Association of *VIPR-1* gene polymorphisms and haplotypes with egg production in laying quails[*]

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Abstract: The laying quail is a worldwide breed which exhibits high economic value. In our current study, the vasoactive intestinal peptide receptor-1 (*VIPR-1*) was selected as the candidate gene for identifying traits of egg production. A single nucleotide polymorphism (SNP) detection was performed in 443 individual quails, including 196 quails from the H line, 202 quails from the L line, and 45 wild quails. The SNPs were genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Two mutations (G373T, A313G) were detected in all the tested quail populations. The associated analysis showed that the SNP genotypes of the *VIPR-1* gene were significantly linked with the egg weight of G373T and A313G in 398 quails. The quails with the genotype GG always exhibited the largest egg weight for the two mutations in the H and L lines. Linkage disequilibrium (LD) analysis indicated that G373T and A313G loci showed the weakest LD. Seven main diplotypes from the four main reconstructed haplotypes were observed, indicating a significant association of diplotypes with egg weight. Quails with the h1h2 (GGGT) diplotype always exhibited the smallest egg weight and largest egg number at 20 weeks of age. The overall results suggest that the alterations in quails may be linked with potential major loci or genes affecting reproductive traits.

Key words: Laying quail, Haplotypes, *VIPR-1* gene, Egg production traits

1 Introduction

In the poultry industry, the aim of modern breeding is to create high-performance poultry lines (Kulibaba and Podstreshnyi, 2012), which has always been of primary interest to breeders and farmers. Many experiments have confirmed that both production and reproductive traits depend on genetic factors (King'ori, 2011; Miazi *et al.*, 2012). In birds, egg-laying is the consequence of a complex cascade of progressive maturational events involving the entire hypothalamic-pituitary-gonadal axis. Many steroid hormones and their receptors involved in this process play pivotal roles throughout vertebrate reproduction and development (Li, 2014). Vasoactive intestinal peptide (VIP), a prolactin (PRL) releasing factor, can promote PRL secretion. As a receptor of VIP, VIPR is activated by VIP to give rise to secretion and release of PRL (el Halawani *et al.*, 1990). The *VIPR* gene has proved to be involved in the regulation of broodiness in avian, as evidenced by the endocrine mechanism of broodiness and expression in vivo and in vitro (Rozenboim and el Halawani, 1993; Kansaku *et al.*, 2001; Chaiseha *et al.*, 2004). The *VIPR-1* gene was expressed in the hypothalamus and pituitary, primarily

in the latter, and only the differential mRNA expression of the *VIPR-1* gene in the pituitary was associated with reproductive changes (You *et al.*, 2001), suggesting that the *VIPR-1* gene is an important candidate gene for egg-laying quails.

The quail egg, an important by-product of laying quails, is believed to be a good tonic and contain high nutrition, also known as "ginseng in animals." In the present study, *VIPR-1* was chosen as the candidate gene to analyze its genetic effects on three quail populations. Based on the two-tailed test method, two single nucleotide polymorphisms (SNPs) of the candidate QTL region were selected to analyze their association with egg production traits.

2 Materials and methods

2.1 Sample collection and preparation

A total of 443 female quails from northeast China were collected for this study, including 196 quails from the H line, 202 quails from the L line, and 45 wild quails. The H and L line populations were raised in the same farm for 10 years and selected due to their differences in feather color and laying performance, with a higher egg number for the H line and a higher egg weight for the L line. The wild quails were caught and raised in the same farm. The egg production traits of 398 quails were recorded throughout the egg production process in terms of body weight for the first egg, age at first laying, egg weight after 12 weeks, and body weight and egg number after 20 weeks of age, but no traits were recorded for the 45 wild quails, because of their low degree of domestication. The blood samples of all the 443 individual quails were collected for further SNP analysis. Genome DNA was extracted from the blood using a blood DNA extraction kit (Lifefeng, China). The DNA samples were dissolved in a Tris-ethylene diamine tetraacetic acid (EDTA) (TE) buffer and stored at −20 °C. All birds were raised in floor pens and had free access to feed and water. Commercial corn-soybean diets, that meet all National Research Council requirements, were used in this research.

