Investigation of association of MC1R gene diversity with feathers colour trait in chicken using DNA sequencing data

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Abstract In chickens, the Extended black (E) locus encodes the melanocortin 1-receptor (MC1R), a G-protein coupled receptor that influence the production of eumelanin and pheomelanin, so that plays a fundamental key in plumage coloration. In this paper, the genetic diversity of a partial coding region of MC1R gene was investigated in Iraqi native chicken breeds using single nucleotide polymorphism (SNP) method. Furthermore, the association between genetic diversity with coat colour was studied as well as. Ninety-five animals were sampled and after extracting DNA, a 676-bp fragment of the MC1R gene was amplified by PCR. For detecting SNP in amplified fragment the DNA sequencing method was used. The result depicted six SNPs, including one synonymous (D78272:g.539G>A) and five nonsynonymous (D78272:g.524C>A, D78272:g.531A>T, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G) nucleotide substitutions. The allele frequencies were 0.5/0.5, 0.46/0.54, 0.5/0.5, 0.5/0.5, 0.65/0.65 and 0.57/0.43 for D78272:g.524C>A, D78272:g.531A>T, D78272:g.539G>A, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G mutations, respectively. Although the significant SNPs have been identified and all of them had high genetic diversity, there was not any association between the observed SNPs and coat colour ($p > 0.05$). It may be additional work can beneficial to clear the role of these SNPs.
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Introduction

The natural, sexual selection, and human breeding programs have role keys in the plumage colors and patterns of domestic avian species (Cheviron et al., 2006). Since genetic markers are benfical for the identification of avian breeds/subspecies, the significant work related to the association between these markers with plumage color have been performed (Hoque et al., 2013). Many genes influenced the plumage color in vertebrates. The E locus is one the key gene linked with color, several alleles were reported for it including E*E (extended black), E*R (birchen), E*WH (dominant wheaten), E*N (wild type), E*B (brown), E*BC (buttercup), and E*Y (recessive wheaten) (Crittenden et al., 1996; Smyth Jr, 1990).

The E locus encodes the melanocortin 1-receptor (MC1R), a seven-transmembrane domain G-protein coupled receptor which is found primarily in melanocytes and affects the production and relative distribution of the eumelanin and pheomelanin pigments(Mountjoy et al., 1992). Following activation by the α-melanocyte-stimulating hormone (α-MSH), MC1R leads to an increase in intracellular cAMP which activates tyrosinase. Tyrosinase finally induces the production and release of eumelanin in melanocytes, resulting in the expression of darker (black to brown) plumage color. A low level of tyrosinase, resulted from MC1R loss of function, can lead to increased deposition of pheomelanin and emergence of lighter (red to yellow) phenotypes (Dávila et al., 2014; Hubbard et al., 2010). Since MC1R is highly conserved among vertebrates and is easily identifiable in a diversity of taxa due to its simple genetic structure (single 1 kb exon), a large volume of studies have been performed to examine the possible relationship between MC1R variations and hair/skin/plumage coloration in a wide variety of unrelated species (Dávila et al., 2014; Hoekstra, 2006; Hoque et al., 2013; Hubbard et al., 2010; Kerje et al., 2003; Mundy, 2005; Ran et al., 2016; Roulin, 2004). Interestingly, the majority of these studies, particularly in birds, have made effort to associate single nucleotide polymorphisms (SNPs), and the corresponding amino acid changes, with observed color polymorphisms (Hoque et al., 2013; Huang et al., 2014; Rahayu et al., 2015; Ran et al., 2016; Theron et al., 2001). Despite the abundance of studies on bird species, the association between MC1R variation and plumage color in the domestic chicken, *Gallus gallus*,

has been examined only in a few breeds. An intercross between the red jungle fowl, (*i.e.* the ancestor of the modern domestic fowl), and White Leghorn chickens revealed that the Extended black (E) locus, controlling the relative amounts of eumelanin and phaeomelanin in chicken melanocytes, is equivalent to MC1R gene (Kerje et al., 2003). The first evidence for the association between MC1R polymorphism and plumage coloration of chickens was provided by Takeuchi et al. (1996). In chickens, the most dominant allele (E) has MC1R in its active form and expresses a uniformly black coloration. By contrast, in the most recessive allele (e^y), MC1R contains amino acid substitutions that cause functional deficiency, thus express a uniformly red-yellow phenotype (Takeuchi et al., 1996). Heo et al. (2011) found three SNPs associated with shank and feather colors in four Korean chicken breeds(Heo et al., 2011). In another study, Hoque et al. (2013) detected nine SNPs of MC1R gene in five Korean native chicken breeds, of which, six SNPs were nonsynonymous. A total of four single nucleotide substitutions, including three nonsynonymous SNPs, were identified between the red jungle owl and White Leghorn chickens (Kerje et al., 2003). It has detected 11 SNPs from *MC1R* gene of 11 Spanish chicken breeds, nine of them resulted in amino acid substitution(Dávila et al., 2014).

