



The Association of *BMPRI1B* and *PRL* Polymorphisms with Egg Productivity and Quality Traits in Japanese Quails

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Abstract

Egg productivity and quality are essential quantitative traits controlled by many genes, including those encoding bone morphogenetic receptor 1B (*BMPRI1B*) and prolactin (*PRL*). This study aimed to identify the polymorphisms of *BMPRI1B* and *PRL* and their association with egg productivity and quality traits in Japanese quail. The phenotypes of 73 female quails raised under an individual cage system were investigated, and their blood samples were analyzed. The phenotypes investigated included egg production, body weight at 1 to 6 weeks, egg weight, egg index, eggshell weight, eggshell thickness, albumen index, yolk index, yolk score, yolk weight, albumen weight, yolk ratio, albumen ratio, and Haugh unit. The polymorphisms of *BMPRI1B* in exon 9 and intron 10 were investigated using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, while a 24 bp indel of *PRL* located in promoter region was directly detected by examining the size of the PCR products (154/130 bp). The association between *BMPRI1B* and *PRL* polymorphisms with egg productivity and quality traits was analyzed using an independent sample t-test. The result showed that the *BMPRI1B* did not have polymorphisms. On the other hand, two genotypes, DD and ID, were found for the 24 bp indel of *PRL*. Moreover, the t-test analysis results indicated no association between the 24 bp indel of *PRL* and egg productivity and quality ($P > 0.05$). In conclusion, *BMPRI1B* was monomorphic, and *PRL* polymorphisms did not affect the egg productivity and quality traits of Japanese quails.

Introduction

Quails are a type of poultry extensively developed using a breeding system that crosses black female quails with brown male quails to obtain a commercial stock of brown female quails based on the phenomenon of crisscross inheritance (Rifki *et al.*, 2018). Quail layers with superior traits have high weight gain, resistance to tropical diseases, and high egg productivity. However, genetic variations significantly affect external and internal egg qualities; thus, this matter is closely related to its reproductive system (Isidahomen *et al.*, 2013). The reproductive

trait in poultry is an important indicator controlled by many genes (Niknafs *et al.*, 2014).

BMPRI1B, located on chromosome 4 and comprising 13 exons closely related to ovulation rate and follicle maturation is expressed by follicular and or theca interna cells in the poultry ovary. A previous study has identified one silent mutation (T35C) in exon 6 and 4 mutations in intron 6. Among the intronic mutation, especially A287G, is highly correlated with egg production at 47–56 weeks (Zhang *et al.*, 2008). Moreover, some *BMPRI1B* polymorphisms found in the quantitative trait loci are

significantly associated with egg productivity and quality (Liu *et al.*, 2011). In addition, *BMPRI1B* polymorphisms are significantly associated with chicken body weight at 2–8 weeks (Awad and El-Tarabany, 2015).

PRL is the gene that specifically controls egg production and growth performance in poultry (Chen *et al.*, 2011; Wang *et al.*, 2011; Mazurowski *et al.*, 2016). *PRL* also regulates the quantity of Graafian follicles in the ovary. Because it is directly related to egg production and quality, many studies have been conducted to identify its polymorphisms (Bhattacharya *et al.*, 2011; Zhang *et al.*, 2015; Shamsalddini *et al.*, 2016; Li *et al.*, 2017). Polymorphisms of the *PRL* are associated with egg production (Li *et al.*, 2013; Sarvestani *et al.*, 2013; Kulibaba, 2015; Tempfli *et al.*, 2015; Mohamed Osman *et al.*, 2017; Yadav *et al.*, 2018). Six mutation sites, C2402T, C2161G, T2101G, C2062G, T2054A, G2040A, and 24 bp insertion-deletion (Indel), are found from direct sequencing; in particular, the 24 bp indel is significantly associated with egg production and hatching traits in chicken (Cui *et al.*, 2006). *PRL* polymorphisms are also discovered in Japanese quails (Yousefi *et al.*, 2012). *PRL* polymorphisms found in the 5'-flanking region in chicken significantly affect poultry productivity (Mohamed Osman *et al.*, 2017). Furthermore, 24 bp indel of *PRL* polymorphisms are closely related to egg production in the native poultry of Yazd province (Begli *et al.*, 2010).

