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Identification of Polymorphism in *HTT* Gene and its Association with Production Performances in Anhui Local Chicken Breeds

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ABSTRACT

The aims of this study were to identify genetic polymorphisms of the huntingtin (*HTT*) gene and to further investigate its association with production performances in two Anhui local chicken breeds. A total of 800 chickens (400 Huangshan black chickens and 400 Huainan partridge chickens) of similar age and weight were evaluated in the present study. We extracted DNA from blood samples and analyzed polymorphisms of *HTT* gene as wells as possible association with egg production performances including age at first egg (AFE), laying rate (LR), egg production (EP), egg weight (EW), average egg weight (AEW). Two polymorphisms (G50831C and G62976A) were detected and located in exon 42 and intron 54 in *HTT* gene, respectively. One of them (g. 50831 G>C) was a missense mutation. Genotype and allele frequency analysis showed that SNP G62976A and SNP G50831C of the *HTT* gene in chicken had some effects on reproduction traits, and the genotype GG was the advantageous genotype. Additionally, four *HTT* haplotypes (H1: GG; H2: GC; H3: AG; H4: AC) and their frequency distributions were estimated using the phase program. Haplotypes combinations constructed on these two SNPs of *HTT* gene were associated with some reproduction traits. In particular, diplotype H1H1 had positive effect on reproduction traits, diplotype H2H4 had negative effect of reproduction traits. In conclusion, our study showed that polymorphisms of the *HTT* gene were significantly associated with egg production traits in two Anhui local chicken breeds, but the suggestions that *HTT* may be considered as a potential molecular marker for the selection of production performance-related phenotypes in local chicken breeds need to be further verified.

INTRODUCTION

Huntington's disease (HD) is an inherited neurodegenerative disease affecting 1 in 10,000 adult (Gonzalez-Alegre and Afifi, 2006; Vonsattel *et al*., 1985). It was characterized by cognitive malfunction, motor deficits and psychiatric dysfunction (Boudreau *et al*., 2009; Roos, 2010; Ross and Tabrizi, 2011) and caused by the repeat polymorphisms of CAG trinucleotide in exon 1 of huntingtin (*HTT*) gene (Andrew *et al*., 1993). The *HTT* gene was one of three identified candidate genes (*HTT,* Sortilin-related VPS10 domain containing receptor 2 and Endothelin 2) of pecking behavior of chicken using the method of genome-wide association studies (GWAS) by some workers. Recently, taking into account that the confirmation and exploitation of underlying candidate genes with remarkable effects on economical traits (Zhang *et al*., 2012) in poultry breeding have inspired us with information in regard to the significance of *HTT* gene, it has been speculated that the polymorphisms of *HTT* gene might play a vital role in economical traits of chicken, including egg production traits.

The *HTT* gene is located on the long arm of chromosomal 4 at position 4p16.3. was identified in 1993 (Huntington's disease collaborative research group, 1993). It encodes huntingtin protein and is ubiquitously expressed in most tissues (Becanovic *et al*., 2015). *HTT* was originally confirmed because of the wild type huntingtin (*wHTT*) protein plays a key role in activity-dependent regulation of synaptic function and the development of nervous system. The *wHTT* protein exerts its effects by protecting against a variety of forms cytotoxicity induced by the toxic mutant huntingtin (*mHTT*) (Leavitt, 2006; Cattaneo, 2001). Remarkable is the decreased levels of wild-type *HTT* (*wHTT*) which leads to the increase in cellular toxicity of *mHTT*. The overexpression of *wHTT* is beneficial for the improvement of striatal neuronal atrophy (Van Raamsdonk *et al*., 2005; Cloes *et al*., 1998). This demonstrated that the balance of wild and mutant-type huntingtin may be a

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momentous regulator of nosogenesis in HD.

Up to now, there is no study on poultry *HTT* gene though it has been extensively studied in human and mouse. The objective of our study was to evaluate mutations in the *HTT* gene in Huangshan black Chicken and Huainan partridge chicken (Anhui native chicken populations, lower egg production) and to investigate the association of these polymorphisms with egg production traits. The candidate gene (*HTT*) was screened for polymorphism using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP). Subsequently, variations in the gene were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing methods.

