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Original Article

Single Nucleotide Polymorphisms of Candidate Genes Related to Egg Production Traits in Vietnamese Indigenous Chickens

■Author(s)

Vinh NT[I] https://orcid.org/0000-0003-4881-7506
Giang NTP[I] https://orcid.org/0000-0002-9158-8844
Linh NV[II] https://orcid.org/0000-0002-7472-1543
Dang PK[I] https://orcid.org/0000-0002-7110-2552
Cahn NX[III] https://orcid.org/0000-0002-7791-6397
Giang NTC[I] https://orcid.org/0000-0002-3501-3587
Doan BH[I] https://orcid.org/0000-0003-0213-2755
Anh NT[I] https://orcid.org/0000-0002-2031-8273
Thinh NH[I] https://orcid.org/0000-0002-5373-6762

[I] Animal Science Faculty, Viet nam National University of Agriculture.
[II] Vietnam Academy of Science and Technology, Hanoi – Viet nam.
[III] Biotechnology Faculty, Viet nam National University of Agriculture.

■Mail Address

Corresponding author e-mail address
Thinh Nguyen Hoang
Faculty of Animal Science, Vietnam National University of Agriculture, Trau Quy, Gia Lam, Ha noi, Viet Nam.
Phone: +84 968643535
Email: nhthinh@vnua.edu.vn

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ABSTRACT

The objective of this work was to detect single nucleotide polymorphisms (SNPs) in two candidate genes known to relate with reproductive traits in Vietnamese chicken. Two indigenous breeds Ri and Mia chickens were used for genotyping 4 SNPs namely intron 1 of growth hormon gene (GH), exon 5 of prolactin receptor gene (PRLR5), a 24 bp nucleotide sequence insertion-deletion at situation -358 in 5'-untranslated region of prolatin gene (PRL24); and at site C2404T of prolactin gene (PRL2402). It was shown that the SNP sites of these candidate genes were identified in two local breeds. Three genotypes of each loci were found (AA, AB and BB for GH; II, ID and DD for 24 bp indel of PRL and CC, CT and TT for PRL2402), except only genotypes of AA and BB of PRLR5 were detected (no heterozygous genotype of AB). The frequencies of the desired genotypes of AA (GH), BB (PRLR5), II (PRL24) and CC (PRL2402) were low in both indigenous Ri and Mia chickens. Based on this study it could be suggested that the study on association between these loci with egg production traits and the selection to increase the desired genotype frequency in poputions are necessary to improve egg production of Ri and Mia chickens.

INTRODUCTION

For many developing countries, indigenous chicken breeds play a key role in poultry production system. They make up from 55% to 70% of the country's total poultry production (Hanh *et al.*, 2007; Desvaux *et al.*, 2008; Phuong *et al.*, 2015). Vietnam is endowed with a variety of indigenous chicken breeds which not only contribute tangible benefits as meat and eggs, but also intangible benefits as gamecocks, savings, cultural, genetic diversity conservation. Meat and eggs from indigenous chickens are always preferred by consumers who are willing to pay a higher price as compared to industrial ones. Moreover, the indigenous chickens have unique combinations of adaptive phenotypes and genotypes that respond to the local environment condition and climate change in which they have developed and evolved. They have obtained important traits such as high adaptability of heat shock and disease resistance and consistence with poor quality feeds and cultural practices, and are attributed essentially for contributing to the sustainable development production in local production systems. However, with egg production the yield is only 55-78 eggs per hen annualy; the egg weight from 35g to 40g; the local chickens begin laying at 22 weeks; they take more time to broody and rear their chicks. With these characteristics, the amount of products from indigenous chicken raising does not meet the needs of consumers and the indigenous diversity is increasingly leading to loss (FAO, 2007).

To improve egg production of indigenous breeds, many methods have been implemented such as selective breeding, enhancing feed

quality; housing; or veterinary support services (D*at et al.*,2005) to enhance growth and reproduction of native chicken. However, the previous selection method is primarily based on the appearance characteristics so the selection's efficiency could be low.