Molecular genetic research in the poultry industry aim to increase egg production and inhibit incubation behavior (Xu *et al.*, 2010). Several studies have shown that *BMPRI1B* and *PRL* are candidate genes for egg productivity and quality traits in poultry because they play a role in poultry production directly or indirectly (Wilkanowska *et al.*, 2014). Furthermore, in chicken ovaries, *BMPRI1B* mRNA levels in granulosa cells are higher than in theca cells, impacting follicular maturation and affecting poultry reproduction (Onagbesan *et al.*, 2003). However, the data regarding the association between *BMPRI1B* and *PRL* with egg productivity and quality traits in Japanese quails are limited. Therefore, this study aimed to identify polymorphisms in *BMPRI1B* and *PRL* and examine their association with the productivity and quality of Japanese quail eggs.

Materials and Methods

Quails and phenotypic data collection

Seventy-three female quails were reared in individual cages as described by Dzuriatmono *et al.* (2019) at the Experimental Farm, Jatikuwung, the Department of Animal Science, Sebelas Maret University. The phenotypes observed were body weight, egg production, and exterior and interior egg qualities. The body weight at 1 to 7 weeks was measured using

an analytic scale (Starco Electrindo, Indonesia). Egg production (%) was average number of eggs produced by quails from the first egg-laying age (at 47 days) to 90 days. The exterior and interior quality of eggs was measured using the eggs collected from 84 to 86-day-old quails every morning at 7 a.m. for three consecutive days. The exterior egg qualities, including egg weight (g), egg index (%), eggshell weight (g), and eggshell thickness (mm), were measured according to Cahyadi *et al.* (2019). The interior egg qualities, including yolk weight (g), albumen weight (g), yolk ratio (%), albumen ratio (%), yolk and albumen indexes (%), and Haugh unit value, were measured in accordance to Yilmaz *et al.* (2011). In addition, yolk color was scored by comparing egg yolk with a Roche Yolk Color Fan with the 1-15 color standards (Robotmatation Co. Ltd., Japan) (Nagarajan *et al.*, 1991).

Blood sample collection and DNA extraction

Blood samples were obtained from the slaughtered quails collected in the 5 ml vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, Plymouth, UK) acting as an anticoagulant. Sterile equipment was used for slaughtering quails and collecting blood samples to minimize possible cross-contamination among the samples. Then, the tubes containing the blood samples were stored at -21°C until DNA extraction.

DNA extraction was carried out using a Wizard Genomic DNA Purification Kit (Promega, Wisconsin, United States). In details, 20 μL of blood sample was put into a 1.5-ml microtube, and 900 μL of Red Blood Cell Lysis Solution was added. The mixture was then homogenized and incubated for 60 minutes at 37°C . Next, the sample was centrifuged in a Hettich Zentrifugen Mikro 22 R (Andreas Hettich GmbH & Co. KG, Germany) at 24,150 g for 20 seconds. Subsequently, 300 μL of Nuclei Lysis Solution and 100 μL Protein Precipitation Solution were added, and the tube was vortexed for 20 seconds and immediately centrifuged at 24,150 g for 3 minutes. Then, the supernatant was transferred into a new 1.5-ml microtube containing 300 μL of isopropanol, inverted for 20 seconds, and centrifuged at 24,150 g for 60 seconds. Next, the supernatant was discarded, and 300 μL of 70% ethanol was added into the tube before centrifuging at 24,150 g for 30 seconds. Subsequently, the tube was dried out, and 100 μL of DNA Rehydration Solution was added. Lastly, the microtube containing the sample was vortexed for 5 seconds and incubated at 65°C for 60 minutes.