MATERIALS AND METHODS

Chicken populations and trait measurement

The Huangshan black chicken and Huainan partridge chicken are meat-and egg-type cultivated breed chickens developed from local chicken breeds in the Anhui Province in China. Each population comprising 400 female individuals from one hatch was used for association analysis in our study. The chickens were raised under the same living conditions and fed on the same feed. All birds were housed individually in laying cages after the laying, and their egg production traits were observed and recorded daily after the laying. Egg production traits in this study included age at first egg (AFE), laying rate (LR), egg production (EP), egg weight (EW), average egg weight (AEW). All experimental animals were carried out in accordance with laws of the People's Republic of China regarding the Ethical Treatment of Experimental Animals.

DNA extraction and PCR amplification

Approximate 2 mL blood per chicken was gained from the wing vein and kept in a vacuum tube which contained anticoagulant acid citrate dextrose (ACD). Total DNA of all samples was extracted using a standard phenol-chloroform method (Sambrook *et al*., 1989). The DNA samples were dissolved in Tris- EDTA (TE) buffer and were stored at -20°C until used. The quality of extracted genomic DNA was checked by gel image systems.

The primers were designed on the basis of DNA sequence which contained missense mutation in the coding regions of *HTT* gene (Accession no. NC 006091.4) using the oligonucleotide design tool Primer 3.0 software (Table I). Primer synthesis was complicated by TaRaKa Biotechnology Co., Ltd. (Dalian, China). PCR amplification was performed in 15 µL volumes containing 0.8 µL of genomic DNA, 0.1 µL of each primer (100 µ/ µL), 7 µL of 2 × Taq PCR Master Mix (TianGen, Biochemical Technology Co. Ltd., Shanghai, China) and 7 µL of ddH_2O. DNA was amplified using a 5 min denaturing step at 95 °C followed by 35 cycles of 95 °C for 30 s, n °C for 30 s (n was the annealing temperature), 72°C for 30 s, and a 10 min final extension at 72 °C.

Identification of polymorphisms and genotyping

PCR products were resolved by PCR-SSCP analysis and the method was performed according to the way described by Orita *et al*. (1989). But owing to the SSCP method is inefficient and the banding pattern of the method is difficult to distinguish when more than one genotype takes place in some polymorphic fragment, we used the PCR-SSCP analysis just to identify Single Nucleotide Polymorphisms (SNPs) in *HTT* gene. After the polymorphisms were tested the PCR products of the different banding pattern were sent for direct sequencing in both directions by TaRaKa Biotechnology Co., Ltd. (Dalian, China). All sequences were analyzed with ChromasPro software (version 1.7.7, ChromasPro, Technelysium, Pty, Ltd) and blast in National Centre for Biotechnology Information (NCBI). Then we use the PCR-RFLP method for genotyping. The PCR were performed as mentioned above. PCR-RFLP reactions were performed in a 10 µL volume system containing 0.2 µL of restriction enzyme (10 U/ µL) (BtsCI and Sau3AI), 1 µL of 10 × NEBuffer, 5 µL of PCR products and 3.8 µL of ddH_2O. The PCR products were digested at 37 °C or 50 °C for 5 h, and then the digested products were separated on a 2.0% agarose gel for 45 min at 180 v.

Statistical analysis

Chi-square (X^2) tests were used to assess whether the study population deviates from Hardy-Weinberg equilibrium. Genotype and allele frequencies were directly calculated. Polymorphism information content (PIC) was performed by PopGene software (Version 1.32, University of Albert, Edmonton, Canada). The associations between SNPs with egg production traits were analyzed by the One-way ANOVA (LSD) procedures of SPSS 20.0. Haplotypes of the *HTT* gene were obtained based on the identified SNPs using the PHASE v2.1 soft (University of Chicago, Chicago, IL, USA) (Stephens *et al*., 2001). Analyses of the associations between the diplotypes and reproduction traits were carried through. The model was just like the single marker association analysis. All the values were considered significant at $P<0.05$ and were expressed as mean±standard error means (SEM).