Several candidate genes are related to reproductive traits, reducing the broody days and broody frequency of native breeds (Jiang *et al.*, 2005; Cui *et al.*, 2006; Bhattacharya *et al.*, 2011). Among these genes prolatin (PRL), prolactin receptor (PRLR) and growth hormone (GH) genes have been widely studied and have significantly improved egg production and reduced broody behaviour (Cui *et al.*, 2006; Rashidi *et al.*, 2012; Stephen *et al.*, 2000; Zhang *et al.*, 2007;). GH gene is highly polymorphic, more than 30 polymorphisms have been identified in chickens. A polymorphism in intron 1 related with egg yield, particularly, egg yeild of the individuals carrying haplotype A1/A1 was 15% higher than others (Feng *et al.*, 1997; Kuhnlein *et al.*, 1997; Stephen *et al.*, 2000; Zhang *et al.*, 2007). Prolactin is a hormone best known for it's role in essential regulator of mammary development, which act to variety of hormones during sexual maturity and pregnancy (Yen *et al.*, 1999; Clevenger *et al.*, 2003). The higher concentration of circulating PRL occur with the increasing broody behavior. Besides, the ovarian follicle growth and egg laying performance were promoted by PRL (Zhang *et al.*, 2015). The 24bp insertion/deletion (indel) mutation at the site of -358 in promotor region of PRL gene affected egg yeild in many native chickens (Jiang *et al.*, 2005; Cui *et al.*, 2006; Begli *et al.*, 2010; Yousefi *et al.*, 2012; Lotfi *et al.*, 2013). Association of C2402T replacement mutation at 5' flanking region of PRL gene with egg production trait was also found in chicken breeds in China, Iran and Ukraine (Cui *et al.*, 2006; Liang *et al.*, 2006; Rashidi *et al.*, 2012; Bagheri *et al.*, 2013; and Kulibaba, 2015). PRLR is a gene that synthesizes receptor polypeptide to receive the action of hormones, from that to start, control and maintain a range of functions of poultry reproduction such as hatching and broody behavious (Cui *et al.*, 2006; Rashidi *et al*, 2012). Polymorphism in exon 5 of PRLR have a positive correlation with age and weight of hens at first egg laying (Rashidi *et al.*, 2012).

Up to now, there are no studies to evaluate the association of GH and PRL and PRLR genes on egg production in two Vietnameses indigenous chicken: Ri and Mia chickens breeds. This study identified a single nucleotide polymorphism in intron 1 of GH, exon 5 of PRLR, C2402T site and a 24bp nuclotide sequence indel of PRL genes in Ri and Mia chickens. This will be the initial scientific basis for further research to improve the egg yield of these two breeds.

MATERIALS AND METHODS

Animals and sample collection

This study was conducted on two populations of female native chicken breeds, namely Ri and Mia. Two breeds were raised in households of Son Tay District – level town (lies 35 km west of the capital Hanoi) and National Institute of Animal Science, Vietnam.

The animals used must represent each breed to ensure the individual has no close relatives. To identify the polymorphism of intron 1 of GH and exon 5 of PRLR gene and C2402T and 24bp indel of PRL gene, a total of 72 Ri chickens and 114 Mia chickens were used in this study.

Individual blood samples were taken from the vein of chicken wing; and collected immediately in anticoagulant tubes (EDTA treated). The collected individual blood samples were transferred to the laboratory and stored at -20^{0}C for futher experiment.

Genomic DNA extraction, PCR amplification reaction and genotyping identification methods

Genomic DNA from blood samples were extracted using the procedure dicribed by Sambook *et al.* (1998) who improved some steps to suit the laboratory conditions. Spectrophotometer and agarose gel electrophoresis methods were used to check the quality and quantity of the extracted DNA. Genomic DNA samples had adjusted to final concentration of 50ng/µl by using TE buffer.

Four pair of primers provided by Feng *et al.* (1997); Rashidi *et al.* (2012); Cui *et al.* (2006) were used to amplify the intron 1 of GH (GH), exon 5 of PRLR (PRLR5), C2402T site (PRL2402) and 24bp indel of PRL (PRL24) gene, respectively. The primer sequences for each marker loci are presented in Table 1. Primer pair GH was used to amplify the 900 bp fragment; primer pair PRL5 was used to amplified the 250 bp fragment; primer pair PRL2402 was used to amplify 439 bp fragment; and primer pair PRL24 was used to amplified 154 or 130 bp fragments.

