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Original Article

Association analysis of candidate gene polymorphisms with egg production in Japanese quails (*Coturnix japonica*)

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Abstract This study was conducted to identify genotypes and evaluate the effect of polymorphisms in growth hormone (GH), prolactin (PRL), bone morphogenetic protein receptor-Type IB (BMPR-1B) and melatonin receptor-type 1C (MTNR-1C) genes on egg production of Japanese quail during 20 weeks of egg laying. PCR-RFLP and PCR-SSCP methods were used to determine the genotype of all genes. Our results showed that the genotypes at loci C2161G in PRL and G294A in MTNR-1C were monomorphic as C and A, respectively. For the A237B in GH and A290T BMPR-1B mutations, two alleles were detected, giving three genotypes at each locus. In addition, the 24-bp difference of two genotype patterns provided an Indel-358 mutation in PRL while three alleles were identified at locus A459TC (MTNR-1C). Taken together, the polymorphic sites did not affect egg production except the A459TC locus in MTNR-1C gene (P=0.002), where quails carrying AA and CC genotypes provided the highest egg number (115.6-116.6 eggs/quail). Thus, the A459TC single nucleotide polymorphism is suggested to apply in other populations and it should be considered as a candidate marker for egg production.

Keywords: Egg production, Japanese quail, genotype, mutation

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Introduction

Quail farming has many advantages such as low investment costs, less diseases, early puberty, long lifespan of egg laying and high nutritional meat value. Practically, quails are raised for egg production and for meat consumption but the first tendency is more popular (Rogerio, 2009). In Vietnam, Japanese quail (*Coturnix japonica*) raising has been developed in recent years but the problem of quail breeding remains the common concern for most producers. For a long time, breeding industry has still been out of the focus and thus current breeds are facing degenerative phenomenon with decreased and unbalanced productivity, making it impossible to maximize breeding potential. Therefore, the issues of selecting and breeding quail lines for high egg yield are essential in the current period.

Advances in candidate gene approach have been widely applied in the determination of genetic relationship as well as selective breeding of domestic animals with desirable traits (Kulibaba and Podstreshnyi, 2012). Researchers are constantly looking for potential genes affecting productivity and quality of traits in poultry. This approach is promising because the identification of genotypes nowadays can be done quickly and inexpensively thanks to the technologies being developed. Selection based on genetic markers offers several benefits including rapid detection, accuracy, improved productivity and increased ability to adapt to the environment of animals (Liu, 2007). There have been reports on the effects of candidate genes on reproductive characteristics of quails such as Growth Hormone (Nie et al., 2002), Prolaclin (Cui et al., 2006), Bone Morphogenetic Protein Receptor-Type IB (Zhang et al., 2008) and Melatonin receptor-Type 1C (Li et al., 2013). Therefore, it can be remarked that exploiting candidate genes to improve egg production is a potential strategy. Within the scope of this study, six polymorphic sites of the mentioned genes were analyzed to determined frequencies and their association with egg yield of Japanese quails.

Materials and Method

Animal: Japanese quails were raised at an experimental farm in Tra Vinh province, of which two populations of 189 and 120 laying quails (6-26 weeks old) were used, respectively. All quails were individually kept in cages for egg collection. During the experiment, they were fed with diets having metabolizable energy of 2.750 kcal/kg and 20% crude protein and they were vaccinated before and during the study. For determination of egg yield and egg weigh parameters, all eggs from individual quails were collected daily during 20-laying weeks and egg weight was recorded by weighing scale of 0.01g accuracy.

All procedures related to birds submitted in the present expreriments were allowed by the local Department of Animal Husbanry and Veterinary Medicines.

DNA extraction: Genomic DNA was isolated from feathers by phenol/chloroform extraction. In detail, quail feathers were taken,

chopped into small pieces and mixed with lysis buffer for incubation overnight at 37°C. In the next step, 300µl phenol: chloroform: isoamyalcohol (25:24:1) was added into the sample, mixed and centrifuged at 10.000rpm for 5 minutes. The supernatant was then transferred into a 2ml tube and added 700µl phenol:chloroform, swirled and centrifuged at 10.000 rpm for 5 minutes. The supernatant was recovered in a new clean tube with 700µl chloroform, swirled and centrifuged at 10.000rpm for 5 minutes. The upper phase was transferred into a new clean tube containing 300µl 1.2M NaCl; 150µl 2M sodium acetate and 1000µl cold ethanol (100%). Mixed gently by hand and centrifuged at 10.000rpm for 5 minutes to collect DNA pellet. DNA was then washed by adding 1000µl ethanol 75%, air-dried and stored in 1X TE buffer (pH8.0). The sample was subjected for OD measurement and diluted into 50ng/µl for further use.

