Study of chicken PMEL17 gene polymorphism using DNA sequencing method in chicken and their effects on protein function

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Abstract: The premelanosome protein 17 gene (PMEL17) plays an important role in plumage/coat pigmentation in a variety of taxa. The transmembrane protein encoded by PMEL17 is responsible for the formation of amyloid fibrils in melanosomes, the striations upon which melanin is deposited. In this paper, the genetic diversity of a partial coding region of *PMEL17* gene was investigated in Iraqi native chicken breeds using single nucleotide polymorphism (SNP) method. DNA was extracted from 117 blood samples and a 428-bp fragment of *PMEL17* gene was sequenced following PCR amplification. A total of eight SNPs, including four nonsynonymous (AY636125:g.1981C>T, AY636125:g.2128C>A, AY636125:g.2140C>T, and AY636125:g.2167A>T) and four synonymous (AY636125:g.1928C>T, AY636125:g.1992C>T, AY636125:g.2127C>T, and AY636125:g.2162G>A) mutations were detected with an allelic frequency of 0.92/0.08, 0.66/0.34, 0.95/0.05, 0.89/0.11, 0.97/0.03, 0.96/0.04, 0.28/0.72, and 0.32/0.68, respectively. The possible impacts of amino acid substitutions on physicochemical properties and three dimensional structures of translated proteins were also investigated. According to the results, one of the nonsynonymous mutations (AY636125:g.2162G>A) significantly altered the isoelectric point and net charge, while the others did not have significant effect on these indices. Given the high genetic diversity of *PMEL17* gene in Iraqi chickens and potential effects of at least one SNP on protein function, this gene may be a suitable marker to investigate its association with plumage color in domestic chicken.
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Introduction

A major goal of evolutionary biology is to identify the genes/loci that underlying phenotypic variations in animal species (Bradley and Lawler, 2011). Domestic chickens (*Gallus gallus*) exhibit a wide range of plumage coloration in comparison with their wild ancestor, the red junglefowl (Kerje et al., 2004). The chicken plumage color is controlled by a variety of genes, including *PMEL17*. This gene encodes the premelanosome protein PMEL17 (also known as gp100, Silv, ME20), a protein that is located in the vesicular structures of premelanosomes and plays an important role in plumage/hair pigmentation by triggering the maturation of premelanosomes into melanosomes (Raposo et al., 2001). In chickens homozygous for the wild type allele, PMEL17 drives the formation of amyloid fibrils in melanosomes, the striations on which melanin is deposited. These chickens exhibit full plumage pigmentation and their phenotype would depend on the genotype at other loci affecting plumage color (Yasumoto et al., 2004). The white plumage color in the White Leghorn, one of the world's most common chicken breeds, arises from the Dominant white (I) mutation resulted from a 9-bp insertion in exon 10 of the *PMEL17* gene. Consequently, chickens homozygous for Dominant white fail to develop mature melanosomes, and therefore, exhibit a completely non-pigmented (white) plumage(Kerje et al., 2004).

During the last decade, the polymorphism and function of *PMEL17* gene has received growing attention because not only this gene plays important roles in plumage/coat color, but also it is suspected to contribute to a variety of social, aggressive, physiological and exploratory behaviors (Bright, 2007; Karlsson et al., 2011; Keeling et al., 2004; Lindqvist et al., 2007; Nätt et al., 2007). For example, the White Leghorns have been reported to have a poorer learning capacity than the red junglefowl, in term of making a connection between food source and symbol and distinguishing the food source from a distance (Lindqvist et al., 2002). It has been reported that, in contrast to the red junglefowl, the gene expression and some behavioral responses to stress in the White Leghorn were transmitted across generations, indicating that the ability to transmit epigenetic and behavioral information between generations are probably favored at the course of domestication

(Lindqvist et al., 2007). Karlsson et al. (2010) reported that the wild type genotype is more active, aggressive, and explorative, but less socially interactive, than the White Leghorn (Karlsson et al., 2010). In some cattle breeds such as Simmental and Hereford, two mutations in *PMEL17* gene have been reported to associate with the coat-colour dilution and hypotrichosis disorder. They include a 3-bp deletion (CTT) at nucleotide 54 in exon 1, which cause the deletion of a leucine from the encoded protein and a C to A nucleotide substitution at codon 612 in exon 11 which results in substitution of alanine by glutamic acid (Jolly et al., 2008). As another example, chickens with the white dominant allele have been shown to be the victim of feather-pecking, less frequently than those expressing the wild recessive allele at *PMEL17* (Bright, 2007; Keeling et al., 2004; Nätt et al., 2007). According to a general view, reduction of plumage pigmentation is among the first responses during domestication, indicating that low pigmented phenotypes are more likely to adapt to dealing with the stress of captivity (Karlsson et al., 2010).