The PCR amplification reaction was performed final concentration 10ng of genomic DNA, 1.3mM $MgCl_2$, 0.15 mM dNTPs, 0.3 µM primers and 1.5U of Taq DNA polymerase (Thermo scientific) and 1x PCR buffer and de-ion water in a 25 µl final volume. PCR reaction conditions of: (1) GH was following program: 95^{0}C

Table 1 – Primer pair sequences and Tm of candidate genes.

Genes	Primer sequences (5'-3')	Tm (°C)	Product size (bp)
GH	F: ATCCCCAGGCAAACATCCTC R: CCTCGACATCCAGCTCACAT	60	900
PRLR5	F: TTGTCTGCTTTGATTCATTTCC R: TGCATTTCATTCTTCCCTTTTT	57	250
PRL24	F: TTTAATATTGGTGGGTGAAGAGACA R: ATGCCACTGATCCTCGAAAACTC	54	154 and 130
PRL2402	F: 5'-CTAAAGGACCTGGAAGAAGGG-3' R: 5'-AACTTGTCGTAGGTGGGTCTG-3'	52	439

for 5 min followed by 28 cycles of 92°C for 30 sec, 60°C for 120 sec, 72°C for 90 sec and final extension of 72°C for 15 min; (2) PRLR5 was at 94°C for 5 min following program: 30 cycles of 94°C for 60 sec, 57°C for 60 sec, 72°C for 60 sec and final extension of 72°C for 15 min; (3) PRL2402 was at 95°C for 2 min, following program: 35 cycles of 94°C for 35 sec, 52°C for 35 sec, 72°C for 45 sec, with an ending step at 72°C for 15 min.

For genotyping of GH, PRLR5 and PRL2402 genes, the PCR products were digested by *MspI, BamHI* and *AluI* enzymes, respectively. 8µL of each amplified DNA fragments was digested at 37°C in 8 hours, in a final volume of 30µL, containing 2 U, 3µL of restriction buffer, and 19.6µL of H_2O, and appropriate restriction enzyme. The DNA fragments of digestive reaction were separated on 2,5% agarose gel.

For genotyping of PRL24 containing the 24bp insertion or deletion, the PCR fragment was separated on 3% agarose gel.

Statistical analysis

The distribution of genotypic frequencies and allelic frequencies of each SNP in two local chicken breeds were counted. Using a chi-squared test (χ^2) comparing the expected and observed genotype count to test Hardy-Weinberg equilibrium in two native chicken breeds. The data was analyzed using SAS software 9.1 (SAS 9.1., 2002)

RESULTS AND DISCUSSION

PCR-RFLP analysis

The electrophoretic profiles of the studied genes are shown in Figure 1. For intron 1 of GH gene, two alelles specific patterns were obtained after *MspI* digestion, allele A including 620 bp and 280 bp DNA fragments and three DNA fragments of 450 bp, 280 bp and 170 bp for allele B. Thus, this gene consequently result in three genotypes, AA (620 and 280 bp), BB (450, 280 and 170 bp) and AB (620, 450, 280 and 170 bp). The results show that the DNA banding patterns of GH was not respectively consistent with what was previously reported by Tanaka *et al.* (1992); Stephen *et al.* (2001); or Kazemi *et al.* (2018). When studying native chicken of Iran, Kazemi *et al.* (2018) show a PCR fragments of 776 bp; and using restriction reaction, with MspI restriction enzyme, detected polymorphism in intron 1 of GH gene. The result obtained three kind of alleles of A, B and C and 6 different haplotype profiles (AA, BB, CC, AB, AC and BC).

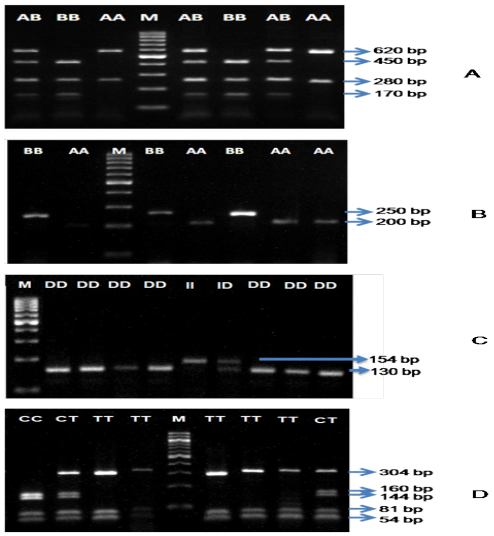

Figure 1 – PCR fragments of GH (A), exon 5 of PRLR (B), 24 bp Insertion-Deletetion PRLR (C) and C2402T PRLR (D) genes; genotypes were shown in image; M: DNA maker.