RESULTS

SNPs of the chicken HTT *gene*

The PCR products with expected size were obtained by the primers mentioned above. Two primer pairs displayed

Table I.- Detail information for primers of the chicken *HTT* gene.

Primer name	Primer sequence	Annealing Temperature (°C)	Location[a]	Length[b]	Genotyping method
P1	F:agtaatgcagctgttgttcga R:tctaccagaaactgtgctgc	62	+50701~+50939	278	SSCP, sequencing and RFLP
P2	F:tgttcagagcccttccattt R:aggtgtccatttcagagcgt	65	+62821~+63203	382	SSCP , sequencing and RFLP

[a], referred to the locations in the *HTT* gene, the first nucleotide of translation start codon was designated as +1.
[b], length of PCR products.

Table II.- Genotypes and alleles frequencies at site NC_006091.4: g. 62976G>A, NC_006091.4: g. 50831G>C of *HTT* gene in two breed chicken.

Breeds	No.	NC_006091.4:g.50831G>C						NC_006091.4:g.62976G>A					
		Allele frequency		Genotype frequency			χ2	Allele frequency		Genotype frequency			χ2
		G	C	GG (N)	GC (N)	CC (N)		G	A	GG (N)	GA (N)	AA (N)	
HS	400	0.902	0.098	0.834(334)	0.316(55)	0.031(11)	16.31	0.895	0.105	0.800(320)	0.190(76)	0.010(4)	0.025
HN	400	0.914	0.086	0.837(335)	0.153(61)	0.010 (4)	0.37	0.897	0.103	0.814(326)	0.166(66)	0.020(8)	3.29

Brackets are the chicken individuals of respective genotypes.

polymorphisms. For primer P1, two alleles (allele G and allele C) and three genotypes (GG, GC, and CC) were observed in two chicken breeds (Fig. 1). For primer P2, three genotypes were detected in the Huangshan black chickens and Huainan partridge chickens named GG, GA, and AA, respectively (Fig. 2). By forward and reverse sequencing, the nucleotide variation at each locus was further confirmed (Fig. 3). Relative to GenBank Accession no. NC_006091.4, a G→C mutation at 50831position nucleotide (NC_006091.4: G50831C) located in exon 42 was a missense mutation (glutamate acid-to-glutamine), a G→A mutation at position 62976 nucleotide (NC_006091.4: G62976A) was located in intron 53.

Genotype frequencies of the HTT *gene in two chicken breeds*

Genotypes and alleles frequencies of *HTT* gene and *P-value* for the Hard-Weinberg equilibrium analysis were showed in Table II. For primer P1, the allele G was the dominant allele and presented the highest allele frequencies between two chicken breeds. The results of χ^2 the Huangshan black chicken breeds were deviated from Hardy-Weinberg equilibrium (*P<0.01*) and the Huainan partridge chicken breeds were accordance with the Hardy-Weinberg equilibrium *(P>0.05*). The average polymorphism information content (PIC) at the two chicken breeds were 0.1572 and 0.1443, respectively. For primer P2, the allele G was the dominant allele and presented the highest allele frequencies between two chicken breeds. The results of χ^2 the SNP were fitted to the Hardy-Weinberg equilibrium in two chicken breeds (*P>0.05*).

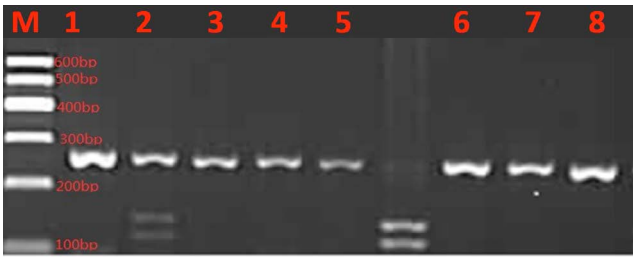

Fig. 1. PCR-RFLP analysis of PCR products amplified with primer set **P1**. **Lanes 1**, **3**, **4**, **5**, GG genotype; **2**, GC genotype; **6**, **7 and 8,** CC genotype.

