ....TA.... ....
H18	...G.....A .... A...T..... ....
H19	...G.....A .... A......... ....
H20	...G.....A ....
H21	...G.....A ....G. ....
H22	...G.....A .....T.... .A........ ....
H23	...G.....A ....TA.... ....
H24	...G.....A ....TA...C ....
H25	G..G.....A ....TA.... ....
H26	...G.....A ....TA.... .G..
H27	...G.....A ......C... .A..TA.... ....
H28	...G.....A ....T..... ....
H29	...G...G.A ....T..... ....
H30	.........A G......... .A.TT..... ....
H31	.....G...A G......... .A.TT..... G...
H32	.........A G......T.. .A.TT..... ....
H33	.........A G......T.. .A.TT....C ....
H34	.........A GG........ .A.TT..... ....
H35	.........A G......TC. .A........ ....
H36	.........A G......T.. .A........ ....
H37	.........A G......... .A........ ....
H38	.........A G.....C... .A........ ....
H39	G........A G......... .A........ ....
H40	.........A G......... ....
H41	.........A G..CT..... ....
H42	..G......A G...T..... ....
H43	.........A G......... ....G.. ....
H44	.........A G......... ..A....... ....
H45	.........A GG........ ..A....... ....
H46	....T...TA G......... .A..T..... ....
H47	....T...TA G......... .A..TA.... ....
H48	....T...TA G......... ....T..... ....
H49	....T...TA G......... ..A...A... ....
H50	.A.......A G......... ....T..... ...G
H51	.........A G......... ....T..... ...G
H52	.........A G......... ....T..... ..GG
H53	.........A G......... A...T..... ...G
H54	.........A G......... ....T...G. ....
H55	.........A ....TA.... ....
H56	.........A ....TA.G.. ....
H57	.........A G......... ....TA.... ....

H = haplotype. The dots represent bases that are identical to the reference sequence (H1).

Of these, 43 were breed-specific (Table 3) and 14 were shared (Table 4). The H1, H5, H7, H13, H20, H23, H28, H30, H32, H37, and H40 haplotypes were shared among breeds; the H44, H48, and H50 haplotypes were shared within breeds (Table 3). The H7, H13, and H23 haplotypes had the highest frequency, while the H9 and H54 haplotypes had the lowest frequency. However, H7 had the highest frequency in the Taibai breed, H13 had the highest frequency in the Haidong breed, and H23 had the highest frequency in the Silkie breed. The Red Junglefowl had 7 breed-specific haplotypes, which was the most of any breed, and the Big Bone and Silkie breeds had 2 breed-specific haplotypes, which was the least of the breeds.

Table 3. *HSP70* haplotypes shared within and between 10 chicken breeds.

Shared haplotypes	Number	Breed									
		DG	HD	TB	SY	ZJ	XH	QY	LS	DW	RJF
H1	6	1						1	2	2	
H5	2	1		1							
H7	8			3	1			1	1	2	
H13	8		5	1		1		1			
H20	2	1					1				
H23	8				5		1		1		1
H28	4	3		1							
H30	3	1			1				1		
H32	2		1			1					
H37	6	1	3				1			1	
H40	4			1		2					1
H44	2					2					
H48	2										2
H50	2								2		

Big Bone (DG), Haidong (HD), Taibai (TB), Silkie (SY), Tibet (ZJ), Xinghua (XH), Qingyuan (QY), Lingshan (LS), Dwarf White (DW), and Red Junglefowl (RJF).

Table 4. Breed-specific *HSP70* haplotypes in 10 chicken breeds.

Breed	Unique haplotypes	Number
DG	H2, H57	2
HD	H4, H31, H36	3
TB	H6, H8, H29, H42, H54	5
SY	H19, H55	2
ZJ	H11, H12, H14, H41, H45	5
XH	H10, H17, H26, H27, H52	5
QY	H3, H16, H24, H35, H51	5
LS	H9, H15, H39, H53	4
DW	H18, H25, H34, H38, H56	5
RJF	H21, H22, H33, H43, H46, H47, H49	7

Big Bone (DG), Haidong (HD), Taibai (TB), Silkie (SY), Tibet (ZJ), Xinghua (XH), Qingyuan (QY), Lingshan (LS), Dwarf White (DW), and Red Junglefowl (RJF).