For exon 5 of PRLR, this stuty has obtained two allele A and B; but just AA genotypes and BB genotypes were indentified in two native chicken breeds. Allele A was an uncut 250bp fragment and allele B was 200 and 50 bp fragments. The heterozygous genotype AB was absent in both Ri and Mia chickens. According to Rashidi *et al.* (2012), the fragment of PRLR5 was detected by digestion with *BamHI* restriction enzyme found in two bands of 195 and 55 bp. Including 195bp and 55 bp fragment for A allele, while uncut fragment 250 bp for allele B because of the lack site for a *BamHI* restriction enzyme activity. However, Rashidi *et al.* (2012) also found there were no heterozygous females identified.

The genotyping of the 24 bp insertion-deletion of PRL gene, three genotypes of II with 154 bp fragment (presence fragment 24 bp), DD with 130 bp fragment (absence fragment 24 bp) and ID with 154 and 130 bp fragments were observed. The banding patterns of PRL24 were consistent to previously reports of (Rashidi *et al.*, 2012; Sarvestani *et al.*, 2012).

For PRLR at site C2402T, three genotypes of CC, TT and CT were obtained. Individuals with 4 fragments of 160, 144, 81 and 54 bp were designated as CC; 3

fragments of 304, 81 and 54 bp were designated as TT; and 5 fragments of 304, 160, 144, 81 and 54 were designated as CT. This result is consistent to previously results which was repored by Cui *et al.*, (2005) and Sarvestani *et al.*, (2012).

Frequencies distribution

The distribution of genotypic frequencies and allelic frequencies of each SNP in the two local chicken breeds tested are presented in Table 2 and 3.

The distribution of alleles and genotypes frequencies of all SNP tended to be the same in both Ri and Mia chikens. Frequencies of expected genotypes for egg production were lower as compared to others. Particularly, in Ri chicken, the frequency of AA (GH gene), BB (PRLR), II (PRL24) and CC (RPL2402) was 0.03; 0.01; 0.07 and 0.06, respectively. In Mia chiken, the frequency of these alleles was 0.02; 0.03; 0.07 and 0.09, respectively. There was no heterozygous genotype AB of RPLR5 detected in both studied populations. The major reason for this is because the PRLR5 gene is located on the Z chromosome, so in this situation there were no heterozygous females (Rashidi *et al.*, 2012). In Ri Chicken, the genotype frequency distributions at every locus were all in Hardy-Weinberg equilibrium ($p>0.05$), except for PRLR5 gene ($p<0.05$). In Mia chicken, the genotype and allele frequency in intron 1 of GH gene were distributions in Hardy-Weinberg equilibrium ($p>0.05$) but the chi-squared test for PRLR5, PRL24 and PRL2402 genes deviated from Hardy-Weinberg equilibrium ($p<0.05$).

For the traits of interest in production animal, the findings of QTLs, genes, SNPs responsible for genetic variation play an important role in genome selection and genomic analysis and animal breeeding (Rothschild & Soller, 1997). In this study, some SNPs of candidate genes have previously been known to improve egg yield in several native chicken breeds and were applied in Ri and Mia Vietnamese native chickens for later use in selection and breeding.

The evaluation of genotypic and allelic frequency have been considered as an important factor for animal breeding and animal selection. Following, the next step in animal selection, is to evaluate the effect of these genotypes to interest phenotype traits which would help to select desirable individuals, with interest in genotype and phenotype traits. Under our study, the alleles associate with egg production had low frequencies in the Ri and Mia chicken population.

Previously, polymorphism of SNPs at intron 1 of Growth Hormone gene and its association with egg production traits was implemented. However, in our study in indigenous Ri and Mia chicken the result was different, with the PCR fragment of 900 bp, two alleles (A allele, B allele) and three haplotypes (AA, AB, BB) were found. The frequencies of AA genotype in Ri chicken population and Mia chicken population were 0.03 and 0.02, respectively.

