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Original scientific paper

ASSOCIATION ANALYSIS BETWEEN GENETIC DIVERSITY OF THE MELANOCORTIN4 RECEPTOR (MC4R) GENE AND PRODUCTION TRAITS IN TURKEY

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Melanocortin receptor (MC4R) plays an important role in central melanocortin system and regulating feeding behavior in humans and birds. The current study was designed to estimate the frequency of MC4R gene polymorphism and investigate if their polymorphisms have association with production traits Turkeys (Meleagris gallopavo) by PCR-SSCP method. For this purpose, a number of 100 turkeys were randomly chosen and blood samples were collected. DNA were extracted from blood samples, and amplified a fragment of 469 bp in size from MC4R gene. Single strand conformation polymorphism technique (SSCP) was used to determine genotypes. Electrophoresis of SSCP-PCR products on 6% polyacrylamide gel indicated that the polymorphism of the MC4R gene. Also, results of electrophoresis showed that three genotypes AA, AB and BB, with frequencies of 0.2397, 0.4998 and 0.2605, respectively. Shanon index, Nei index and observed heterozygosity with frequencies of 0.6929, 0.4998 and 0.5208, respectively.Significant associations ($P<0.01$) were obtained for genotypes with live body weight. But no significant relationship between the genotypes with egg performance (egg mass, mean egg weight and the number of eggs) is found. These results suggest that, the turkey *MC4R* gene can be selected as the major candidate gene for the carcass traits such as body weight, and at future more study is required to confirm these results in Turkey.

Key words: MC4R, Turkey, Polymorphism, Production Traits

INTRODUCTION

Turkeys are a great species of birds from genus “Meleagris” that are native to North America. In the global dimension of turkey breeding, industrial production, along with domestic

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poultry, ducks, fowl and quail, plays an important role in increasing the economic and nutritional status of different populations (BEYGEI, 2011). Native breeds are national capital and their maintenance is of particular importance. According to the FAO, native genetic resources are at high risk due to high levels of Crossbreeding in Commercial breeds. Obesity in humans is an undesirable condition and has become an important health issue in the whole world (DOAK *et al*., 2012). But in animals that are kept and raised for meat production, it is important as a desirable trait. So far, many genes it is known that they are associated with traits related to meat production and weight gain, some of which are considered as candidate gene, that the *MC4R* gene is also in the category of major genes affecting the traits of production.

Research on mutations in useful genes (candidate genes) and their relationship with economic traits to determine the genetic basis of the production traits and to develop the DNA test as a water Selection of breeding projects was done (DE VRIES *et al*., 1998). Melanocortin 4 receptor (MC4R) is a member of the G-protein coupled receptor family. This receptor plays a crucial role in melanocortin system and the regulation of feeding behavior in humans and rodents (CALTON *et al*., 2009; HUANG *et al*., 2010), interacting with several other factors to regulate energy homeostasis or reacting with neuropeptides released from pro-opiomelanocortin and agouti-related peptide neurons (BUTLER, 2006). Feeding behavior and BW are mainly regulated by the central melanocortin system (WARDLAW, 2001).The central melanocortin system appears to play an important role in the regulation of energy balance in birds, as it does in mammals. Many studies have been involved in the fields of association analysis between candidate gene SNPs with animal growth and body composition traits (CHUNG *et al*., 2005; MENG *et al*., 2005; XU *et al*., 2005).

Mutations of the *MC4R* gene are associated with the appetite, obesity and growth in pig, mice and human (DOAK *et al*., 2012; JOSH *et al*., 2014; PANPAN *et al*., 2016). There is little information available on the function of turkey *MC4R* gene. Therefore, the purpose of this study was 2-fold: 1) to identify polymorphisms that may affect gene function associated with egg performance and BW in *MC4R*, and 2) to design a PCR-SSCP test for genotyping purposes in East Azerbaijan (Iran) native Turkeys.