Establishment of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay: based on published sequences, primer pairs were prepared to amplify GH, PRL2, BMPR-IB, MTNR-1C[a] genes as shown in Table 1. Each polymerase chain reaction (PCR) was performed in a final volume of 10µL containing 25ng of quail genomic DNA, 0.25M each primer, 0.25M each dNTP, 1X PCR buffer, and 1U *Taq* DNA polymerase. PCR products were digested with restriction enzymes overnight at 37°C. The restriction fragments were separated on 3% agarose gel stained with ethidium bromide.

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP): PCR products amplified by the MTNR-1C[b] primer was denatured and electrophoresed on 10% polyacrylamide gel. To perform DNA denaturation, 8µl of PCR product was mixed well with 10µl loading buffer. The mixture was then heated to 95°C and maintained for 10 minutes to separate the DNA strands into single strands of DNA. Quickly transfered the mixture tube to the ice (about 3°C) and kept at -20°C for 10 minutes. Before electrophoresis, the gel was cooled at 7°C for 30 minutes. The DNA mixture was loaded on gel and run at 10°C for 6 hours at 30 W. After that, the gel was stained in 250ml of TAE 1X solution containing 25µl of ethidium bromide (25mg) in 30 minutes. The bands were recorded from the gel under the UV light.

Statistical analysis: genotype frequencies of candidate genes and the Hardy-Weinberg Equilibrium (HWE) were estimated using the method of Rodriguez *et al*. (2009). The association between genotype and egg production was analyzed based on General Linear Model of Minitab software version 16.0: $Y_{ij} = \mu + G_i + \xi_{ij}$ (where Y_{ij}: traits observed; μ: general mean, G_i: influence of genotype; ξ_{ij}: random error).

Results and Discussion

In the current study, five single nucleotide polymorphisms (SNPs) and one 24-nucleotide Indel (Insertion/Deletion) of four candidate genes were identified in the population of Japanese laying quails. The number of bands, PCR-RFLP and PCR-SSCP band size, genotype and allele

frequencies of polymorphisms on GH, PRL, BMPR-IB and MTNR-1C genes are shown in Figure 1 and Table 2.

Generally, all mutations did not follow the Hardy-Weinberg law (P<0.05). The imbalance might be due to: (i) the sample size was not large enough and (ii) individuals in the population were not randomly mated. For the GH/*Msp*I polymorphism, two alleles and three genotypes were detected with frequencies of 0.3 (AA), 0.4 (AB) and 0.3 (BB). The present outputs supported the report of Johari *et al.* (2013) on the Q-R quail population where two alleles were found and the correspoding genetic structure was 0.38AA+0.45AB+0.17BB. At the PRL/*Csp*6I (C2161G) and MTNR-1C[a]/*Mbo*I (G294A) loci, only one allele (either C or A) was found in the population, therefore their frequency was considered to 1.0 and further analysis was not performed. Additionally, for the PRL-Indel mutation, two alleles were D (130 bp) and I (154 bp) with corresponding frequencies of 0.31 and 0.69, respectively. Similar results were previously reported by Lotfi et al. (2013) in Japanese quails with frequency ranging from 0.48 (allele D) to 0.52 (allele I).

Table 1. Information regarding the polymorphisms studied

Gene	SNP/ Genbank No	Sequencing primer (5'-3')	Ta (°C)	Enzyme	PCR product (bp)	References
GH	A237B/ EF452679.1	F: ATCCCCAGGCAAACATCCTC R:CCTCGACATCCAGCTCACAT	52	*Msp*I	776	(Nie et al., 2002)
PRL-1	Indel-358/ AB011438.2	F: TTTAATATTGGTGGGTGAAGAGACA R: ATGCCACTGATCCTCGAAAACTC	54	-	130 or 154	(Cui et al., 2006)
PRL-2	C2161G/ AB011438.2	F: AGAGGCAGCCCAGGCATTTTAC R: CCTGGGTCTGGTTTGGAAATTG	57	*Csp*6I	439	(Cui et al., 2006)
BMPR-1B	A290T/ EF530593	F: CCATAGCAAAACAGATTCAG R: TCAGGA CAGTTTGGTAGATT	53	*Hind*III	575	(Zhang et al., 2008)
MTNR-1C[a] (PCR-RFLP)	G294A/ JQ249896	F: GGTGTATCCGTATCCTCTAA R: GACAGTGGGACAATGAAGT	55	*Mbo*I	372	(Li et al., 2013)
MTNR-1C[b] (PCR-SSCP)	A459TC/ XM_015860811.1	F: TGCCAGATAAGTGGGTTCCT R: AGCGTCCAGGTCAGACAGAT	55	-	164	This study