Genetic diversity analysis

Haplotype diversity and nucleotide diversity of the chicken *HSP70* gene that were determined in 10 breeds in this study were 0.9740 and 0.0016, respectively, and the average number of nucleotide differences of all breeds was 4.1998 (Table 5). This revealed a high haplotype diversity of the *HSP70* gene in the 10 breeds, which approached 1 in the Qiingyuan and Xinghua breeds. The variation in the nucleotide diversity and the average number of nucleotide differences among breeds were similar. However, the Silkie breed had the low-

est haplotype diversity, nucleotide diversity, and average number of nucleotide differences, which were 0.7222, 2.5000, and 0.0010, respectively, while Red Junglefowl had the highest nucleotide diversity and average number of nucleotide differences, which were 4.800 and 0.0018, respectively.

Table 5. Haplotype diversity and nucleotide diversity of the *HSP70* gene in 10 chicken breeds.

Breed	Sample	Number of haplotypes	Haplotype diversity	Average number of nucleotide differences	Nucleotide diversity
ZJ	11	9	0.9636	3.6727	0.0014
DG	10	8	0.9330	3.4444	0.0013
HD	12	6	0.8030	3.0606	0.0012
LS	11	9	0.9636	4.6546	0.0018
QY	8	8	1.0000	4.3571	0.0017
SY	9	5	0.7222	2.5000	0.0010
TB	12	10	0.9545	3.7273	0.0014
XH	8	8	1.0000	4.4643	0.0017
DW	10	8	0.9556	4.1778	0.0016
RJF	11	10	0.9818	4.8000	0.0018
Total	102	57	0.9740	4.1998	0.0016

Big Bone (DG), Haidong (HD), Taibai (TB), Silkie (SY), Tibet (ZJ), Xinghua (XH), Qingyuan (QY), Lingshan (LS), Dwarf White (DW), and Red Junglefowl (RJF).

AMOVA

AMOVA of the chicken *HSP70* gene in 10 breeds revealed that variance within breeds only accounted for 5.37% of the total variance in the chicken *HSP70* gene, but the variance between breeds accounted for 94.63% of the total variance; the fixation index value was 0.05372 ($P < 0.01$; Table 6). These results indicated that there was significant genetic differentiation of the chicken *HSP70* gene between breeds. Further analysis of the genetic differentiation of the *HSP70* gene between breeds was performed (Table 7), and the genetic differentiation of the *HSP70* gene among breeds was significantly different.

Table 6. AMOVA of the *HSP70* gene in 10 chicken breeds.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation (%)	Fixation index (F_{ST})
Between breeds	9	6.575	0.0263	5.37	0.0537**
Within breeds	92	42.601	0.4630	94.63	
Total	101	49.176	0.4893		

d.f. = degrees of freedom. **Denotes a highly significant difference ($P < 0.01$).

Table 7. Fixation index (F_{ST}) values and the corresponding P values of the *HSP70* gene among 10 chicken breeds.

	DG	HD	TB	SY	ZJ	XH	QM	LS	DW	RJF
DG		0.0000	0.1982	0.0000	0.0270	0.3694	0.2072	0.1982	0.2522	0.0360
HD	0.1113**		0.0360	0.0000	0.0180	0.0540	0.1441	0.0000	0.0180	0.0000
TB	0.0233	0.0896*		0.0090	0.1892	0.2342	0.8018	0.1802	0.5856	0.1622
SY	0.1609**	0.2350**	0.1321**		0.0000	0.1261	0.0180	0.0180	0.0180	0.0270
ZJ	0.0513*	0.0755*	0.0186	0.1528**		0.2522	0.4595	0.0450	0.0270	0.4234
XH	0.0095	0.0752	0.0238	0.0774	0.0189		0.9910	0.4595	0.4685	0.7297
QY	0.0221	0.0545	-0.0188	0.1298*	0.0076	0.0000		0.7568	0.9910	0.4955
LS	0.0248	0.1177**	0.0186	0.0891*	0.0364*	0.0076	-0.0158		0.5676	0.3964
DW	0.0264	0.1002*	-0.0053	0.1399*	0.0404*	0.0105	-0.0287	-0.0150		0.0721
RJF	0.0422*	0.1087**	0.0246	0.0985*	0.0109	-0.0020	0.0094	0.0192	0.0312	