2.2 Primer design and PCR amplification

Based on the *VIPR-1* gene sequences of the quail (Zhou *et al.*, 2012), chicken, and turkey (Gene ID: 395329 and 7433), four pairs of primers (Table 1; named as P1, P2, P3, P4) were designed to amplify the target regions for the SNP genotyping using the Primer 5.0 program (Clarke and Gorley, 2001). The detailed information for these primers is listed in Table 1.

Polymerase chain reaction (PCR) was carried out in a total volume of 15 μl consisting of 40 ng genomic DNA, 0.5 pmol each primer, 1.5 μl 10× buffer, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L dNTP mixture, and 1.5 U Taq DNA polymerase (Fermentas, Canada). The conditions of PCR were as follows: 95 °C for 5 min, followed by 35 cycles of 94 °C for 35 s, annealing temperature (T_m) for 35 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min.

2.3 Polymorphism screening and sequencing

Genomic DNA from quails was used as the template to amplify the target fragments using the four pairs of primers, and the purified PCR products were sequenced commercially (AuGCT, China). The sequences were aligned to find the base variations using ClustalW (http://www.ebi.ac.uk/Tools/msalclustalw2). A total of 443 samples were subjected to genotyping using the PCR-restriction fragment length polymorphism (PCR-RFLP). For the PCR-RFLP profile,

Table 1 Primers used for analysis of the *VIPR-1* gene in quail

Primer	Product size (bp)	Primer sequence (5'→3')	Amplified region	Annealing temperature (°C)
P1	958	F: GCGTTCTATGGCACAGTTA	Exons 4–5	54.0
		R: AAAGCAATGTTCGGGTTCT		
P2	1492	F: GCTGCTGGTGGAAGGGTTA	Exons 6–7	58.7
		R: CCGTCCAAGCAGTGATGAA		
P3	2101	F: GTGTTCATCACTGCTTGGA	Exons 7–8	53.4
		R: AATGGAGACCAAAATAGGA		
P4	1660	F: GTGGTGGCTGTCCTCTACT	Exons 11–12	54.0
		R: CACTGCCCTGCTCATTAA		

8.0 µl of PCR products were digested with 5 U restriction endonuclease (NEB, America) for 6 h at 37 °C or for 30 min at 65 °C, followed by separation through using electrophoresis on a 1.0% (0.01 g/ml) agarose gel with ethidium bromide in a 1× Tris-acetate-EDTA (TAE) buffer.

2.4 Statistical analysis and haplotype construction

Pairwise tests for linkage disequilibrium (LD) were performed for each SNP using the SHEsis online software platform (http://analysis.bio-x.cn/myAnalysis.php). Haplotypes were constructed based on using two SNPs for all of the 443 quails using the PHASE 2.1 program. The genotypic frequencies were calculated for each individual and the Hardy-Weinberg equilibrium was analyzed using the Chi-square test of PopGene Version 1.3.1. The egg production traits were compared among the genotypes. The association between the SNPs and different traits in 398 quails were analyzed using SPSS 13.0 with the model $Y=\mu+G+L+G\times L+e$, where Y is the dependent variable (analyzed traits), μ is the overall mean, G is the genotype with a variation for the candidate gene, L is the quail population, $G\times L$ is the interaction between the genotype and quail population (a fixed effect), and e is the random error. The differences between genotypes were determined by least square analysis.

3 Results

3.1 Polymorphism identification and detection

The sequences amplified with four pairs of primers (named as P1, P2, P3, and P4) were aligned among the three quail populations, and two SNPs (G373T and A313G mutations in exons 4–5 and exons 6–7, respectively, of *VIPR-1* gene) were found. These polymorphisms can be detected by PCR-RFLP and sequencing. The PCR products were digested with two restriction enzymes (*Bsr*DI for P1 and *Hpy*CH4IV forP2). The G to T transversion for the 373 locus in exons 4–5 and the A to G transition at the nucleotide position 313 in exons 6–7 expressed three genotypes: GG, GT, TT and GG, AG, AA, respectively (Fig. 1).