F: Forward primer; R: Reverse primer; Ta: Annealing temperature

MTNR-1C[a] : Primer used for PCR-RFLP; MTNR-1C[b]: Primer used for PCR-SSCP

The associations of GH/*Msp*I (A237B), PRL-1 (Indel-358), BMPR-1B/*Hind*III (A290T) and MTNR-1C[b] (A459TC) with egg weight and egg production are presented in Table 3. Among the polymorphic sites, only A459TC SNP showed significant impact on egg yeild (P=0.002). At this locus, quail with AA and CC genotypes provided highest egg number in five laying months (115.6-116.6 eggs/quail) and the lowest productivity was in quail carrying CT genotype (105.6 eggs/quail).

Similar to the present study, in PRL gene there was no association between the Indel-358 locus with egg weight (Lotfi et al., 2013). As shown in Table 3, in all SNPs studied egg weight ranged from 11.4 to 12.0 g/egg, which were slighly higher than those reported by Men and Dinh (2012) (10.4-10.9 g/egg). Previously, it was pointed out by Vali et al. (2006) and Doan and Thanh (2010) that average egg weight of Japanese quails were 11.2 and 11.7 g,respectively.

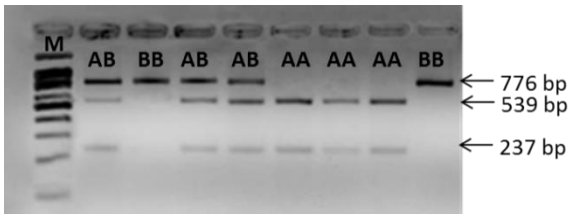
Table 2. Allele and genotype frequencies of polymorphic sites in Japanese quail

Gene/locus	Observed population					Expected population			HWE
	Genotype			Allele		Genotype			
GH/*Msp*I, A237B	AA	AB	BB	A	B	AA	AB	BB	
(n=196)	0.30	0.40	0.30	0.50	0.50	0.25	0.50	0.25	0.006
PRL-1, Indel-358	II	ID	DD	I	D	II	ID	DD	
(n=189)	0.40	0.58	0.02	0.69	0.31	0.47	0.43	0.10	0.000
BMPR-1B/ *Hind*III	AA	AT	TT	A	T	AA	AT	TT	
A290T (n=122)	0.48	0.36	0.16	0.66	0.34	0.44	0.44	0.12	0.034

MTNR-1C[b]	AA	AT	TT	CT	CC	AC	A	T	C	AA	AT	TT	CT	CC	AC	
A459TC (n=131)	0.18	0.00	0.10	0.14	0.29	0.30	0.32	0.17	0.51	0.11	0.11	0.03	0.18	0.26	0.33	0.000

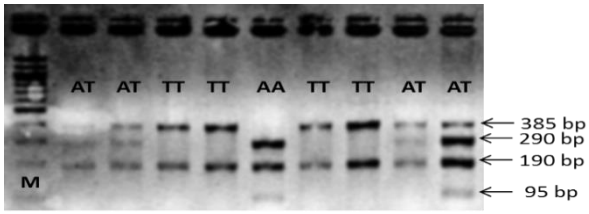
HWE: Hardy-Weinberg Equilibrium

In chicken, the BMPR-1B primer was designed by Zhang *et al*. (2008) to amplify a PCR product of 581 bp including a part of exon 6, intron 6 and a part of exon 7. The authors identified five SNPs, in which the A287G was recognized by *Hin*III restriction enzyme. This result was later confirmed in Noi chicken by Vu and Ngu (2016). However, in quail this mutation was slightly changed to A290T with similar regconition size of enzyme. Supportedly, the A290T was detected by El-Tarabany *et al*. (2014) in two meat and egg quail lines but the PCR product was shorter compared to that of chicken (575 vs. 581 bp). Moreover, the A287G locus was found to affect egg production in chicken from week 47 to 56. It was previously indicated by Onagbesan et al. (2003) that the activity of BMPR-1B gene was closely related to ovarian folliculogenesis and supposed to influence egg productivity in poultry. Nevertheless, in the present SNP observed in quail, the linkage between allele and egg number was not established in the population.



