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A polymorphism in the 5'-flanking region of the melanocortin-4 receptor gene is associated with carcass traits in quails

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ABSTRACT

The melanocortin-4 receptor gene (*MC4R*) has been implicated in regulation of feeding behavior and body weight. Its gene sequence and protein function exhibits high evolutionary conservation and have been studied extensively in humans, mice, pigs and chickens. Quail *MC4R* is nearly identical to chicken *MC4R*, but little is known about its function. Thus, we investigated whether polymorphisms in the *MC4R* 5'-flanking region were associated with quail carcass traits. Chinese recessive white feather layer quails (n,233) and French Shaweimate meat-type quails (n,307) were assessed for body weight and carcass traits for use in statistical correlation analysis with genomic sequence data. Only one SNP was detected in the *MC4R* 5′-flanking regions of each quail 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.

Key words: Carcass traits, Genomic sequence data, Polymorphism, Quail *MC4R*

The melanocortin peptidergic system is a critical regulator of appetite and energy homeostasis (Wardlaw 2001). As such, individual components of the melanocortin system are implicated in body weight regulation (Benoit *et al.* 2000), especially the melanocortin receptors. The melanocortin receptor family consists of 5 subtypes that are characterized by distinct patterns of tissue expression (Gantz *et al.* 1993 and 1994). *MC4R* and *MC3R* are robustly expressed in the brain, and believed to play distinct but complementary roles in body weight regulation.

MC4R belongs to the superfamily of G-protein coupled receptors (GPCRs) (Andersson 2003). Most often present as a trans-membrane neuron receptor, MC4R was recently detected in a secreted peptide form in the ventromedial hypothalamus (Yeo *et al.* 2000). One of the principal ligands of *MC4R* is the alpha melanocyte stimulating hormone (α-MSH). Once bound to α-MSH, MC4R activates anorexigenic signals that ultimately lead to decreased food intake and decreased body weight (Sinha *et al.* 2004). Stimulation of the *MC4R* gene also resulted in inhibition of food intake. Targeted disruption of the *MC4R* gene in mice led to the obese phenotype and development of obesity-related disorders, including hyperinsulinemia and hyperglycemia (Huszar *et al.* 1997). Direct sequencing of the *MC4R* gene in families with severely-obese members indicated that some *MC4R* mutations were strictly found in individuals with the obese phenotype and not in the non-obese members (Dubern *et al.* 2001).

Gene expression distribution analysis by PCR detection of *MC4R* mRNA revealed that the chicken *MC4R* gene expression profile is significantly more diverse than the brain-restricted profile seen in humans (Takeuchi *et al.* 1998). These findings suggested that the *MC4R* gene may have a distinct function in birds, as opposed to the presumably more specialized function in mammals. The *MC4R* gene likely retains its key function in body weight regulation in birds, but may utilize a different mechanism or contribute to a unique metabolic component related to body weight. This hypothesis is particularly intriguing for the poultry industry, which is primarily concerned with improving body mass while limiting economic impact to the producers.

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Little is known about the quail *MC4R* gene, its biological function, or nucleotide mutations that affect such. Taking into consideration the well-characterized features of chicken growth and development, along with the *MC4R* gene expression profile (in the brain and several peripheral tissues), it is reasonable to propose *MC4R* involvement in the growth and carcass traits of quails. Therefore, we investigated the *MC4R* gene sequences in two common breeds of quail to identify any single nucleotide polymorphisms (SNPs) present in the 5'-flanking region and to determine whether those SNPs were associated with desirable growth and carcass traits. We expect our findings to provide novel insights into the quail *MC4R* gene that will benefit future studies into this and other quail genotypes and support the development and application of molecular breeding.

MATERIALS AND METHODS

Experimental animals and management: The present study examined laying quails and meat-type quails by using two common breeds. The F1 of laying quails was established by crossing Chinese white feather quails and Korea quails and produced 233 quails (male:female, 155:78). The F1 of the meat-type quails were bred from French Shaweimate quails, and totaled 307 (male: female, 164:143). Thus, a total of 540 quails from 2 breeds were used in our analysis.

All birds had *ad lib.* access to feed and water. The diet consisted of commercial corn-soybean-based feed that met all NRC requirements (Nation Research Council 1994).

Phenotypic measurements: Carcass trait measurements included body weight (BW), carcass weight (CW), semi-eviscerated weight (SEW), eviscerated weight (EW), breast muscle weight (BMW), leg muscle weight (LMW), heart weight (HW), liver weight (LW), gizzard weight (GW), and abdominal fat weight (AFW).

DNA preparation, primer design, and PCR amplification: Venous blood samples were obtained from all birds immediately after sacrifice and body weight measurement. Genomic DNA was extracted from all whole blood samples using standard methods. Isolated DNA from each bird was individually dissolved in sterile water to obtain a concentration of 50 ng/μ and stored at–20°C.