MATERIALS AND METHODS

Sampling and genomic DNA extraction

This experiment was carried out at the Poultry Research Station of Khodafarin of Agricultural Science and Natural Resources (East Azerbaijan, Iran). 100 female Turkey (*Meleagris gallopavo*) were used in this study. Sampled turkeys are of the same age and have been hatched from an incubation period and fed according to the standard conditions. At 113 days, body weight was measured on live turkeys that were fasting for 12 h with free access to water. In the first laying period (25 week), the number of eggs produced, as well as the weight of eggs produced by each bird, was recorded in one season.

Blood samples (3 mL) were obtained and stored in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Genomic DNA was extracted from 10 µl bloods by Salting out Extraction of DNA based on IRANPUR and ESMAILIZADEH (2010) protocol. Quality and quantity of extracted DNA was measured on 0.8% Agarose gel prepared in 0.5 X TBE buffer (45 mMTris base, 45 mM boric acid, 1mM EDTA, pH 8.0), and visualized with Ethidum Bromide (1.0 mg ml-1) and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA).

Amplification of promoter MC4R gene

The primer used for the amplification of a fragment of *MC4R* gene (469 bp). The primer sequences were as follows:

MC4RF:5′-TTCACCCAGCATCGTGGAAC-3′

MC4RR:5′-TGTTATGATACTGGAGGGCGT-3′

One of the main components of the polymerase chain reaction is primer in the multiplication reaction that manages the starting point of replication, and in addition to the concentration of Mg ion, it should also have an optimal binding temperature, which in most molecular experiments find the optimum connection temperature with the test and error frequency or gradient are performed by Thermo Cyclic devices. Therefore, to determine the optimal temperature of the primer with a Gradient, the thermocycler (T Gradient) was used. It should be noted that used by Amplifx software (version 1.5.3-WIN-EN-3) for primers design. The reaction PCR was performed at 25 µl final volume including 100 ng template DNA, 0.5 µl of each primers, 2.5 µl of 10 × PCR buffer, 4 µl of 1.25 mMdNTP (BioFluxbiotech, http://biofluxbiotech.com), 1 µl of 50 mM MgCl2 (Cinna Gen, Tehran, Iran), 1 U Taq DNA Polymerase (Cinna Gen, Tehran, Iran) by using of a 96-well Eppendorf Master cycler Gradient. The following cycles were applied for the *MC4R* gene amplification: initially denatured at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for30 sec, annealing 45 sec at 53°C (it was determined according to temperature gradiant technique, (image 2), and extension 45 sec at 72°C; and a final extension of 10 min at 72°C.

SSCP

SSCP method was used only to identify genotypes. PCR products were mixed with 8 µL denaturing loading dye [95% (w/v) deionized formamide, 0.05% (w/v) xylene Cyanol, 0.05% (w/v) bromophenol blue, and 0.02 M EDTA] in a total volume of 15 µL. The mixture was denatured at 95°C for 15 min and then snap-chilled on ice. The total volume was electrophoresed on 6% polyacrylamide gel with performed in 0.5X TBE buffer at room temperature (18°C) and a constant 75 V and were stained using silver nitrate according to the protocol described by BASSAM *et al*. (1991). Genotypes were recorded according to the band patterns. The 100 bp ladder was used as molecular size marker.

Statistical analysis

Allele frequency, genotype frequency, efficient allelic number (NE), observed and expected heterozygosities and Nei's (HE= 1 - Σ P2i, where Piis the frequency of allele i) and chi-square tests were calculated using the POPGENE32 version 1.31 program (YEH *et al*., 1999). They were calculated by using of PopGene software. Association between MC4R genotypes and production traits were tested by using of the GLM procedures of SPSS11.0 with the following model:

$$Yij = \mu + Si + Mj + eij$$

Yij: dependent variable,
µ: population mean,
Si: fixed effects of ith hatch,
Mj: fixed effect associated with the genotype
eij: random error,

The values were presented as least square means ± standard error means. Least square mean differences were tested for significance (P>0.05) by using of the Duncan test.