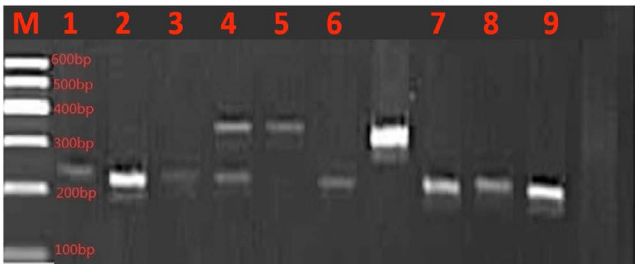

Fig. 2. PCR-RFLP analysis of PCR products amplified with primer set **P2**. **Lanes 1**, **2**, **3**, GG genotype; **4**, GA genotype; **5**, **8**, AA genotype.

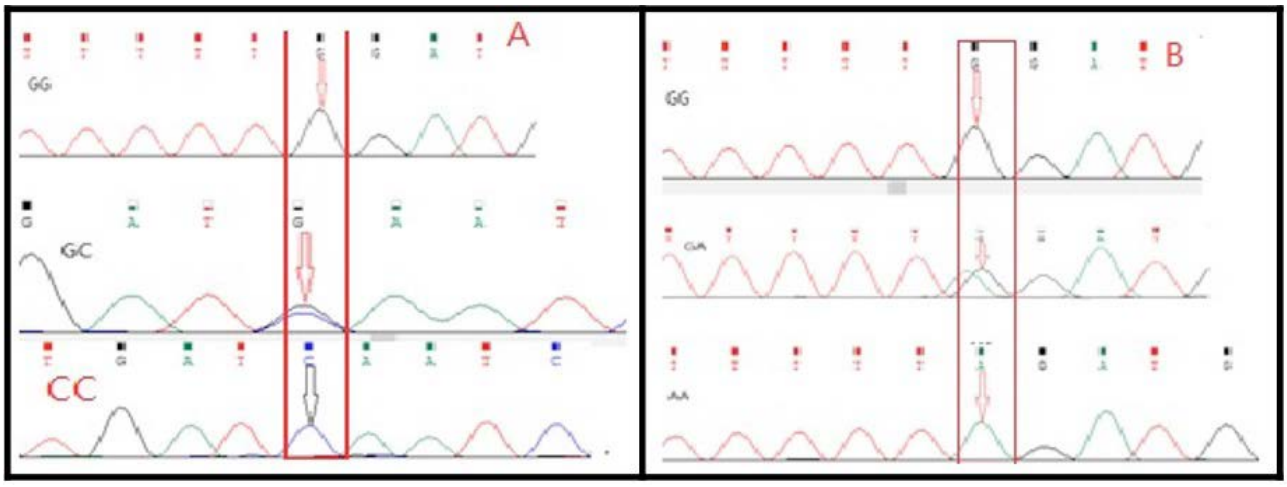

Fig. 3. Sequencing analysis of the *HTT* gene. **A**, **B**, Partial sequencing results of the P1, P2 loci, respectively; arrows indicate mutation sites.

Table III.- Relationships between genotypes and reproduction traits of the 2 polymorphic sites in Huangshan black chickens.

Traits	SNP G50831C			SNP G62976A		
	GG	GC	CC	GG	GA	AA
AFE(d)	210.72±1.10	211.66±0.04	219.00±1.01	210.63±1.15	210.92±1.90	211.00±0.12
LR(egg/d)	0.28±0.006^{a}	0.28±0.002^{ab}	0.21±0.001^{b}	0.31±0.004^{a}	0.30±0.001^{ab}	0.24±0.002^{b}
EP(egg)	45.79±0.93^{a}	44.97±0.75^{ab}	33.71±0.46^{b}	50.28±0.70^{a}	48.08±0.68^{ab}	38.33±0.38^{b}
EW(g)	2163.94±45.15^{a}	2092.93±32.59^{ab}	1567.85±21.68^{b}	2385.31±34.36^{a}	2252.87±53.01^{ab}	1745.00±25.86^{b}
AEW(g)	47.26±0.19	46.54±0.49	45.61±0.84	47.44±0.20	46.86±0.37	45.50±0.17

Abbreviations: AFE, age at first egg; LR, laying rate; EP, the total number of eggs; EW, egg weight; AEW, average egg weight.
^{a,b} Different lower case superscript were significantly different by the One-way ANOVA means in a row (P<0.05).
Data is reported as mean ± SE.