Given the contribution of *PMEL17* genotype to a wide variety of biological, social and exploratory behaviors, besides direct effects on plumage coloration, the study of genetic diversity of *PMEL17* in chicken breeds and its consequences on the function and structure of the encoded protein may be an interesting subject of study. Therefore, the objective of the present study was to investigate the genetic diversity of the partial coding regions of the *PMEL17* gene in Iraqi native chicken breeds using single nucleotide polymorphism (SNP) method. In addition, the possible impact of amino acid substitutions on physicochemical properties and structure of the *PMEL17* protein was investigated.

Materials and methods

Animals

We totally collected 117 blood samples from the external jugular vein of Iraqi native chicken breeds. All samples used in this study were obtained from Abu Ghraib's research station (Baghdad, Iraq) 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, Iran.

DNA extraction and amplification and sequencing

Genomic DNA was extracted from whole blood samples using salting out method (Miller et al., 1988) and stored at -20 °C until being used for PCR amplification. Part of coding region of the *PMEL17* gene (428 bp) was amplified using the primer pairs: F:5'- CCGTCACCCACACCTACCTG -3' and R:5'-CGGAGCATCACCACCTGAG-3', which were designed based on the chicken *PMEL17* gene encoding sequence (GenBank, Accession Number AY636125.1).

The PCR was carried out 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, under the following thermal cycle conditions: 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 transillumination.

All PCR products were subjected to sequence analysis using ABI 3730 XL DNA Analyzer (BioNeer, Daejeon, South Korea). The chicken *PMEL17* nucleotide sequences were edited and aligned using ClustalW program available in Bioedit software and then, SNPs were detected and each animal was genotyped for SNPs. Genotypic, allelic frequencies and Hardy–Weinberg equilibriums were examined by the use of GenAlEx 6.41 software.

Protein sequence and structural analysis

In order to analyze the sequence and structure of proteins, the sequence of chicken mRNA, translated from *PMEL17* gene, was recaptured from NCBI GenBank databases (GenBank Accession Number AY636125.1). BioEdit software was used to detect the mutations and nucleotide sequences of the translated proteins. Also, we used GPMAW and ProtParam tools, available in ExPASy website, to analyze the possible impact of amino acid substitutions on physicochemical parameters of the chicken *PMEL17* proteins. I-TASSER software was used to design the three-dimensional (3D) structure of the PMEL17 protein. Furthermore, we used the MEGA4 software, to draw the phylogenetic tree of the relevant nucleotide sequences.

Results and Discussion

The coat/plumage color of animals is controlled by the function of a diverse array of genes including *TYR, TYRP1, TYRP2* (*DCT*), *PMEL17, SLC45A2* (*MATP, AIM-1*), and *MC1R*, all of which are regulated by the microphthalmia-associated transcription factor (MITF) (Gunnarsson et al., 2009). The *Dominant white* locus (*PMEL17*) is one of the major loci affecting feather pigmentation in domestic chickens. In the wild type recessive allele of this gene (i/i), the encoded PMEL17 protein polymerizes into amyloid fibrils (striations) upon which the melanin pigments are deposited. Mutations in the *PMEL17* gene (either insertions, deletions, or/and single nucleotide substitutions) have been reported to associate with

plumage/coat pigmentation and diverse behavioral variations in mammals and birds (Bright, 2007; Brunberg et al., 2006; Keeling et al., 2004; Kerje et al., 2004; Martínez-Esparza et al., 1999; Nätt et al., 2007). In mice, for example, a single nucleotide substitution (G to A) can generate a premature stop codon that truncates the last 25 amino acids of the encoded protein and results in expression of silver coloration (Martínez-Esparza et al., 1999). A missense mutation in exon 11 of *PMEL17* gene of horse has been found to change the second amino acid in the cytoplasmic region from arginine to cysteine (Arg618Cys), resulting in expression of silver phenotype (Brunberg et al., 2006). In homozygous (I/I) and heterozygous (I/i) chicken genotypes, the mutant *PMEL17*, experiencing a 9-bp insertion in exon 10, inhibits feather pigmentation, resulting in expression of white phenotype (Kerje et al., 2004). The *Smoky* is another allele giving a grayish phenotype and is recessive to *Dominant white* (I/I), but partially dominant to the wild-type allele (i/i). As a mutant of a line of White Leghorn, the *Smoky* allele shares the 9-bp insertion in exon 10 with *Dominant white*, but experiences a second mutation, where the deletion of 12 nucleotides in exon 6 has resulted in elimination of four amino acids from the mature protein and partial restoration of plumage pigmentation. Similarly, a deletion of 15 nucleotides from the Dominant white allele yields a new allele, the Dun (I*D), which results in expression of dun coloration (Kerje et al., 2004).