Allele and genotype frequencies were calculated by using of Pop Gene, 1.31 software. A chi-square ($\chi 2$) test was performed to test the goodness of fit to Hardy–Weinberg equilibrium expectations for the distribution of genotypes.

RESULTS

The frequencies of alleles and genotypes for this gene are shown in Table 1.

Table1. Parameters of genetic variation MC4R genes in Turkey

Parameters	Value	Allele frequencies	Value	Genotypic frequencies	Value
Number of actual alleles (Na)	2	A	0.4896	AA	0.2397
Number of affective alleles (Ne)	1.9991	B	0.5104	AB	0.4998
Shannon´s Information Index (I)	0.6929			BB	0.2605
Nei´s Index	0.4998				
Observed Heterozygosity	0.5208				
Expected Heterozygosity	0.52024				
Expected Homozygosity	0.4976				
Observed Homozygosity	0.4792				

Based on present results, the frequencies of A and B alleles were 0.4896 and 0.5104, respectively. Frequencies of AA, AB and BB genotypes were 0.2397, 0.4998 and 0.2605, respectively. Observed heterozygosity value was 0.5208. Expected heterozygosity value was 0.5204. Effective number of alleles (Ne) was 1.9991 and Shannon's Information index (I) was 0.6929Association of gene polymorphism with production trait were analyzed by using of the GLM procedure of SPSS11.0. The results are presented in the Table 2.

In the turkey population; association analysis demonstrated that the *MC4R* polymorphism was significantly associated with body weight, with the BB genotype associated with higher body weight than the AA and AB genotypes. Meanwhile, there was no significant difference in egg performance, among the genotypes. The electrophoretic profile of PCR products and SSCP analysis of *MC4R* gene are shown in Figure1, Figure 2.

Table 2. Effect of MC4R gene on genotypes performance of Turkeys ((least square mean and standard error).

MC4R genotypes	Trait			
	Egg Mass(ns)	Egg Number(ns)	Mean egg Weight(ns)	Body Weight
AA	3903.342±1441.11	50.580±18.40	77.028±3.28	4733 ± 79^{b}
AB	4120.05±1233.90	53.000±16.14	77.875±1.02	4139 ± 73^{b}
BB	3787.418±1509.90	49.727±19.90	76.445±3.91	5453 ± 93^{a}

Within rows different superscripts (a – b) indicate significant differences (P <0.05). Ns: Non significant

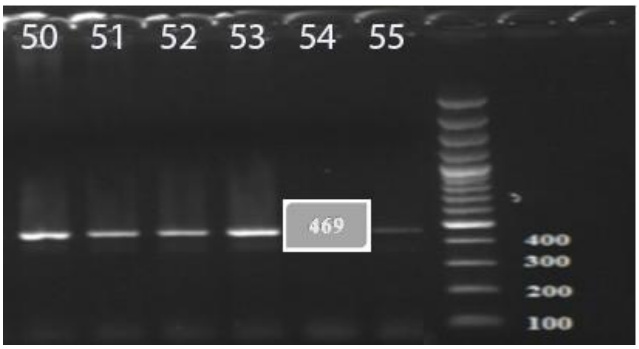

Fig. 1. Thermal gradient electrophoresis to determine the optimum temperature for primer connection with Marker PUC19 and PCR Products (469 bp fragment).

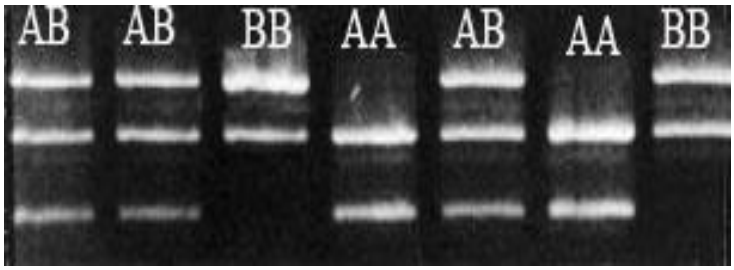

Fig. 2. SSCP analysis of the MC4R gene on 6% polyacrylamide gel in native Turkeys.