Table IV.- Relationships between genotypes and reproduction traits of the 2 polymorphic sites in Huainan partridge chickens.

Traits	SNP G50831C			SNP G62976A		
	GG	GC	CC	GG	GA	AA
AFE(d)	158.23±1.29	158.30±1.62	158.78±0.73	158.58±0.65	158.62±0.38	158.62±0.38
LR(egg/d)	0.55±0.001	0.54±0.007	0.47±0.002	0.57±0.005	0.56±0.002	0.51±0.006
EP(egg)	65.41±0.61^{a}	65.07±0.87^{ab}	58.21±0.65^{b}	68.66±0.64^{a}	67.61±0.27^{ab}	61.61±0.52^{b}
EW(g)	2833.33±19.43^{a}	2812.62±19.99^{ab}	2508.17±18.38^{b}	2983.24±23.09^{a}	2904.75±16.46^{ab}	2642.03±21.71^{b}
AEW(g)	43.32±0.27^{a}	43.22±0.15^{ab}	43.09±0.23^{b}	43.45±0.24	42.96±0.19	42.88±0.21

For Abbreviations see Table III.
^{a,b} Different lower case superscript were significantly different by the One-way ANOVA means in a row (P<0.05).
Data is reported as mean ± SE.

The average PIC at the two chicken breeds were 0.1703 and 0.1646, respectively. All the genotypes demonstrated low polymorphism.

Association of polymorphisms with egg production traits

The associations between genotypes and five reproduction traits in two chicken breeds were summarized in Tables III and IV. For Huangshan black chickens, the results indicated that LR, EP and EW were significantly associated with *HTT* genotypes at SNP G50831C ($P<0.05$). Chickens with GG genotypes at nt50831 had a higher value for LR, EP, EW than genotypes CC ($P<0.05$), and there was no significant difference between

Table V.- Haplotypes constructed with 2 SNPs and frequencies in two chicken breeds.

Haplotype	Site		Breeds	
	62976	50831	HS Frequency	HN Frequency
H1	G	G	0.817	0.840
H2	G	C	0.078	0.057
H3	A	G	0.085	0.074
H4	A	C	0.020	0.028

Abbreviations: HS, Huangshan black chickens; HN, Huainan partridge

Table VI.- Associations between diplotypes and egg production traits in Huangshan black chicken.

Diplotypes	N	Frequency (%)	Traits				
			AFE(d)	LR (egg/d)	EP(egg)	EW(g)	AEW(g)
H1H1(GG+GG)	277	0.6915	210.70±1.22	0.30±0.00[53] a	50.02±1.08	2277.95±15.19[a]	45.54±0.48[a]
H1H2(GG+GC)	34	0.0847	209.04±3.21	0.29±0.0070[a]	48.00±1.32	2265.22±13.[6]0a	47.19±0.53
H2H2(GG+CC)	9	0.0237	218.80±1.53	0.26±0.0048	44.20±1.56	1835.00±34.81	41.52±0.21
H1H3(GA+GG)	58	0.1458	211.32±2.46	0.29±0.0014	46.76±2.31	2207.29±19.26[a]	47.20±0.47
H1H4(GA+GC)	15	0.0373	216.88±3.08	0.18±0.0021[b]	46.00±1.35	2177.25±15.14[a]	47.33±0.36[a]
H2H4(GA+CC)	3	0.0068	207.50±1.50	0.17±0.0013[b]	43.08±1.21	1105.00±15.00[b]	25.65±0.33[b]
H3H3(AA+GG)	1	0.0034	211.00±0.00	0.23±0.00	42.00±0.00	1515.00±0.00	36.07±0.00
H3H4(AA+GC)	3	0.0068	205.00±1.60	0.25±0.0034	49.50±1.50	1860.00±22.50	37.57±0.44
H4H4(AA+CC)	0	0	--	--	--	--	--

For Abbreviations see Table III.
[a,b] Different lower case superscript were significantly different by the One-way ANOVA means in a row ($P<0.05$).
Data is reported as mean ± SE.