Amplification of *BMPRI1B* and *PRL*

Genomic DNA was used as the template for amplifying *BMPRI1B* and *PRL* using GeneAmp 9700 PCR Thermal Cycler (Thermo Fisher Scientific, Singapore) with the Taq PCR Master Mix Kit

(Qiagen, Hilden, Germany). The primer pairs and PCR programs were presented in Tables 1 and 2, respectively. The number of cycles of PCR was set at 35. Next, the PCR products were ran using 3%

agarose gels which is previously stained by ethidium bromide (EtBr) using Electrophoresis System EX® (Advance, Japan) and visualized using a Glite UV Gel Documentation System (Pacific Image, Taiwan).

Table 1. Primer pairs and PCR product size of *BMPR1B* and *PRL* investigated in this study

| Gene | Oligonucleotide primer (5' – 3') | PCR Product | SNP name | Location |
|---------------|---|-------------|-------------------------------------|-----------------|
| <i>BMPR1B</i> | F: CCATAGCAAAACAGATTCAG R: TCAGGACAGTTTGGTAGATT | 162 bp | rs316948669 ^a (C35T) | Exon 9 |
| | F: AAAGGTCGCTATGGGGAAGT R: ATGCAGCTATGCCTCACCTT | 437 bp | rs740296587 ^b (A287G) | Intron 10 |
| | F: TGCCACCTGTGTTCAAGTATC R: GCGGCTCTTACCCAACCTTTC | 536 bp | rs739326357 ^c | Exon 10 |
| <i>PR</i> | F: TTTAATATTGGTGGGTGAAGAGACA R: ATGCCACTGATCCTCGAAACTC | 154/130 bp | 24 bp Indel ^d | Promoter region |

^{a,b} Zhang et al. (2008); ^c This study; ^d Cui et al. (2006)

Table 2. PCR programs for amplification of targeting fragment of *BMPR1B* and *PRL*

| <i>BMPR1B</i> | <i>PRL</i> |
|---------------------------------------|---------------------------------------|
| Initial denaturasi (94°C) → 3 minutes | Initial denaturasi (94°C) → 5 minutes |
| Denaturation (94°C) → 30 seconds | Denaturation (94°C) → 30 seconds |
| Annealing (60°C) → 30 seconds | Annealing (54°C) → 30 seconds |
| Extension (72°C) → 30 seconds | Extension (72°C) → 30 seconds |
| Final extension (72°C) → 10 minutes | Final extension (72°C) → 5 minutes |

Genotyping of *BMPR1B* and *PRL*

BMPR1B was genotyped using PCR-RFLP with HphI for single nucleotide polymorphism (SNP) rs316948669 and HindIII for SNP rs740296587 (Thermo Fisher Scientific). The PCR products were digested for 6 hours in a 31-μL reaction containing 10 μL of PCR Product, 18 μL of nuclease-free water, 2 μL each of 10 × Buffer B and 10 × Buffer R, and 1 μL of HphI or HindIII. The PCR-RFLP results were visualized using electrophoresis with a 3% agarose gel. SNP rs739326357 was not genotyped due to the lack of restriction enzyme sites. Meanwhile, the indel mutation, a 24 bp indel of *PRL*, was genotyped directly by observing the PCR product sizes.

Calculation of genotype and allele frequencies

Frequencies of genotype and allele were measured by

Equation 1

$$x_{ii} = \frac{n_{ii}}{N} \times 100\%$$

and Equation 2

$$x_i = \frac{2n_{ii} + \sum n_{ij}}{2N} \times 100\%$$

where X_{ii} was the genotype frequency of the ii-th, X_i was the allele frequency of the i-th, n_{ii} was the sum of individuals with genotype ii, n_{ij} was the sum of individuals with genotype ij, and N was the sum of all individual samples (Nei and Kumar, 2000).