DISCUSSION

Growth is a very complex trait that is regulated by a number of genes and pathways, and more than 1500 quantitative trait loci have been found to be associated with poultry growth traits (WAHLBERG *et al.*, 2009; GU *et al.*, 2011).During recent years, in poultry industry, recognizing the genetic aspects and the major genes that influence the regulation of feeding behavior has attracted the attention of genetic and genetic researchers.

Therefore, work towards the genetic improvement of growth traits is essential, and the genetic improvement in poultry growth traits has made great progress during the past few years. However, most of the work was conducted using traditional breeding methods. Marker-assisted selection (MAS) is a new method based on molecular markers, which can shorten the breeding process and save a lot of time and money (LU and WU, 2002). Therefore, it is essential to exploit and identify new markers that could be used in breeding work.

The results of least square analysis also confirm the significant association between the polymorphisms and body weight trait. So the *MC4R* gene is the major candidate gene for the turkey growth and carcass traits. The further study of the *MC4R* gene would lay a good foundation for turkey molecular breeding and production. Briefly, this study is the first to show

an association between DNA variations in the *MC4R* gene in turkey. In turkey breeding, selection of growth traits has been important, and efficient genetic improvement in these quantitative traits may be augmented through marker-assisted selection using high density genetic maps. Through this study, the role of *MC4R* in growth traits suggests that it may be an important genetic marker for the related traits of turkey. We should enlarge the sample size for further analysis. Most of reported data are limited to native or commercial chickens in studies of QIU *et al*. (2006), TAO *et al*. (2008), HUO *et al*. (2006), WANG *et al*. (2009), QING ZHU *et a*l. (2009), and HUI and CHUN-YU (2006).

HUO *et al*. (2006) detected a G315T mutation in the encoding region of the chicken MC4R gene using PCR–SSCP and sequencing methods, and there was significant association between this polymorphic site and body weight, carcass weight, and breast muscle weight at 5 and 7 weeks of age. Two SNPs (c.923G → T and c.944C → T) have been recently identified in the chicken MC4R gene, and haplotypes were significantly associated with meat quality traits (WANG *et al*., 2009). HUI *et al*. (2006) designed to investigate the associations of a *MC4R* gene polymorphism on chicken growth and body composition traits in broiler lines divergently selected for abdominal fat. A SNP (G54C) was found in CDS region of chicken *MC4R* gene. The analysis of the least squares and variance revealed a significant association between the G54C SNP and BW, carcass weight (CW) and shank length (SL)at 7 week of age, and there were significant differences in different genotypes ($p<0.05$).

The polymorphism resulted in three genotypes defined as AA, AB and BB. Birds with BB genotype had significantly higher BW and CW than birds with AB genotype ($p<0.05$). Birds with BB genotype had significantly higher SL than AA genotype birds ($p<0.05$). The results from protein secondary structure prediction and tertiary structure prediction showed that it appeared a helix in 13th amino acid and two strands at 14th and15th amino acid in mutant protein, respectively. It may be induce the change of the activity or function of *MC4R* gene in poultry. Turkey *MC4R* is nearly identical to chicken *MC4R*, but little is known about its function. Thus, we investigated whether polymorphisms in the *MC4R* were associated with production traits. In the goose, a 996 bp *MC4R* gene has been obtained by HUANG *et al*., (2010), two synonymous *MC4R* SNPs (c.108G → A and c.627C →T) were identified in Landes geese, and association analyses indicated that these mutations might be important in affecting several carcass traits. A significant association was observed for c.108G →A and carcass weight, breast muscle percentage, and leg muscle percentage. The c.627C→ T marker was significantly associated with body weight, carcass weight, semi-eviscerated weight, and eviscerated weight.