We amplified and sequenced a 428-bp fragment of the coding region of *PMEL17* gene in a total of 117 Iraqi native chicken breeds and compared the sequences with the NCBI reference sequence for this gene (Accession Number AY636125.1) using Bioedit software. Sequence analysis revealed eight single nucleotide substitutions (SNPs) within the amplified region, including AY636125:g.1928C>T, AY636125:g.1981C>T, AY636125:g.1992C>T, AY636125:g.2127C>T, AY636125:g.2128C>A, AY636125:g.2140C>T, AY636125:g.2162G>A, and AY636125:g.2167A>T. (Fig. 1).

Fig. 1. Comparative alignment of conceptualized nucleotide sequence of PMEL17 gene in Iraqi native chicken with NCBI reference sequence AY636125.1.

Table 1. Allele frequencies of the PMEL17 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	
AY636125:g.1928C>T	0.97	0.03	p<0.01
AY636125:g.1981C>T	0.92	0.08	p<0.01
AY636125:g.1992C>T	0.96	0.04	p<0.01
AY636125:g.2127C>T	0.28	0.72	p<0.01
AY636125:g.2128C>A	0.66	0.34	p<0.01
AY636125:g.2140C>T	0.95	0.05	p<0.01
AY636125:g.2162G>A	0.32	0.68	p<0.01
AY636125:g.2167A>T	0.89	0.11	p<0.01

HWE = Hardy-Weinberg equilibrium

The allele frequencies as well as the Chi square ($\chi 2$) tests of Hardy-Weinberg equilibrium (HWE) for these mutations have been listed in Table 1. The allele frequency were estimated as 0.97/0.03, 0.92/0.08, 0.96/0.04, 0.28/0.72, 0.66/0.34, 0.95/0.05, 0.32/0.68 and 0.89/0.11 for AY636125:g.1928C>T, AY636125:g.1981C>T, AY636125:g.1992C>T, AY636125:g.2127C>T, AY636125:g.2128C>A,

AY636125:g.2140C>T, AY636125:g.2162G>A, and AY636125:g.2167A>T mutations, respectively (Table 1). The results revealed that three SNPs (*i.e.* AY636125:g.2127C>T, AY636125:g.2128C>A, and AY636125:g.2162G>A) had high genetic diversity among individuals, while the other mutations (AY636125:g.1928C>T, AY636125:g.1981C>T, AY636125:g.1992C>T, AY636125:g.2140C>T, and AY636125:g.2167A>T) had low genetic diversity within *PMEL17* gene (Table 1). In addition, none of the mutations were in HWE (P<0.01) (Table 1).

Out of the eight SNPs detected, four mutations (AY636125:g.1928C>T, AY636125:g.1992C>T, AY636125:g.2127C>T, and AY636125:g.2162G>A) were nonsynonymous, resulting in amino acid substitution of proline to serine, threonine to methionine, proline to leucine, and aspartic to asparagine, respectively (Table 2). The four other mutations (AY636125:g.1981C>T, AY636125:g.2128C>A, AY636125:g.2140C>T, and AY636125:g.2167A>T) were synonymous in term of amino acid substitution (Table 2). Simulation of the three-dimensional structures for *PMEL17* protein (Fig. 2) as well as analysis of phylogenetic tree for *PMEL17* gene revealed that the chicken *PMEL17* mRNA sequence had low similarity to other species (Fig. 3).

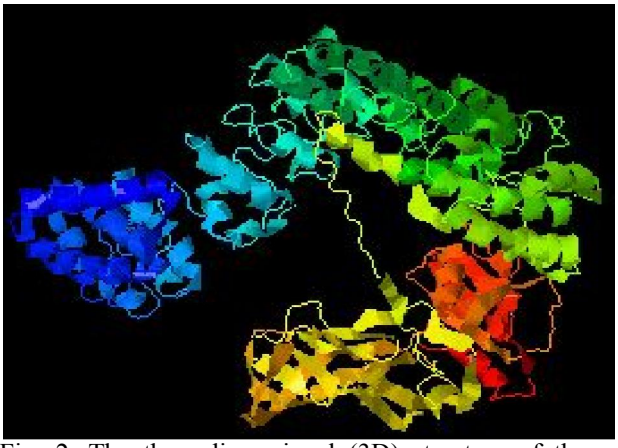

Fig. 2. The three-dimensional (3D) structure of the PMEL17 protein.

Table 2. The effect of nucleotide substitutions on amino acid of chicken PMEL17 protein.