Association between *PRL* gene with productivity and egg quality traits

The association of *PRL* with egg productivity and quality traits was examined using an independent sample t-test (R-studio, $\alpha = 0.05$). The mathematical model used was (Astuti, 1981):

$$t = \frac{M_1 - M_2}{\sqrt{\frac{SS_1 + SS_2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

where M_1 was the average score of group 1, M_2 was the average score of group 2, SS_1 was the sum of the square of group 1, SS_2 was the sum of the square of group 2, n_1 was the subject number of group 1, and n_2 was the subject number of group 2.

Results

Amplification and Genotyping of *BMPR1B* and *PRL*

A total of 3 SNPs of *BMPR1B* were investigated. The PCR products were successfully amplified, as indicated by the clear DNA bands. Two SNPs, rs316948669 and rs740296587, were located in exon 9 and intron 10, as indicated by the 162 and 437 bp bands, respectively (Figure 1A). The rs739326357 SNP of *BMPR1B* was also successfully amplified, as indicated by the 536 bp product (Figure 1B).

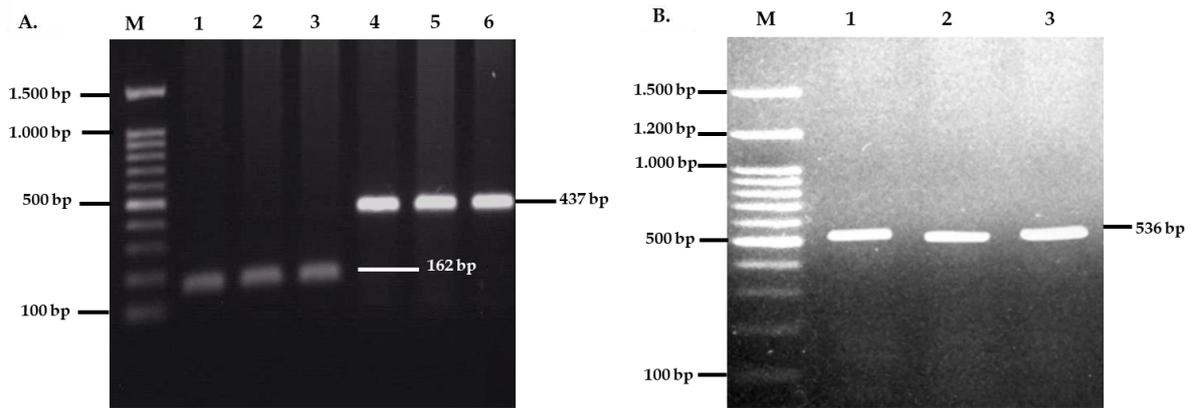


Figure 1. Amplification of *BMPR1B* polymorphisms. A is PCR products for rs316948669 and rs740296587 SNPs in exon 9 and intron 10. B is PCR product for rs739326357 SNP in exon 10. M is 100 bp marker ladder; 1 to 6 are individual quail samples.

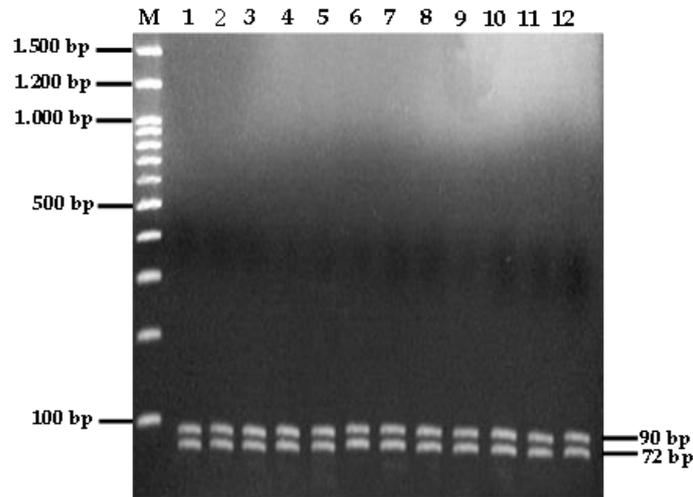


Figure 2. Genotyping of rs316948669 of the *BMPR1B*. M is 100 bp marker ladder; 1 to 12 are individual quail samples.