Table VII.- Associations between diplotypes and egg production traits in Huainan partridge chickens.

Diplotypes	N	Frequency (%)	Traits				
			AFE(d)	LR (egg/d)	EP(egg)	EW(g)	AEW(g)
H1H1(GG+GG)	286	0.7148	159.19±1.23	0.56±0.009[a]	61.15±1.16[a]	2645.37±15.4[8]a	43.26±0.21
H1H2(GG+GC)	40	0.1007	159.25±0.85	0.51±0.002[a]	61.13±11.26[a]	2337.90±16.88	38.23±0.65
H2H2(GG+CC)	1	0.0034	159.38±1.65	0.45±0.001	54.00±1.21	2457.00±13.22	45.50±0.35
H1H3(GA+GG)	44	0.1107	160.23±0.98	0.47±0.002	60.67±1.74[a]	2608.60±18.34[a]	42.99±0.47
H1H4(GA+GC)	19	0.0470	161.31±1.24	0.41±0.003[b]	49.14±1.49[b]	2097.64±16.79[b]	42.68±0.95
H2H4(GA+CC)	1	0.0034	159.85±1.38	0.45±0.002	54.00±1.15	2333.80±12.85	43.21±0.68
H3H3(AA+GG)	6	0.0133	159.32±1.48	0.52±0.005	60.50±1.84	2440.38±18.07	40.34±0.59
H3H4(AA+GC)	0	--		--	--	--	
H4H4(AA+CC)	3	0.0067	161.35±1.51	0.48±0.004	58.00±1.11	2445.35±14.45	42.16±0.17

For Abbreviations see Table III.
[a,b] Different lower case superscript were significantly different by the One-way ANOVA means in a row ($P<0.05$).
Data is reported as mean ± SE.

chickens with GG and GC (*P>0.05*). For g.62976G>A, the genotypes of at nt62976 were significantly associated with LR, EP and EW (*P<0.05*), but not associated with the other reproduction traits (*P>0.05*). The LR, EP and EW of chicken with the GG genotypes was significantly higher than those of chickens with the AA genotypes (*P<0.05*), there was no significant difference between chickens with GA and AA (*P>0.05)*. For Huainan partridge chickens, the results demonstrated that traits EP, EW and AEW were significantly associated with *HTT* genotypes at SNP G50831C (*P<0.05*), chickens with GG genotypes had significantly higher EP, EW and AEW than those of CC genotypes (*P<0.05*). There were no significant differences for other traits among genotypes. For g.62976G>A, the genotypes of at nt62976 were significantly associated with EP, EW (*P<0.05*), but not associated with the other reproduction traits (*P>0.05*). The EP and EW of chicken with GG genotypes was significantly higher than those of chickens with the AA genotypes (*P<0.05*), there was no significant difference between chickens with GA and AA (*P>0.05*). The G allele at these two SNPs had a favorably positive effect on LR, EP and EW.
chickens.

Haplotypes associations of diplotypeswith egg production traits

In the present study, there were four haplotypes in two chicken breeds. A total of 8 diplotypes were detected in all samples. The frequencies of haplotypes of both breeds are listed in Table V. The ANOVA analysis indicated that there were significant associations between diplotypes and reproduction traits in two breeds (Tables VI and VII). Huangshan black chickens with H1H1 diplotype had a significant positive effect on LR and EW than H1H4 and H2H4 diplotypes (*P*<0.05). H2H2 diplotype had significant higher AEW than H1H4 diplotype (*P*<0.05). Huainan partridge chickens with H1H1 and H1H2 diplotypes had higher LR than H1H3 diplotype (*P*<0.05). H1H1, H1H2 and H1H3 diplotypes were significantly higher than diplotype H1H4 in EP (*P*<0.05). H1H1 and H1H3 diplotypes had higher EW than H1H4 diplotype (*P*<0.05).