3.2 Allele and genotype distribution in quail populations

Allele and genotype frequencies found at exons 6–7 of the *VIPR-1* gene indicate that the G allele is
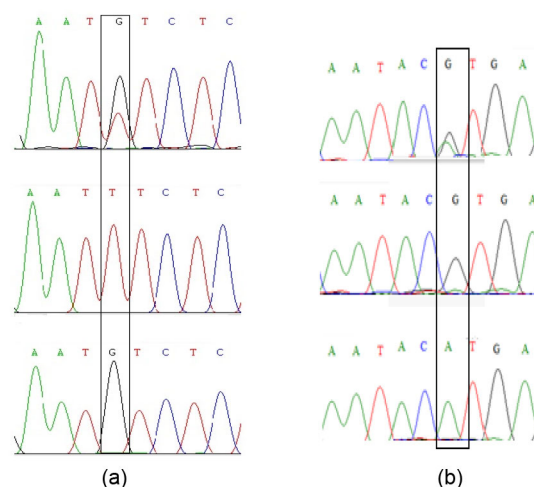
Fig. 1 Sequences amplified by primers P1 and P2 containing the G373T (a) and A313G (b) mutations, respectively

dominant in all the three quail populations at the A313G locus, while G is dominant at G373T for both the H line and wild quails, but not for the L line. The alleles of A313G and G373T in the three quail populations did not deviate from the Hardy-Weinberg equilibrium ($P<0.05$; Tables 2 and 3).

3.3 Association analysis of SNPs with egg production traits

Table 4 shows the results of the general linear model analysis of association between the *VIPR-1* gene polymorphisms and egg production traits. For the G373T locus or A313G locus in the H and L lines, the egg weight of the genotype GG was significantly higher ($P<0.05$) than that of the genotype TT (AA). However, for the G373T locus in the H line, the egg number of the genotype TT at 20 weeks of age was significantly higher ($P<0.05$) than that of the genotype GG. Likewise, for the A313G locus in the L line, the egg number of the genotype AG at 20 weeks of age was significantly higher ($P<0.05$) than that of the genotype GG. Obviously, G is the dominant gene in egg weight and T may be the dominant gene in the egg number at 20 weeks of age.

3.4 Linkage disequilibrium and haplotype analyses

LD analysis of the three quail populations showed that the two SNPs were in a weak LD (Table 5), indicating that the G373T and A313T loci were not linked and this region may not be inherited as a unit. Through estimating the SNP haplotype frequencies, four haplotypes with frequencies higher

Table 2 Distribution of G373T genotypic and allelic frequencies in all populations

Population	Genotype frequency			Allele frequency1		χ^2
	TT	TG	GG	T	G	
H line (n=196)	0.2092	0.5408	0.2500	0.4792	0.5208	0.2678
L line (n=202)	0.2475	0.5842	0.1683	0.5417	0.4583	1.2962
Wild quail (n=45)	0.1778	0.4889	0.3333	0.4259	0.5741	0.0318[*]

[*] P<0.05, the SNP locus in this population deviated from the Hardy-Weinberg equilibrium

Table 3 Distribution of A313G genotypic and allelic frequencies in all populations

Population	Genotype frequency			Allele frequency		χ^2
	AA	AG	GG	A	G	
H line (n=196)	0.1480	0.4592	0.3929	0.3750	0.6250	0.0516
L line (n=202)	0.1238	0.4802	0.3960	0.3646	0.6354	0.0270[*]
Wild quail (n=45)	0.2000	0.5111	0.2889	0.4600	0.5400	0.0170[*]

[*] P<0.05, the SNP locus in this population deviated from the Hardy-Weinberg equilibrium

Table 4 Association of SNP of *VIPR-1* gene with egg production traits in the H and L lines