Next, the 3 SNPs of *BMPR1B* were genotyped; however, they were not polymorphic (Figure 2). Regarding rs316948669, only the AB genotype was identified, as indicated by the 90 and 72 bp DNA bands. In addition, the sequence analysis of the 162

bp fragment revealed a novel SNP, a G/A substitution (Figure 3) that was a synonymous mutation (Gly > Gly). However, there were no restriction enzyme sites to genotype this SNP.

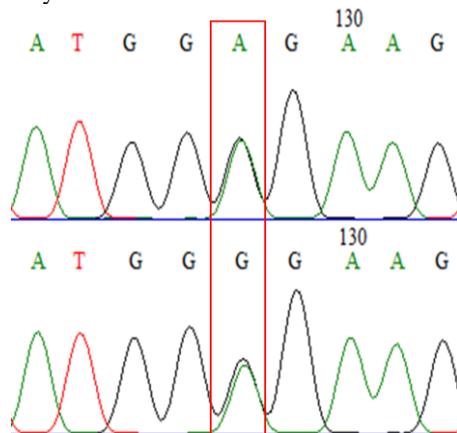


Figure 3. A novel SNP of *BMPR1B* identified in this study. Red bar indicates G/A substitution.

The amplification of the 24 bp indel of *PRL* was also successful, as indicated by the 130 and or 154 bp DNA band. This study revealed two genotypes of the 24 bp indel of *PRL*: the homozygous DD genotype

indicated by the 130 bp band and the heterozygous ID genotype indicated by the 130 and 154 bp bands (Figure 4).

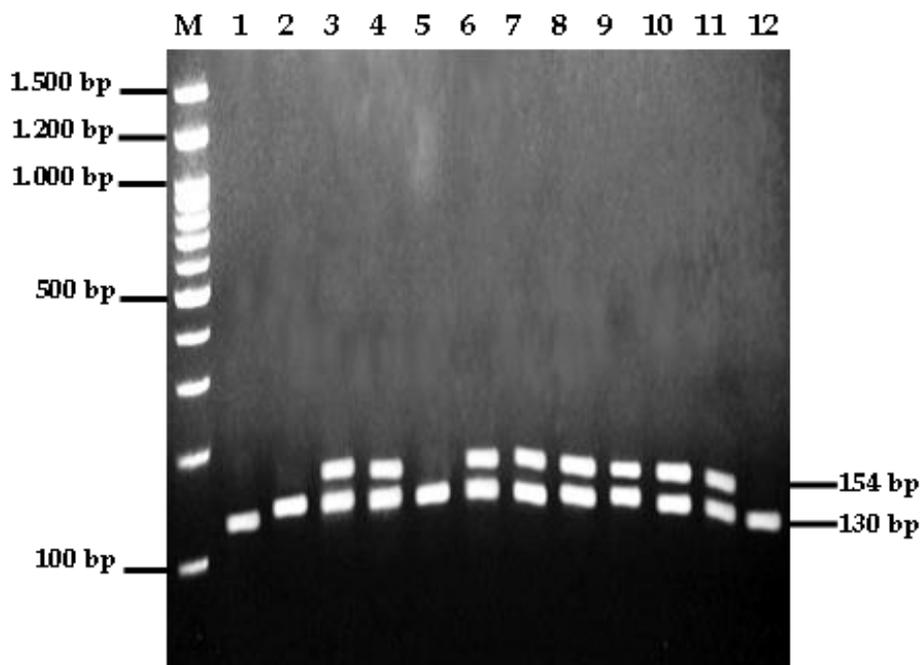


Figure 4. Genotyping of 24 bp indel of *PRL*. M is 100 bp marker ladder; 1, 2, 5, and 12 are individual quails with homozygous DD genotype; 3, 4, 6 to 11 are individual quails with heterozygous ID genotype.