The *MC4R* 5'-flanking region PCR primers were designed according to the quail *MC4R* gene sequence deposited in GenBank (Accession No. GU165835). Three pairs of primers were designed using Oligo Primer Analysis software (version 6.0) and synthesized by invitrogen. The primer sequences and size of the amplified fragments are shown in Table 1. PCR reaction mixtures (25 μl) included 50 ng of genomic DNA, 25 pmol of each primer, 2.5 μl 10×PCR buffer, 2 μl dNTP and 1.5 U *Taq* DNA polymerase. Thermal cycling conditions were: denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55.9°C for 30 sec, 72°C for 30 sec; and extension at 72°C for 10 min.

Table 1. Primers to amplify quail MC4R gene

Primer	Sequence (5'-3')	Size of PCR products (bp)
P1	Forward: AAGCTTGCGCACATCCAAGT Reverse: GCTGCCGAGCAGAAACTAAT	229
P2	Forward: GCAGCAGTCACTTGAGCATT Reverse: GCGCTCTCAAAGATGCAGAT	201
P3	Forward: CTTTGAGAGCGCAAACCAGT Reverse: TGCTGGGTGAAATTCATCTT	210

PCR-single strand conformation polymorphism (SSCP) analysis and DNA sequencing: The PCR product was diluted 1:5 with loading buffer (98% formamide, 10 mmol/l EDTA, pH 8.0, 0.025% xylene cyanol FF, 0.025% bromophenol blue, and 2% glycerol). After denaturation by incubation at 98°C for 10 min, the mixture was immediately chilled on ice for 10 min and then loaded onto a 16% acrylamide/bisacrylamide (arc: bis, 29:1) gel. Bands were resolved by electrophoresis at 10 V/cm for 16 h, and detected by using the standard silver staining method. For each polymorphism, three PCR products were amplified and purified. Sequencing was carried out by the ABI377 sequencer.

Statistical analysis: To determine associations between the SNPs and carcass traits, the PROC-GLM procedure in the SAS statistical software (version 8.2) was used. The linear model was as follows: $Y,\mu+G_i+S_j+e_{ij}$, where y is carcass traits, i is mean number of population, G_i is the effect of genotype, S_j is the effect of sex, and e_{ij} is the residual effect. The X^2 test was used to determine significant differences in allele frequencies. A P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Polymorphisms in the 5'-flanking region of quail MC4R gene: PCR amplification and SSCP analysis were carried out targeting the 5'-flanking region of the quail *MC4R* gene with three pairs of primers. Only the 229 bp fragment produced by primer pair P1 exhibited polymorphisms in its sequence: 407A>G in laying quail and 543A>G in meat-type quail. The PCR-SSCP method was then developed to genotype these particular breed-specific mutations (Fig. 1). Three genotypes were detected in laying quail, and designated as *AA* (A407A), *AB* (A407G), and *BB* (G407G). Similarly, 3 genotypes were detected in meat-type quail, designated as *CC* (A543A), *CD* (A543G), and *DD* (G543G).

Allele frequency of MC4R gene in distinct quail breeds: The 2 breeds of quails were screened by the PCR-SSCP method to determine the allele frequencies in each. The χ^2 test results indicated that there were no significant differences in the allele frequencies detected among laying quails ($P>0.05$; Table 2). In contrast, the allele frequencies detected among meat-type quails were significantly different ($P<0.01$, Table 2).

Table 2. Allele frequencies among two breeds of quails

Breed	Number	Genotype frequency			Allele frequency		X^2
		AA	*BB*	*AB*	*A*	*B*	
Laying quails	233	0.154(36)	0.322(75)	0.524(122)	0.416	0.584	1.898^{ns}
Meat-type quails		*CC*	*DD*	*CD*	*C*	*D*	
	307	0.391(120)	0.4(123)	0.209(64)	0.495	0.505	104.354^{**}

Numbers in parentheses represent numbers of quails for each genotype;X^2 using Hardy-Weinberg test of genotype in different populations (breeds); ns, no significant difference (P>0.05); ** very significant difference (P<0.01).