These results showed that MC1R gene has a crucial key in the coat colour of chicken. Iraqi native chicken has different colors and since MC1R gene is one of the fundamental genes in the coat colour of chicken, so the objective of the present study was to investigate the genetic diversity of the partial coding regions of MC1R gene and its association with coat colour in Iraqi native chicken.

Materials and methods

Animals

A total of 95 individuals, belonging to Iraqi native chickens, were obtained from Abu Ghraib's research station (Baghdad, Iraq). Blood samples were collected from the external jugular vein and stored at -20 °C for DNA extraction. This study was approved by the Institutional Animal Care and Use Committee of the University of Tehran.

DNA extraction and amplification

Genomic DNA was extracted from the whole blood samples using salting out method (Miller et al., 1988) and stored at -20 °C until being used for PCR amplification. Based on the chicken *MC1R* gene sequence present in the Genbank with accession number of D78272.1, one pair of primers (F:5'-ATCCCCTCTGCCTCGTGAC-3' and R:5'-GTGATTAAGACGGTGCTGGAGAC-3') was designed to amplify a 676-bp fragment of the coding region of chicken *MC1R* gene using polymerase chain reaction (PCR). The PCR was performed in a 30 µL reaction mixture, containing 15 µL of PCR Master Mix, 1 µL of each primer, 2 µL of DNA and 11 µL of nuclease free water. The PCR temperature profiles consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 63 °C for 30 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. Electrophoresis of PCR products was performed in 1% (w/v) agarose gel in parallel with 100 bp DNA marker, in 1x TAE buffer at a fixed voltage of 90 V for 30 min. After ethidium bromide staining, the products were visualized by ultraviolet trans illumination.

Sequencing and Analysis

All PCR products were subjected to sequence analysis using ABI 3730 XL DNA Analyzer (BioNeer, Daejeon, South Korea). The chicken *MC1R* nucleotide sequences were aligned using ClustalW program available in Bioedit software and then, SNPs were identified and each animal was genotyped for SNPs. Genotypic, allelic frequencies and Hardy–Weinberg equilibriums were estimated using GenAlEx 6.41 software. The analysis of associations of the animal genotypes with coat colour were done in the Genmod of SAS 9.1 (Institute, 1985)

Results and Discussion

Table 1 dipected allele frequency and the Chi square (χ2) test of Hardy-Weinberg equilibrium (HWE) of these SNPs. The observed mutations revealed high genetic diversity within MC1R gene in analyzed population (Table 1). Among the observed SNPs, D78272:g.732A>G mutation showed the least frequency. The allele frequency of D78272:g.524C>A, D78272:g.531A>T, D78272:g.539G>A, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G mutations were observed 0.5/0.5, 0.46/0.54, 0.5/0.5, 0.5/0.5, 0.65/0.65 and 0.57/0.43, respectively (Table 1).

The all amplified fragments (e.g. 95 samples) were initially sequenced and Bioedit software was used to compare these sequences with NCBI reference sequence D78272.1. the result of sequence analyzez showed six SNPs in the partial coding regions (676 bp) of the MC1R gene in Iraqi native chicken. These polymorphisms are D78272:g.524C>A, D78272:g.531A>T, D78272:g.539G>A, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G. (Fig. 1).

Table 1. Allele frequencies of the MC1R gene and the test Hardy–Weinberg for level of significance of the deviation within population.

The position of SNP	Allele		value (HWE)
	Wild allele	Mutant allele	
D78272:g.524C>A	0.5	0.5	p<0.01
D78272:g.531A>T	0.46	0.54	p<0.01
D78272:g.539G>A	0.5	0.5	p<0.01
D78272:g.730C>A	0.5	0.5	p<0.01
D78272:g.732A>G	0.65	0.35	p<0.01
D78272:g.783A>G	0.57	0.43	p<0.01

Fig. 1. Comparative alignment of conceptualized nucleotide sequence of MC1R gene in Iraqi native chicken with NCBI reference sequence D78272.1.

Furthermore, all of the six detected SNPs were not in HWE (P<0.01) (Table 1). There was not any association between different genotypes and coat colour in Iraqi native chicken (p> 0.05). Since the size population is low, it can decerse the capture of quantitative trait locus associated with coat color in this study.