Genotype and allele frequencies

As the SNPs of the *BMPRI3* SNPs investigated were monomorphic, their genotype and allele frequencies could not be calculated. On the other hand, the values of the genotype and allele frequencies and the Hardy-Weinberg equilibrium (HWE) of the 24 bp indel in *PRL* in the Japanese quail population were calculated

(Table 3). The calculation of the HWE using a chi-square analysis showed that the quail population was in equilibrium. In addition, the genotype frequency analysis demonstrated that the frequency of the ID genotype was higher than DD. The allele frequency calculation showed that the D allele was higher than the I allele.

Table 3. Genotype and allele frequencies of 24 bp indel of *PRL*

| Genotype | Genotype frequency | He | Ho | X ² |
|----------|--------------------|------|------|--------------------|
| DD | 0.11 (n=8) | 0.31 | 0.11 | 0.64 ^{ns} |
| ID | 0.89 (n=65) | 0.49 | 0.89 | |
| II | 0 (n=0) | 0.20 | 0 | |
| Allele | Allele frequency | | | |
| D | 0.55 | | | |
| I | 0.45 | | | |

He is expected heterozygosity value; Ho is observed heterozygosity value; ^{ns} is not significant (P = 0.42).

Association between 24 bp indel of the *PRL* with productivity and egg quality traits

The association between the 24 bp indel of *PRL* with egg productivity and quality traits in Japanese quails was examined (Table 4) by analyzing 7 egg productivity and 12 egg quality traits. Statistical

analysis indicated no association between the 24 bp indel of *PRL* and egg productivity or quality traits. However, a suggestive significance difference was found between *PRL* with yolk color score (P = 0.09), in that the quails with the DD genotype had better yolk color scores than those with the ID genotype.

Table 4. Association between 24 bp indel of the *PRL* and productivity and egg quality traits

| Traits | Genotype | | P-Value |
|-------------------------|----------------|----------------|-------------------|
| | DD | ID | |
| Egg production (%) | 59.05 ± 6.06 | 60.87 ± 10.66 | 0.47 |
| BW1 (g) | 24.00 ± 2.24 | 23.48 ± 3.28 | 0.56 |
| BW2 (g) | 42.38 ± 5.55 | 43.95 ± 6.61 | 0.48 |
| BW3 (g) | 70.80 ± 10.81 | 71.41 ± 11.25 | 0.88 |
| BW4 (g) | 96.67 ± 12.17 | 98.45 ± 12.66 | 0.71 |
| BW5 (g) | 120.81 ± 14.54 | 123.64 ± 14.94 | 0.62 |
| BW6 (g) | 137.72 ± 14.79 | 139.74 ± 12.93 | 0.72 |
| Egg weight (g) | 10.23 ± 1.16 | 9.94 ± 0.83 | 0.50 |
| Egg index (%) | 79.43 ± 2.19 | 79.10 ± 2.52 | 0.71 |
| Eggshell weight (g) | 1.35 ± 0.22 | 1.35 ± 0.17 | 0.99 |
| Eggshell thickness (mm) | 0.38 ± 0.12 | 0.34 ± 0.16 | 0.35 |
| Albumen index (%) | 26.22 ± 1.56 | 26.14 ± 2.47 | 0.91 |
| Yolk index (%) | 35.66 ± 4.08 | 36.97 ± 2.75 | 0.40 |
| Yolk color score | 7.04 ± 0.28 | 6.81 ± 0.71 | 0.09 [†] |
| Yolk weight (g) | 3.10 ± 0.42 | 3.06 ± 0.37 | 0.80 |
| Albumen weight (g) | 5.79 ± 0.73 | 5.53 ± 0.57 | 0.38 |
| Yolk ratio (%) | 30.25 ± 1.66 | 30.77 ± 2.70 | 0.46 |
| Albumen ratio (%) | 56.55 ± 2.96 | 55.67 ± 3.01 | 0.45 |
| Haugh unit | 98.86 ± 0.72 | 98.74 ± 2.27 | 0.76 |

[†] Indicates suggestive-significant effect ($P < 0.1$); BW1 is Body Weight at a week of age; BW2 to BW6 is Body Weight at 2 to 6 weeks of age.