F_{ST} values are shown below diagonal and P values are shown above diagonal. **Denotes highly significant differences ($P < 0.01$). *Denotes significant differences ($P < 0.05$). Big Bone (DG), Haidong (HD), Taibai (TB), Silkie (SY), Tibet (ZJ), Xinghua (XH), Qingyuan (QY), Lingshan (LS), Dwarf White (DW), and Red Junglefowl (RJF).

DISCUSSION

Phenotypic differences between species can be due to genetic changes that alter gene products as well as their expression level (Hoffmann and Willi, 2008; McManus et al., 2010). Mutations in gene sequences that change gene expression by altering the morphology and physiology have been reported (Chen et al., 2007; Graze et al., 2009). Chen et al. (2011) reported that changes in the AT content of the promoter markedly affect *HSP70* expression of *HSP70*, which may play an important role in regulatory evolution. Therefore, other changes in the chicken *HSP70* gene sequence might also explain the evolution of this gene. In this study, 34 SNPs and 2 indel mutations in the chicken *HSP70* gene were identified. Thirty SNPs were transitions and the other 4 were transversions. The transition incidence was 7.4 times higher than the transversion incidence. The high incidence of transitions may be due to the location of cytosine residues in the CpG dinucleotide and could be easily changed to thymine (Wang et al., 1998). The average density of SNPs in the chicken genome was reported to be 5:1000 (Wong et al., 2004). In this study, the average density of SNPs in the chicken *HSP70* gene was 13.1:1000, indicating that the structural polymorphism of the *HSP70* gene was very rich. On the other hand, 24 SNPs in the coding region of the chicken *HSP70* gene were located in the ATP enzyme active region, polypeptide combining region, and C-terminal region, and some SNPs changed the amino acid sequence. Theses SNPs may affect the binding of peptide substrates with *HSP70* or the activation of *HSP70* (Favatier et al., 1997).

Our calculated nucleotide diversity of the chicken *HSP70* gene was lower than that of Berlin and Ellegren (2004). This conflicted with the high SNP density of the *HSP70* gene (13.1 SNPs/kb). A possible reason for this was that nucleotide variation could only occur two times at each SNP site, so the SNP density was relatively higher than the nucleotide diversity. Fifty-seven haplotypes were identified in this study; of these, 14 were shared within and between breeds and 43 were breed-specific. In the native Chinese breeds, the highest haplotype diversity approached 1 (Qingyuan and Xinghua), and the lowest haplotype diversity was 0.7222 (Silkie). This indicated that the haplotypes of the *HSP70* gene in native Chinese chickens were rich, and we believed that the rich diversity of SNPs was the direct reason for the high degree of haplotype diversity. AMOVA of the *HSP70* gene also indicated that nucleotide mutations in the *HSP70* gene were rich in native Chinese chickens. However, most of the variations were breed-specific. Therefore, we hypothesized that there was a clear genetic differentiation in the *HSP70* gene between breeds and that the sequence of the chicken *HSP70* gene that changed during the long history of evolution and natural and artificial selection may also play a role in this process.

Further analysis revealed that the genetic difference between Xinghua and other breeds was small; however, the genetic difference between Silkie and other breeds (except Xinghua) was large. Additionally, the nucleotide diversity and haplotype diversity were the lowest in the Silkie breed; this might be caused by sampling or was probably due to the inclusion of the Silkie breed in the long process of domestication and improvement, in which artificial selection can lead to beneficial allele fixation or flock expansion.

CONCLUSIONS

Our results demonstrate the abundant structural polymorphism of the chicken *HSP70* gene. The underlying mechanism of this polymorphism may be due to natural and artificial selection.

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Supplementary material

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