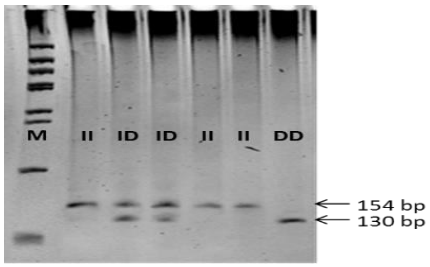
GH/*Msp*I, A237B

AA: 539/237 bp; AB: 776/539/237 bp; BB: 776 bp



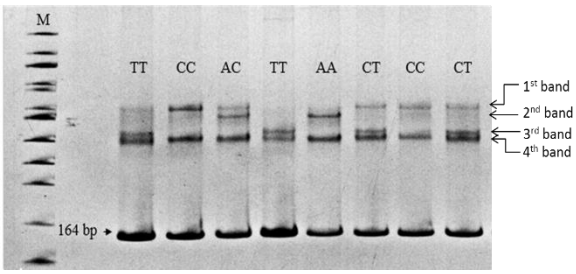
BMPR-1B/ *Hind*III, A290T

AA: 290/190/95 bp; AT: 385/290/190/95 bp; TT 385/190 bp



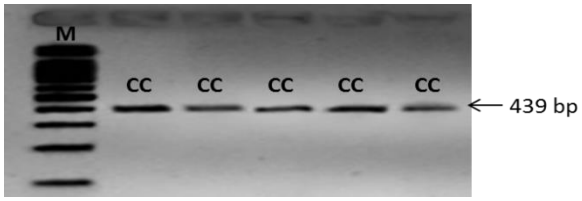
PRL, Indel-358/PCR

II: 154 bp; ID: 154/130 bp; DD: 130 bp



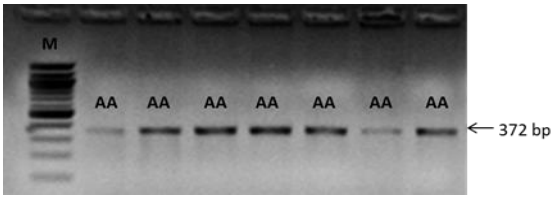
MTNR-1C[b]/SSCP, A459TC

CC: band 1&4; TT: band 3&4; CT: band 1,3&4; AC: band 1,2&4



PRL/*Csp6*I, A2161G

CC: 439 bp; CG & GG: not available



MTNR-1C[a]/*Mbo*I, G294A

AA: 372 bp; AG & GG: not available

Figure 1. Gel electrophoresis of PCR-RFLP and PCR-SSCP profiles of candidate genes (M: 100-bp DNA marker, Fermentas)

Table 3. Association of SNPs with egg weight and egg yield in 20 laying weeks

Gene/locus	Genotype	n	Egg weight (g)	Egg yield
GH/*Msp*I, A237B	AA	63	11.8±0.1	120.3±1.7
(population 1)	AB	74	11.7±0.1	122.8±1.5
	BB	59	11.9±0.1	124.1±1.7
	P		*0.381*	*0.283*
PRL-1, Indel-358	II	75	11.9±0.1	123.7±1.5
(population 1)	ID	110	11.7±0.1	121.3±1.3
	DD	4	12.0±0.4	129.5±6.7
	P		*0.285*	*0.265*
BMPR-1B/*Hind*III, A290T	AA	54	11.5±0.1	111.6±1.5
(population 2)	AT	36	11.6±1.0	113.2±1.9
	TT	15	11.4±0.1	111.7±2.9
	P		*0.498*	*0.786*
MTNR-1C[b], A459TC	AA	20	11.5±0.1	116.6±2.2[a]
(population 2)	TT	11	11.4±0.2	107.0±0.3[ab]
	CT	11	11.8±0.2	105.6±2.9[b]
	CC	29	11.6±0.1	115.9±1.9[a]
	AC	29	11.5±0.1	109.8±1.8[ab]
	P		*0.438*	*0.002*

[a,b] Different letters within a column indicate significant differences at the 5% level.

Being different from the other SNPs, three alleles namely A, T and C at locus A459TC were identified in the MTNR-1C gene formulating five genotypes, of which the AT genotype was absent in the population. Melatonin (N-acetyl-5-methoxytryptamine) secreted from the pineal gland is an indole hormone that is involved in many functions including seasonal changes in reproduction (Adachi et al., 2002; He et al., 2014). Moreover, it helps activate many receptors in the ovary and signaling pathways on various different cells including theca and granulosa cell (Soarces et al., 2003). According to Sundaresan et al. (2009), MTNR-1C gene was highly expressed in granulosa cells, therefore it is considered to effect on the process of egg formation and egg productivity in poultry. This statement was confirmed in the present work with significantly different egg number produced from quails of different genotypes.

Conclusion

In the populations studied, the A459TC (MTNR-1C) mutation was associated with egg production, of which those baring AA and CC

genotypes provided higher egg number in 20 laying weeks. This SNP is suggested to apply in other populations and it could be considered as a potential marker for egg production in Japanese quails.

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