Discussion

This study is the first to evaluate the association between *BMPRI1B* and *PRL* and egg productivity and quality traits in Japanese quails. SNPs were not found in exon 9, intron 9, and exon 10 of *BMPRI1B*, indicating that the study population might have undergone natural selection or come from the same parents (inbreeding). In addition, the relatively small number of samples used did not lead to diversity in the quail population. However, this result was consistent with a previous study by Hidayati *et al.* (2016), which did not find any polymorphism of *BMPRI1B* in populations of Arab chickens, Indonesian native chickens, and laying hens. On the other hand, three genotypes of an intronic SNP (A287G) of *BMPRI1B* have been successfully identified in Fayoumi and Rhode Island Red chicken populations (Awad and El-Tarabany, 2015). In addition, A287G SNP of *BMPRI1B* is significantly associated with egg production in synthetic broiler lines (Zhang *et al.*, 2008). Therefore, in different species and populations, the gene may have different effects on egg productivity and quality traits in poultry. The lack of research on *BMPRI1B* in Japanese quails may also lead to the lack of polymorphisms identified since the targeting fragment of the gene and sequence references are based on the chicken genome.

Meanwhile, a 24 bp indel was identified in *PRL* in the Japanese quail population. Two genotypes, DD and ID, were observed. The frequency of the D allele was

greater than the I allele, at 0.55 and 0.45, respectively, consistent with previous studies (Cui *et al.*, 2006; Yousefi *et al.*, 2012). Moreover, a previous study reported that three genotypes of the 24 bp indel of *PRL* has been investigated in chickens (Xu *et al.*, 2011). Cui *et al.* (2006) also revealed that frequency of D allele was 0.80, 0.95, 0.78, and 0.83, respectively for Taihe Silkies, Yangshan White Rock, and Nongdahe chicken populations, and no D allele was detected in White Leghorn population. In addition, Alipanah *et al.* (2011) observed a *PRL* SNP in Zabol native chickens. The value of minor allele frequency was 0.45, suggesting that the quail population was polymorphic. According to Nei and Kumar (2000), a population is considered polymorphic when the minor allele frequency is more than 1% and less than 99%. Therefore, low allele frequency may be lost due to genetic drift and natural selection from one generation to the next (Smith and Baldwin, 2015). In addition, the high or low cases of inbreeding or the diversity of a population can be determined by calculating the expected heterozygosity value (H_o) and the observed heterozygosity value (H_e) based on the chi-square analysis in the HWE (Akramullah *et al.*, 2020). Based on the known *PRL* genotype and allele frequencies, the quail population in this study followed the HWE; in other words, there was no selection, mutation, migration, or non-random mating (Allendorf *et al.*, 2013).

Here, association analysis could only be conducted between *PRL* and the phenotypic traits observed because no variations were found in

BMPR1B. The association analysis showed that the 24 bp indel of *PRL* did not affect egg productivity and quality traits in Japanese quails. Previously, an intronic A412G SNP of the *PRL* was found to be significantly associated with egg production in two Chinese Jinding and Youxian laying ducks (Bai *et al.*, 2019). On the other hand, no association was found between the C2402T SNP of *PRL* and egg production in chickens. However, haplotype analysis of *PRL* SNPs revealed that the C2402T SNP interacted with other SNPs, significantly increasing *PRL* expression and reducing egg production (Cui *et al.*, 2006). A lack of significant association between *PRL* and egg productivity and quality traits in Japanese quails suggests an opportunity to evaluate more polymorphisms of this gene. Therefore, a larger quail population and breeding design are needed to obtain

more precise results.

Conclusion

Three targeted SNPs in *BMPR1B* were found to be monomorphic, and a novel SNP was identified in exon 9. In addition, two genotypes of the 24 bp indel of *PRL*, DD and ID, were observed. However, there was no association between these genotypes and egg productivity and quality characteristics. Therefore, future studies with larger quail populations and investigation of more SNPs are necessary to achieve an in-depth analysis.

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