DISCUSSION

Egg production performance determines the economic benefits in laying hen production system owing to its effects on productivity (Zhu and Jiang, 2014). Inspecting the balanced selection and the metabolism in chickens for tune performance has become a heated topic in research (Ou *et al*., 2009). The candidate gene method is turning to be increasingly powerful for detecting candidate genes and single nucleotide polymorphisms with dramatic effects on economic characteristics (Kanehisa *et al*., 2002; Cogburn *et al*., 2008; Kuhn *et al*., 2008).

As mentioned earlier, *HTT* gene expressed in most of tissues, encoded a protein involved in multitudinous cellular processes, including RNA splicing, trafficking, endocytosis and cellular homeostasis (Harjes and Wanker, 2003). Mutant *HTT* may damage innate immune cell function (Andre *et al*., 2016). Furthermore, *HTT* gene may influence body weight by regulating metabolism. Mice with overexpressed wild-type *HTT* show increased body weight and increased weights for some organisms (Pouladi *et al*., 2010; Van Raamsdonk *et al*., 2006). Also the level of body weight can affect the egg production (Karplus and Schulz, 1985). Despite the mechanism by which *HTT* gene polymorphisms related to reproduction traits remaining unclear, by combining the present and previous data, we can propose a hypothesis that *HTT* gene polymorphisms usually have highly negative effects on egg performances.

In the present study, we sequenced all the PCR products of P1and P2 loci and confirmed 2 SNPs of the *HTT* gene. One missense mutation (50831 G→C) in *HTT* gene was detected, which was located in exon 42 and could mutate glutamic acid mutation into glutamine. Another SNP 62976G>A, located in intron 53 and therefore does not affect the protein sequence of aromatase. However, mutations in introns sometimes can be associated with regulatory sequences. Also, being an intronic SNPs, the likelihood of a direct influence on the egg reproduction traits is rather small. However, variations in intronic SNPs could have other effects such as influencing splicing or regulatory processes by affecting the binding of transcriptions factors to the gene (Gao *et al*., 2010, Oczkowicz *et al*., 2012). This hypothesis needs to be affirmed.

The association analysis of single SNPs demonstrated that polymorphisms of *HTT* gene is significantly associated with reproduction traits in Anhui local chicken breeds. The results revealed that the GG genotype was associated with greater EW and higher AEW, which coincides with the positive correlation between EW and AEW. However, some studies had indicated that associations of haplotypes and diplotypes with significant economic traits were more precise than single SNPs (Daly *et al*., 2001; Zhang *et al*., 2008). We used the software PHASE v2.1 to get information on the interactions between SNPs. According to the association of haplotypes and diplotypes with reproduction traits, we found that the H1 haplotype had the highest frequencies and H4 had the lowest frequencies in these two chicken breeds. In addition, in Huangshan black chickens, the H1H1 dyplotypes had greater LR, EP and EW; the H2H2 had later AFE and higher AEW. In Huainan partridge chickens, the highest LR, EP and EW were detected with diplotype H1H1; the H4H4 diplotype

had later AFE and the H2H2 diplotype had greater AEW.

CONCLUSIONS

Our results not only illustrated that polymorphisms of the *HTT* gene were significantly associated with egg production traits in two Anhui local chicken breeds, but also broadened the scope of study on egg reproduction traits. However, despite the mutational population had inferior phenotype in our study, some limitations of this study need to be considered. First of all, the limited population and less statistical data didn't enable us determine how the interactions between miscellaneous SNPs affect the reproduction traits. Moreover, potential interactions between genetic and environmental factors could not be assessed because of the lack of data. Generally speaking, future studies with lager chicken sample sizes as well as functional evaluation of the *HTT* gene polymorphisms are strongly recommended.

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Conflict of interest statement

We declare that we have no conflict of interest.

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