FU *et al*. (2012) reported that only one SNP was detected in the *MC4R* 5′-flanking regions of each quail type (laying and meat-type): 407A>G in laying quails and 543A>G in meat-type quails. The mutation in laying quails produced three genotypes: AA, BB, and AB; the A and B allele frequency was 0.416 and 0.584, respectively. Statistical analysis of variance revealed that *MC4R* polymorphism was associated with breast muscle weight (BMW), leg muscle weight (LMW) and heart weight in laying quails. The mutation in meat-type quails produced three genotypes as well: CC, DD, and CD; the C and D allele frequency was 0.495 and 0.505, respectively. Statistical analysis of variance revealed that the *MC4R* polymorphism was associated with body weight, carcass weight, semi-eviscerated weight, eviscerated weight and heart weight in meat-type quails. Our findings suggest that the *MC4R* gene could be a qualitative trait locus or linked to a major gene that affects carcass traits in quails. ZHA MING-HENG *et al*. (2011) reported that the results of SSCP in Quail showed that obvious polymorphisms were

detected and three genotypes, AA, AB and BB were detected. One single nucleotide polymorphisms (SNPs) site, a synonymous mutation of T→C at 855 bp was detected, when comparing the sequencing results with quail *MC4R* gene sequence in GenBank. The frequencies of genotype AA, AB and BB were 0.1933, 0.6867 and 0.1200 respectively. The frequencies of allele A and B were 0.5367 and 0.4633 respectively. At the present study the frequency of A (0.4896) and B (0.5104) and frequencies of AA, AB and BB genotypes, 0.2397, 0.4998, 0.2605, respectively were approximately close to and are similar to those reported for a MC4R polymorphism in birds . Therefore, the effects of *MC4R* mutations in birds could be similar to those already observed in mammals. The bovine MC4R gene was mapped to BTU 24 by radiation hybrid mapping (HAEGEMAN *et al*., 2001; THUE *et al*., 2001; VALLE *et al*., 2004) .Two nucleotide changes by single stranded conformation polymorphism (SSCP) and sequencing. The substitutions proved to be a T to C and G (allele **B**) to A (allele **A**) resulting, respectively, in a conservative valine to alanine substitution (Val 145 Ala) and an alanine to threonine (Ala 172 Thr). Using PCR-RFLP, 13 different cattle breeds were screened for the presence of the Ala 172 Thr substitution. With the exception of one Red Pied animal, allele **A** could only be detected in Red Holstein animals (HAEGEMAN *et al*., 2001).

This study BB genotype produced higher body weight than AA and AB genotypes, indicating that allele B could be the favorite for body weight or linked with the QTLs of interest and suggesting that the enrichment of allele B in a population by molecular selection may be helpful for the enhancement of body weight in turkey. The frequency of allele B was 0.5104 , the frequency allele A was 0.4896,so the allele B was a preponderant gene in turkey population , but the frequency of heterozygous genotype (AB:0.4998) was higher compared to homozygous (AA:0.2397) and (BB:0.2605) genotypes. Therefore, it may be assumed that the *MC4R* gene affected body weight by regulating of appetite in turkeys. N_E is import genetic parameter that is used to show the size of intra-population genetic variation (TAO *et al*., 2008) showed in Table 1. In this study, the least square analysis showed that the BB turkey had significant body weight than AA and AB ($P < 0.01$) but no significant between the genotypes and egg performance. So, we presumed that the *MC4R* gene influenced the body weight but ineffective on activity of reproduction. The result of least square analysis confirmed the significant association between the *BB* genotype and body weight. But little is done about the structure and regulation elements of the *MC4R* gene. Further analyses of the effects of *MC4R* polymorphisms are essential to confirm the association between the alleles and carcass traits in other breeds or lines.