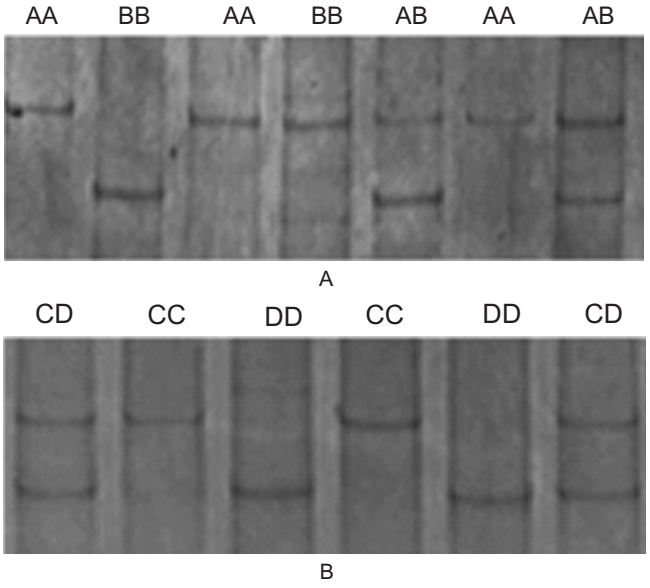

Fig. 1. PCR-SSCP patterns for 5'-flanking region of *MC4R* gene in (A) laying quails and (B) meat-type quails. Three genotypes were detected in laying quail, and designated as *AA* (A407A), *AB* (A407G), and *BB* (G407G). Similarly, three genotypes were detected in meat-type quail, designated as *CC* (A543A), *CD* (A543G), and *DD* (G543G).

Association of MC4R gene SNPs with carcass traits: MC4R gene polymorphisms in the 5'-flanking regions of each breed correlated to carcass traits (Table 3). In laying quails, the polymorphism was significantly associated with breast muscle weight, leg muscle weight, and heart weight. In meat-type quails, the polymorphism was significantly associated with body weight, carcass weight, semi-eviscerated weight, eviscerated weight, and heart weight. Furthermore, laying quails with the BB genotype had significantly higher BMW and LMW than birds harboring the AA or AB genotype. For the meat-type quails, the CC and DD genotypes were associated with significantly higher BW, CW, SEW, and EW, as compared to birds harboring the CD genotype. Moreover, the CC genotype was associated with significantly higher LW than in birds with DD and CD genotypes.

In mammals, the *MC4R* gene functions to control appetite, body weight, energy metabolism and obesity (Srinivasan *et al.* 2004, Ma *et al.* 2004). As such, it has garnered much attention as a potential drug target to manipulate feeding behavior. Likewise, the *MC4R* gene in birds may be manipulated to produce more robust animals and benefit the commercial poultry industry. In this study, we investigated the quail *MC4R* gene to determine its contributions to growth and carcass traits. We began our search for *MC4R* polymorphisms by using the SSCP technique, which is considered a useful tool for preliminary DNA polymorphism studies. Sensitivity of this technique is negatively correlated with the length of the analyzed DNA fragment (Shorczyk *et al.* 2007); therefore, we devised three primer sets to target the 5' flanking region sequence and generate fragments of sufficient length (<250 bp) for detecting polymorphisms. By this approach, we were able to detect a single SNP (407A>G) in laying quails and one (543A>G) in meat-type quails.

The 5'-flanking sequence of a gene generally functions as a region of transcriptional regulation, with binding sites for general, tissue and condition-specific transcription factors. In addition, this region can interact with proteins that modify the epigenetic configuration, including removal of methyl groups to facilitate access to the DNA structure by various regulatory proteins. Thus, sequence mutations in the 5'-flanking region of a gene can have profound effects on, either robustly promoting or silencing, gene transcription. In this study, we detected two breed-specific polymorphisms within the 5'-flanking region of the quail *MC4R* gene that may significantly affect expression and the phenotypic traits associated with *MC4R* protein function, specifically body weight. The results of least square analysis confirmed that a significant association existed between the *AA* genotype of laying quails and breast muscle weight, leg muscle weight, and heart weight and between the CD genotype of meat-type quails and body weight, carcass weight, semi-eviscerated weight, eviscerated weight, and heart weight, and between the CC genotype of meat-type quails and heart weight. Unfortunately, little is known about the *MC4R* gene structure and its regulatory elements, so the exact mechanism underlying the carcass trait associated polymorphisms detected in our study remains unclear.

According to our study, the polymorphisms in the 5'-flanking region have little to no effect on the abdominal fat weight. This is consistent with previously published results from a similar study in chicken (Qiu *et al.* 2006), in which a