SNP	Line	Genotype	Association of SNP with egg production traits				
			Body weight at first egg	Age at first laying	Egg weight	Body weight at 20 weeks of age	Egg number at 20 weeks of age
G373T	H	TT (n=41)	142.80±8.00	45.50±1.90	10.01±0.33b	153.60±12.95	86.90±3.28a
		GT (n=106)	142.62±8.29	45.69±3.25	10.07±0.52b	150.35±13.15	85.92±6.05a
		GG (n=49)	140.58±11.22	43.83±2.41	10.92±0.68a	152.08±12.62	81.75±7.07b
	L	TT (n=50)	139.79±8.00	46.36±1.90	10.78±0.99b	156.86±12.95	82.00±3.28
		GT (n=118)	134.00±8.29	46.67±3.25	10.88±0.99b	155.75±13.15	80.67±6.05
		GG (n=34)	141.50±11.22	49.13±2.41	10.83±1.15a	158.88±12.62	79.75±7.07
A313G	H	AA (n=29)	145.86±5.79	44.86±2.48	9.79±0.27b	147.43±14.72	84.43±7.25
		AG (n=90)	140.36±8.12	45.77±2.93	10.24±0.63ab	150.82±12.75	85.09±5.33
		GG (n=77)	142.84±10.43	44.63±2.97	10.46±0.70a	153.68±12.33	86.26±6.53
	L	AA (n=25)	134.33±6.35	48.17±2.48	10.22±1.36b	160.17±14.41	75.50±4.81b
		AG (n=97)	138.52±10.33	45.70±3.96	10.51±0.94b	155.57±14.80	86.26±4.40a
		GG (n=80)	140.11±13.64	47.95±4.73	11.34±0.69a	157.53±8.90	77.11±9.94b

Data are expressed as mean±SD. Values followed by different alphabets differ significantly (P<0.05).

than 2% were identified separately in the H line and L line, including h1 (GG, 43.75%), h2 (GT, 18.75%), h3 (TA, 29.17%), and h4 (GA, 8.33%) in the H line and h1 (GG, 35.42%), h2 (GA, 10.41%), h3 (GT, 28.13%), and h4 (TA, 26.04%) in the L line. Based on the four haplotypes, seven diplotypes were obtained, with two of them having a frequency higher than 20% in the L line (Tables 5 and 6). Association analysis revealed that the diplotypes were significantly linked with egg weight in the H and L lines (P<0.05). Quails with the h1h2 (GGGT or GGGA) diplotype always exhibited the smallest egg weight but the largest egg number at 20 weeks of age. A significant difference (P<0.05) was observed between the diplotypes and egg weight in the L line, and quails with the h1h1 (GGGG) or h1h3 (GGGT) always exhibited the largest egg weight, but the egg number at 20 weeks of age is not significantly different (P>0.05).

Table 5 Linkage disequilibrium tests of G373T and A313G loci in the *VIPR-1* gene

Line	D'	r^2
H	0.472	0.145
L	0.005	0
W	0.499	0.159

D' is above and r^2 is below the diagonal in the H, L, and W (wild) lines

4 Discussion

With the rapid development of molecular genetics, scientists can obtain large amounts of genomic information for accurate estimation of an animal's genetic and breeding values, which facilitates the research on candidate genes and their effects on phenotypic manifestations, and has established the basis for a marker-associated selection process (MAS)

Table 6 Association analysis between diplotypes and egg production traits in the H and L lines

Line	Diplotype	Association between diplotypes and egg production traits				
		Body weight at first egg	Age at first laying	Egg weight	Body weight at 20 weeks of age	Egg number at 20 weeks of age
H	h1h1 (n=33)	139.63±13.39	43.00±2.51	10.78±0.66a	150.13±13.10	80.38±7.71b
	h1h2 (n=41)	145.00±7.85	46.00±2.87	10.24±0.70b	156.60±2.25	88.10±4.72a
	h1h3 (n=49)	139.33±8.49	46.00±3.72	10.00±0.37b	146.67±0.32	85.25±5.82ab
	h1h4 (n=16)	142.50±5.97	45.50±1.00	11.20±0.71a	156.00±12.36	84.50±5.45ab
	h2h3 (n=24)	141.00±9.51	45.50±2.17	10.07±0.38b	155.67±16.29	85.50±2.88ab
	h3h3 (n=12)	145.00±6.00	46.00±1.73	9.87±0.32b	149.67±7.37	88.67±3.51a
	h4h3 (n=16)	146.50±6.45	44.00±2.83	9.73±0.26b	145.75±19.70	82.50±9.00ab
L	h1h1 (n=17)	141.25±3.20	48.25±2.06ab	11.63±0.81a	160.75±9.50	77.00±4.24cd
	h1h2 (n=12)	144.33±8.96	50.00±3.61a	9.90±1.00b	152.00±8.00	84.00±5.29abc
	h1h3 (n=46)	140.00±15.28	47.73±5.92ab	11.26±0.37a	158.18±8.87	79.64±9.96bc
	h1h4 (n=51)	141.83±10.55	44.50±3.06b	10.65±1.02ab	155.25±14.73	87.08±4.93a
	h2h4 (n=21)	134.40±7.09	47.80±2.59ab	10.18±1.52b	157.80±14.75	75.00±5.20cd
	h3h3 (n=17)	139.25±18.01	48.25±3.59ab	11.25±1.20ab	152.50±8.58	70.25±12.55d
	h3h4 (n=34)	131.38±6.78	45.88±4.52ab	10.54±0.81ab	157.38±17.86	85.88±3.36ab