The coat color is one of the important characteristics for identifying breed in domestic animal species. So the association between host genetics and coat color was much suited for detecting SNPs linked with this trait. Recently, some SNPs related to different phenotypes (ranging from the dominant extended black to the recessive yellow) have been identified in the chicken *MC1R* gene (Kerje et al., 2003; Takeuchi et al., 1996). For example, the distribution of the SNP, 69T>C, has been studied in some Korean native breeds(Hoque et al., 2013). Based on this study, the CC genotype is abundant in yellow Korean native chicken (80%), red Korean native chicken (66.7%), and white Leghorn (100%) chicken breeds, whereas the TT genotype is common in black Korean native chicken (56.7%) and black silky (80%) breeds (Hoque et al., 2013). It had reported that the ED and E+ alleles are associated with black coat colour and a combination of red or reddish brown/black coat colors, respectively in cattle (Klungland et al., 1995). In lines with different plumage color, 8 SNPs are associated with 4 haplotypes in the coding region of MC1R gene in chickens(Tixier-Boichard et al., 2006). It has been observed that the expression of MC1R was significantly different at 56 d of age in a cross between chickens of white and black color, but it was not different in other days. Some SNPs of the MC1R gene are associated with the E locus and may be associated with feather pigmentation. Moreover, it has reported that there was a close association between MC1R polymorphism and the E locus (Okimoto et al., 1999). Also, our sequence analysis revealed that five of the observed six SNPs were non-synonymous (e.g. D78272:g.524C>A, D78272:g.531A>T,

D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G) and one was synonymous (e.g. D78272:g.539G>A), as D78272:g.524C>A, D78272:g.531A>T, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G, reflecting an arginine into serine replacement, a threonine into serine replacement, an alanine into aspartic replacement, a lysine into glutamic replacement and a serine into glycine replacement, respectively (Table 2).

Table 2. The effect of nucleotide substitutions on amino acid of chicken MC1R protein.

Nucleotide substitution	Position	Changed amino acid	The type of SNP
C/A	D78272:g.524C>A	Serine converted into an arginine	nonsynonymous
A/T	D78272:g.531A>T	Threonine converted into a serine	nonsynonymous
G/A	D78272:g.539G>A	Glycine	synonymous
C/A	D78272:g.730C>A	Alanine converted into an aspartic	nonsynonymous
A/G	D78272:g.732A>G	Lysine converted into a glutamic	nonsynonymous
A/G	D78272:g.783A>G	Serine converted into a glycine	nonsynonymous

These results imply that these mutations can affect the function of MC1R protein, suggesting that these polymorphisms can be used as molecular markers for plumage color in chicken. It has been suggested that six nonsynonymous SNPs (namely p.M71T, p.E92K, p.A126I, p.T143A, p.C213R, and p.H215P) are strongly associated with plumage colors in chicken. Davila et al. (2014) detected a total of 11 SNPs in a 1,056-bp fragment of MC1R gene, of which two were synonymous (C69T and C834T) and others were nonsynonymous (T212C, G274A, G376A, T398AC, G409A, A427G, C637T, A644C, and G646A). The nonsynonymous mutations resulted in amino acid substitutions of Met72Thr, Glu92Lys, Val126Ile, Leu133GlnPro, Ala137Thr, Thr143Ala, Arg213Cys, His215Pro, and Val216Ile, respectively. A nonsynonymous SNP (Glu92Lys) resulting in activation of MC1R gene and production of eumelanin has been also detected by Takeuchi et al. (1996). Hoque et al. (2013) identified two nonsynonymous SNPs in Korean native chickens resulting in amino acid substitutions of Val126Ile and Ala143Thr. According to previous studies, the polarity of amino acids can affect the signal transduction of the MC1R gene and ultimately lead to color variations. Although there was not any association between the observed SNPs and coat colour, our finding identified six SNPs for MC1R gene in Iraq indigenous chicken and it appears that the considering theirs association with other biophysical and biochemical indexes in chicken is beneficial.

Conclusion

The result of the present study sowed a high genetic diversity for the MC1R gene in Iraqi native chickens; the analysis of DNA sequencing could identified six mutations. The allele frequency of D78272:g.524C>A, D78272:g.531A>T, D78272:g.539G>A, D78272:g.730C>A, D78272:g.732A>G and D78272:g.783A>G mutations were observed 0.5/0.5, 0.46/0.54, 0.5/0.5, 0.5/0.5, 0.65/0.65 and 0.57/0.43, respectively. Furthermore, all of the six detected SNPs were not in HWE ($P<0.01$) (Table 1). Although the significant SNPs have been identified and all of them had high genetice diversity, there was not any association between the observed SNPs and coat colour ($p> 0.05$). It appears that further studies can beneficial to detect the function of these SNPs.

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