Differences in the level of single nucleotide polymorphism in different species of poultry may be due to differences in sample sizes, differences in genetic potential of species and implementation of breeding programs. Improving the performance of production traits in animals can be achieved through improved management, nutrition and genetic improvement, of these cases, the use of animals that are genetically superior, in terms of cumulative genes, the best way to increase the efficiency of animal production. Selection based on the genome of animals requires a DNA test that is costly and time-consuming. Therefore, increasing our knowledge about the great polymorphisms of genes and their relationship with important economic traits leads to the identification of effective alleles and their application as molecular markers in Selection programs. Therefore, before using a SNP for MAS, more studies are needed, such as increasing the population of the study population, using multiple species simultaneously and the same maintenance (DU *et al*., 2013).

To ensure the conclusion, more samples should be examined and all mutations examined in all gene areas to better understand the mechanisms of regulation of the hormonal and metabolic regulation of this gene. The genetic mechanisms of most genes are very complex and require more effort in a long time to understand the mechanisms. However, in selecting genetic markers, a broader genetic scan should be conducted at the DNA level of farm animals so that identifying potential mutations and investigating their relationship with important productive traits, identifies important SNPs and includes animal breeding programs. On the other hand, while preserving the native genetic masses of the country which are in full compliance with the environment and disease of the region the necessity of accurate record keeping of quantitative traits is important in order to further study the increase in the production capacity of these animals.

CONCLUSION

Molecular characterization of genes regulating the production traits can be useful for genetic improvement of poultry. This study provides preliminary result on genotype and allele frequencies of the *MC4R* gene in native turkeys. Allele B was observed to be higher as frequent as that of allele A. Significant associations were obtained for genotypes with live body weight. But no significant relationship between the genotypes with egg performance is found.. However, follow-up studies using larger sample size is highly recommended to confirm the effect of this gene on the Production traits in native turkeys. Establishment of a breeding population of purified strains of native turkeys is also suggested in order to obtain much more reliable results. Nevertheless, this study provided baseline information for future extensive *MC4R* gene research on native turkey in Iran.

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ASOCIATIVNA ANALIZA IZMEĐU GENETIČKOG DIVERZITETA MELANOCORTIN4 RECEPTORA (MC4R) I PROIZVODNIH OSOBINA ĆURKE

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Izvod

Melanocortin receptor (MC4R) igra važnu ulogu u centralnom sistemu melanokortina i reguliše ponašanje u ishrani ljudi i ptica. Ovaj rad je dizajniran da proceni učestalost polimorfizma MC4R gena i ispita da li njihovi polimorfizmi imaju povezanost sa proizvodnim osobinama divlje ćurke (*Meleagris gallopavo*) metodom PCR-SSCP. Za ovu svrhu je slučajno odabrano više od 100 ćuraka i uzeti su uzorci krvi. DNK je ekstrahovana iz uzoraka krvi i amlifikovan je fragment od 469 bp od MC4R gena. Elektroforeza proizvoda SSCP-PCR na 6% poliakrilamidnom gelu pokazala je polimorfizam MC4R gena. Takođe, rezultati elektroforeze su pokazali da su tri genotipa AA, AB i BB, bili sa sa frekvencijama od 0.2397, 0.4998 i 0.2605. Izračunat je Shanon indeks, Nei indeks i utvrđena je heterozigotnost sa frekvencijama od 0.6929, 0.4998 i 0.5208. Značajne asocijacije ($P < 0.01$) su dobijene kod genotipova sa svežom telesnom težinom. Međutim, ne postoji značajna veza genotipova sa performansama jaja (masa jaja, težina jajeta i broj jaja). Ovi rezultati sugerišu da MC4R gen ćurke može biti odabran kao glavni kandidatni gen za osobine trupa kao što je telesna težina, a u budućnosti je potrebno više istraživanja da bi se potvrdili dobijeni rezultati.

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