Data are expressed as mean±SD. Values followed by different alphabets differ significantly (*P*<0.05)

(Kulibaba and Podstreshnyi, 2012). However, the research on the quail at the genetic level is just in the initial stages and little information is available currently (Roussot *et al.*, 2003; Kayang *et al.*, 2004; Beaumont *et al.*, 2005; Mo *et al.*, 2013; Recoquillay *et al.*, 2015).

Laying quail is an important economic animal. In the present study, the H and L lines were selected for different breeding goals, with a higher egg number for the former and a higher egg weight for the latter. Additionally, we chose the *VIPR-1* as the candidate gene and found two SNPs (A313G and G373T). Gene frequency calculation found that different groups in the same mutation locus have a different dominant gene, with the G allele dominant at the A313G locus in all of the three quail populations and at the G373T locus in the H line and wild quails. Meanwhile, the G allele is dominant in the wild quail just like in the H line. Thus, we can speculate that the wild quail has a larger egg production potential, which has not been reaped due to lack of domestication and a suitable breeding environment. However, the T allele has a higher ratio than the G allele at the G373T locus in the L line, which is different from the A313G locus and might have resulted from the interaction between genetic loci, especially artificial selection.

Furthermore, the polymorphisms of the *VIPR-1* gene were found to be associated with egg weight of the quails in both the H and L lines, and a significant association was also found between genotypes (diplotypes) and egg numbers in the H line at 20 weeks of age, suggesting that the H line has a greater potential in egg production. Therefore, we inferred that the allele A (T) is dominant as an egg production trait, and the allele G is dominant for egg weight in the two mutations of the *VIPR-1* gene. We analyzed the G allele and found it is higher in the H line than in other populations, which may have resulted from reproduction of special groups.

Hence, our findings in the present study demonstrate that G373T and A313G of the *VIPR-1* gene can be used for selecting the high yield or egg weight strain in quails. Additionally, the *VIPR-1* gene could be used not only as an important candidate gene for egg production in quails but also as a genetic marker in molecular marker-assisted selection for quail reproduction traits.

In summary, two SNPs (A313G and G373T) have been identified in the exons 4–5 and exons 6–7 of the quail *VIPR-1* gene, which are likely to be the causative mutations of two SNPs in their reproduction traits. Therefore, *VIPR-1* is probably an important gene in egg production traits of quails and can also be used as a marker-assisted selection for traditional breeding.

Compliance with ethics guidelines

Yue-jin PU, Yan WU, Xiao-juan XU, Jin-ping DU, and Yan-zhang GONG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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<u>中文概要</u>

题　目：*VIPR-1* 基因多态性及其单倍型与蛋用鹌鹑产蛋性能的相关性研究

目　的：蛋用鹌鹑 *VIPR-1* 基因单核苷酸多态性（SNP）研究。

创新点：首次开展蛋用鹌鹑 *VIPR-1* 基因多态性研究，发现了两个与蛋重相关的多态性位点。

方　法：以 *VIPR-1* 基因设计引物，进行聚合酶链式反应–限制片段长度多态性分析，获得基因突变位点。并通过关联性分析，研究基因多态性与繁殖性状之间的相关性。

结　论：在个体数为 443 个（202 只 L 个体、196 只 H 个体和 45 只野生鹌鹑个体）的群体中发现两个突变位点（G373T 和 A313G），且两位点间存在微弱的连锁不平衡。相关性分析表明，SNP 多态性与鹌鹑蛋重之间存在明显的相关性。组成的 7 个主要二倍型中，含有 h1h2（GGGT）的个体总是表现为较小的蛋重及较大的产蛋数。

关键词：血管活性肠肽；多态性；单倍型；*VIPR-1* 基因；产蛋性能相关性