Table 3. Effects of *MC4R* gene polymorphisms on quail growth and carcass traits

Traits[1]	*MC4R* polymorphism sites			
	Laying quails (407A>G)	Genotype (n) Mean±SD	Meat-type quails (543A>G)	Genotype (n) Mean±SD
BW (g)	AA (36)	131.90±16.04[a]	CC (120)	159.70±14.69[a]
	BB (75)	131.15±12.89[a]	DD (123)	154.13±14.35[a]
	AB (122)	128.51±12.31[a]	CD (64)	159.64±13.64[b]
	P,NS		P,0.0192	
CW (g)	AA (36)	122.58±14.76[a]	CC (120)	147.73±15.10[a]
	BB (75)	121.62±16.61[a]	DD (123)	142.76±14.47[a]
	AB (122)	120.19±11.47[a]	CD (64)	147.44±12.44[b]
	P,NS		P,0.0149	
SEW (g)	AA (36)	104.75±10.37[a]	CC (120)	133.66±12.31[a]
	BB (75)	105.06±9.95[a]	DD (123)	129.79±11.95[a]
	AB (122)	101.96±9.74[a]	CD (64)	133.51±10.57[b]
	P,NS		P,0.0221	
EW (g)	AA (36)	86.90±9.5[a]	CC (120)	111.46±10.51[a]
	BB (75)	87.60±9.5[a]	DD (123)	107.28±14.13[a]
	AB (122)	84.79±9.62[a]	CD (64)	111.28±9.43[b]
	P,NS		P,0.0141	
BMW (g)	AA (36)	10.19± 1.47[b]	CC (120)	13.50±2.18[a]
	BB (75)	10.37±1.53[a]	DD (123)	13.08±1.89[a]
	AB (122)	9.82±1.39[b]	CD (64)	13.37±2.23[a]
	P,0.0329		P,NS	
LMW (g)	AA (36)	7.42±0.94[b]	CC (120)	9.35±1.15[a]
	BB (75)	7.63±0.96[a]	DD (123)	9.05±1.28[a]
	AB (122)	7.25±0.25[b]	CD (64)	9.36±1.13[a]
	P,0.0351		P,NS	
HW (g)	AA (36)	1.24±0.17[b]	CC (120)	1.78±0.09[b]
	BB (75)	1.41±0.08[a]	DD (123)	1.64±0.04[a]
	AB (122)	1.22±0.16[b]	CD (64)	1.60±0.08[a]
	P,0.0459	P,0.0343		
LW (g)	AA (36)	2.82±1.34[a]	CC (120)	4.23±1.18[a]
	BB (75)	2.80±1.366[a]	DD (123)	4.07±1.11[a]
	AB (122)	2.68±1.22[a]	CD (64)	4.22±1.20[a]
	P,NS		P,NS	
AFW (g)	AA (34)	1.47±1.06[a]	CC (120)	1.06±0.56[a]
	BB (71)	1.33±1.15[a]	DD (123)	0.98±0.68[a]
	AB (95)	1.53±1.11[a]	CD (64)	1.25±0.82[a]
	P,NS		P,NS	
GW (g)	AA (36)	2.95±0.52[a]	CC (120)	3.69±0.50[a]
	BB (75)	2.89±0.47[a]	DD (123)	3.64±0.51[a]
	AB (122)	2.81±0.44[a]	CD (64)	3.66±0.38[a]
	P,NS		P,NS	

BW,body weight; CW,carcass weight; SEW, semi-eviscerated weight; EW, eviscerated weight; BMW, breast muscle weight; LMW, leg muscle weight; HW, heart weight; LW, liver weight; AFW, abdominal fat weight; GW, gizzard weight

NS means P>0.05; SD, Standard deviation; [a, b], means within a column with no common superscript differ significantly (P<0.05).

C'!T mutation in the 5'-flanking region of chicken MC4R gene was significantly associated with body weight and leg muscle weight (P<0.05). Likewise, in this previous study, no significant association was found to exist between the genotypes and abdominal fat weight in chicken. These findings in birds are strikingly different from the findings from mammalian studies, which show that *MC4R* is a principal regulator of obesity (Kim *et al.* 2000 and Ma *et al.* 2004). This apparent difference in function may be a reflection of the distinctive expression profiles that have been observed in chicken and human tissues (Lubrano-Berthelier *et al.* 2004). Interestingly, a study in pigs reported an apparent association of *MC4R* gene missense mutation with back fat content, growth, and food intake behaviors (Kim *et al.* 2000). Nucleotide sequence alignment has revealed that the porcine *MC4R* gene is highly homologous with that of the chicken (sharing 97% identity) (Fu *et al.* 2011). It is probable that the single variant amino acid residue of the *MC4R* mutation (or a closely linked mutation) causes a significant change in *MC4R* function. These results support the functional significance of a pig *MC4R* missense mutation and suggest that comparative genomics based on model species may be equally important for application to farm animals as they are for human medicine (Kim *et al.* 2000). Investigations of the human *MC4R* gene suggested that a particular missense mutation could contribute to individual variability in body mass and abdominal fat distribution (Rosmond *et al.* 2001). Based on these findings, the authors proposed that targeted inactivation of *MC4R* could cause obesity.

In conclusion, our study demonstrated that mutations in the 5'-flanking region of quail *MC4R* gene contribute to variance of growth and carcass traits in different breeds. Thus, the *MC4R* gene may be an important target of molecular breeding to produce meatier quails.

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