Cui *et al.* (2006) found the distribution of expected allele I (PRL24) and C (PRL2402) appears quite differently in Chinese chicken breeds: 0.02 and 0.02 (Taihe Silkies local chicken), 0.05 and 0.05 (Yangshan local chicken), 0.17 and 0.42 (Nongdahe local chicken), 0.22 and 0.35 (White Rock exotic chicken); 1.00 and 1.00 (White Leghorn exotic chicken). Arcorrding Cui *et al.* (2006), exotic chicken (White Leghorn chicken) breed can produce around 320 eggs per year because they do not present broodiness; two Chinese native Taihe Silkies and Yangshan chicken have strong incubation and lay almost 90 eggs and less than 70 eggs per

Table 2 – Distribution of genotypic and allelic frequencies of candidate genes of Ri chickens breed.

Breeds	Number of hens	Allelic frequencies		Genotypic frequencies			X^2 (5.99)	*p* value
		Allele 1	Allele 2	11	12	22		
GH	72	A: 0.24	B: 0.76	0.03 (2)	0.43 (31)	0.54 (39)	2.06	0.36
PRLR5	72	A: 0.99	B: 0.01	0.99 (71)	0	0.01 (1)	71.96	<0.01
PRL24	59	I: 0.19	D: 0.81	0.07 (4)	0.25 (15)	0.68 (40)	1.80	0.41
PRL2402	59	C: 0.22	T: 0.78	0.09 (5)	0.27 (16)	0.64 (38)	2.36	0.31

Note: Figures in brackets are the number of genotypes. X^2 = chi-square test.

Table 3 – Distribution of genotypic and allelic frequencies of candidate genes of Mia chickens breed.

Breeds	Number of hens	Allelic frequencies		Genotypic frequencies			X^2 (5.99)	*p* value
		Allele 1	Allele 2	11	12	22		
GH	114	A: 0.18	B: 0.82	0.02 (2)	0.33 (38)	0.65 (74)	1.09	0.58
PRLR5	114	A: 0.97	B: 0.03	0.97 (111)	0	0.03 (3)	78.95	<0.01
PRL24	45	I: 0.12	D: 0.88	0.07 (3)	0.11 (5)	0.82 (37)	10.43	<0.01
PRL2402	45	C: 0.11	T: 0.89	0.07 (3)	0.09 (4)	0.84 (38)	14.08	<0.01

Note: Figures in brackets are the number of genotypes. X^2 = chi-square test.

year, respectively. The local chicken line (Nongdahe chicken), which was produced by China Agricultural University, lays 190 eggs per year. Frequency of allele I was found low in Ukrainian meat-line chicken (0.14) and high in egg-line chicken (0.73) (Kulibaba *et al.*, 2012). When study in Fars native chickens (a high frequency of broodiness, producing 20-70 eggs during each laying period - 8 months) of Iran, Sarvestani *et al.* (2012) show two alleles were found for the SNP of C-2402T with frequency of 0.34 for T and 0.66 for C. The 24-bp indel at the site -358 in promoter region of prolactin gene was shown to be polymorph with the observed genotypic frequency of 0.42 in II, 0.45 in ID and 0.13 in DD.

For some indigenous chicken breeds in Vietnam, Nguyen *et al.* (2018) found no individuals with homozygous genotype II (PRL24) and CC (PRL2402) was found in Lien Minh native chicken breed, with relatively low egg yield. Vu and Ngu (2016) observed low frequency (0.03) of CC (PRL2402) in native Noi chicken.

Study of Rashidi *et al.* (2012) with polymorphism exon 5 of PRLR of Iran indigenous chicken show there were no heterozygous females and the distribution of BB genotype was lower (0.28) than AA genotype (0.72).

Under the present study, the expected genotype frequencies of candidate genes in indigenous Ri and Mia chickens were low compared to other genotypes. This is entirely consistent with the egg production characteristics of indigenous chickens. Therefore, breeding programs need to be done to select chickens carrying the desired genotypes for enhancing egg production of indigenous chickens.

CONCLUSION

In conclusion, 4 SNPs of the GH, PRLR and genes were identified in both indigenous Ri and Mia chickens. Low frequencies of expected genotypes of AA (GH), BB (PRLR5), II (PRL24) and CC (PRL2402) was observed in both breeds. These loci should be used for purpose of futher studies on the realtionship between genotype or allele with egg reproduction traits in Ri and Mia chickens.

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CONFLICT OF INTEREST

We declare that there is no conflict regarding the results discussed in the paper.

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