Nucleotide substitution	Position	Changed amino acid	The type of SNP
C/T	AY636125:g.1928C>T	Proline converted into a serine	Nonsynonymous
C/T	AY636125:g.1981C>T	Serine	Synonymous
C/T	AY636125:g.1992C>T	Threonine converted into a methionine	Nonsynonymous
C/T	AY636125:g.2127C>T	Proline converted into a leucine	Nonsynonymous
C/A	AY636125:g.2128C>A	Proline	Synonymous
C/T	AY636125:g.2140C>T	Proline	Synonymous
G/A	AY636125:g.2162G>A	Aspartic converted into a asparagine	Nonsynonymous
A/T	AY636125:g.2167A>T	Alanine	Synonymous

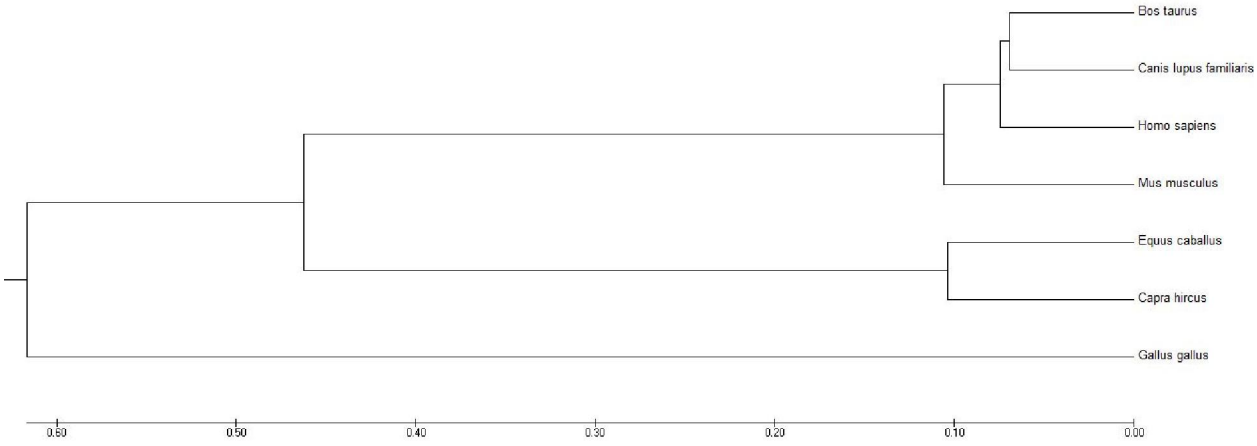

Fig. 3. Phylogeny tree of PMEL17 gene sequences were drawn using the ClustalW and MEGA4 methods of sequence alignment.

In addition, we used the GPMAW and ProtParam tools to explore the potential effects of amino acid substitution in PMEL17 protein on physicochemical parameters of the chicken *PMEL17* gene. According to the results, one of the nonsynonymous mutations (AY636125:g.2162G>A) caused significant changes in isoelectric point and net charge, while the others did not have significant effects on these parameters (Table 3). The hydrophobic index was not considerable affected by any of above mentioned mutations (Table 3). In case of the AY636125:g.2162G>A mutation, substitution of aspartic, as a polar and charged amino acid, with asparagine, as a polar and uncharged amino acid, increased the isoelectric point from 4.57 to 4.62, and the net charge from -22 to -21, but did not alter hydrophobic index (0.03) (Table 3). Altogether, we found eight single nucleotide polymorphisms in the partial coding region of *PMEL17* gene of Iraqi chickens, most of which had high genetic diversity. These results imply that the polymorphism in this gene can be used as a reliable molecular marker for identification of plumage color in chickens.

Table 3. The effect of amino acid substitutions on physicochemical parameters of chicken PMEL17 protein.

Position	Amino acid substitutions	Isoelectric point	Net charge	Hydrophobic index
AY636125:g.1928C>T	Proline > Serine	4.57 > 4.57	-22 > -22	0.03 > 0.03
AY636125:g.1992C>T	Threonine> Methionine	4.57 > 4.57	-22 > -22	0.03 > 0.04
AY636125:g.2127C>T	Proline > Leucine	4.57 > 4.57	-22 > -22	0.03 > 0.04
AY636125:g.2162G>A	Aspartic > Asparagine	4.57 > 4.62	-22 > -21	0.03 > 0.03

Conclusion

The White Leghorn chicken is among the most common breeds throughout the world and identification of polymorphisms in genes associated with this plumage coloration has been the subject of many studies during the last decade. We sequenced a 428-bp fragment of *PMEL17* gene in Iraqi native chickens and detected eight SNPs, including four nonsynonymous (AY636125:g.1981C>T, AY636125:g.2128C>A, AY636125:g.2140C>T, and AY636125:g.2167A>T) and four synonymous (AY636125:g.1928C>T, AY636125:g.1992C>T, AY636125:g.2127C>T and AY636125:g.2162G>A) mutations. The mutation AY636125:g.2162G>A significantly changed the isoelectric point and net charge of the encoded protein, while the others did not had significant effects on these indices. Given the high genetic diversity and allelic frequency of *PMEL17* gene, this gene can be used as a reliable molecular marker associated with plumage color in chickens.

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