

ASSOCIATION OF POLYMORPHISM IN PROLACTIN (*PRL*) PROMOTER REGION AND PROLACTIN RECEPTOR (*PRLR*) EXON 5 WITH EGG PRODUCTION AND EGG QUALITY TRAITS OF *DARAG* PHILIPPINE NATIVE CHICKEN

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ABSTRACT

Prolactin (*PRL*), through the mediation of its receptor (*PRLR*), initiates and sustains the broodiness of chickens, leading to reduced egg production. Association of polymorphisms in the *PRL* promoter region and *PRLR* exon 5 with egg production and egg quality traits of *Darag* were then investigated. Hens (n=41) were genotyped using indel and SNP markers. Indel analysis identified three *PRL* genotypes: II with 154 bp, ID with 154 and 130 bp, and DD with 130 bp. The DD genotype and the D allele had the highest frequencies at 0.54 and 0.77, respectively. The ID genotype had heavier egg and yolk weights, and higher egg length ($P<0.05$) than DD genotype, while total egg number and shell weight tend ($P<0.10$) to be different between genotypes. In most indigenous breeds of chickens, the D allele is more predominant than the I allele. Further, the ID genotype of *PRL* is more favorable than DD genotype in certain egg quality traits. However, for *PRLR*, all hens were monomorphic (AA) at the genotyped loci in exon 5. In conclusion, the association of ID genotype of *PRL* with egg quality was established, and thus, *PRL* genotypes may be used as a basis for selection.

Keywords: *PRL*, *PRLR*, *Darag*, egg number, egg quality

INTRODUCTION

The domestication of native chickens has long been a cornerstone of rural Philippine agriculture. Among these, the *Darag* native chicken of Panay Island, Western Visayas, stands out for its unique qualities. Renowned for its distinctive taste and lean meat, *Darag* chicken is rich in protein, potassium, and unsaturated fatty acids while being low in fat. It also has higher levels of aspartic acid, contributing to its superior flavor (Cocjin *et al.*, 2001). Despite these advantages, *Darag* chickens produce only about 110 eggs per year, significantly less than the 250-300 eggs per year from commercial hybrids (FAO, 2003). This disparity highlights the need to improve the productivity of this valuable native genetic resource. One approach is to explore polymorphic sites in candidate genes for possible association with the trait of interest. Upon identifying the favorable genotype, these candidate genes

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become a basis for effective selection strategies in marker-assisted selection (Kulibaba & Podstreshnyi, 2012).

Prolactin (PRL), a hormone released by the anterior pituitary, is considered essential for initiating and sustaining broodiness in birds (Alipanah *et al.*, 2011). PRL impedes the release of hormones responsible for follicular development and stimulating egg release, resulting in reduced egg production (Reddy *et al.*, 2006). Increasing levels of PRL in native birds were also observed to reduce the duration of egg-laying sequences. The importance of *PRL* in avian reproduction makes it a suitable candidate gene for improving laying performance of chickens. Polymorphic sites in different parts of the *PRL* gene were identified and tested for association with egg production.

The promoter region of *PRL* serves as an excellent model for investigating the gene activity and its effect on hormone regulation in chicken. An association between an insertion-deletion (indel) in the promoter region of *PRL* in chicken and egg production was observed (Cui *et al.*, 2006). Their study revealed a large difference in the allelic frequency between White Leghorn, a commercial layer breed, compared to the native breeds in the population. In the 25-bp indel analysis, three genotypes, II, ID, and DD, were detected. Interestingly, in White Leghorn, only genotype II was present, while native chicken breeds had ID and DD genotypes only. Their study revealed that the genotype ID had a significant association with egg production from 17 to 72 weeks of age. Others have shown that *PRL* was associated with egg quality traits including egg weight, Haugh unit, albumen weight, yolk percentage, and albumen percentage in naked neck chickens at 40 weeks of age (Bhattacharya *et al.*, 2010) and eggshell thickness and weight with *PRL*:129G>A intronic SNP in Iraqi indigenous chickens (Ali *et al.*, 2024).

PRL primarily exerts its biological effects via prolactin receptor (PRLR), a member of the type I cytokine receptor family, situated on chromosome Zp23-22. PRLR occupies a central position in the PRL signal transduction cascade, making it critical for the onset of broodiness as well (Bole-Feysot *et al.*, 1998). The receptor levels in target tissues are modulated in response to changes in circulating PRL concentrations. Polymorphisms in *PRLR* exon 5 were seen to be associated with egg number of indigenous chickens of Mazandaran province, Iran, where the AA genotype had five more eggs than BB genotype based on laying time (Rashidi *et al.*, 2012).

It is important to validate the reported associations between various DNA variants and egg production and egg quality traits found in other chicken breeds like *Darag*. These DNA markers can be used to select for improved laying performance. Thus, the objectives of the study include identifying polymorphisms in the *PRL* promoter region and *PRLR* exon 5 and associating these with total eggs produced at 40 and 52 weeks of age (EN40, and EN52) and egg quality traits (length, weight of egg and its components, shell thickness) of *Darag* native chicken.

MATERIALS AND METHODS

All animal-related procedures were examined and authorized by the Institutional Animal Care and Use Committee at the University of the Philippines Los Baños (IACUC protocol number: CAFS-2019-003). *Darag* native chickens were raised and managed using standardized procedures at the University of the Philippines Los Baños, Brgy. Putho

Tuntungin Los Baños, Laguna Native Animal Farm. Individual egg production of 41 *Darag* hens was monitored from the start of lay until 40 weeks (EN40) and 52 weeks of age (EN52), while egg weight, length, width, yolk weight, shell weight, and shell thickness were determined at 40 weeks of age. Blood samples were used to extract genomic DNA with a GF1 Blood Extraction Kit (Vivantis Technologies, Malaysia).

Primers used for *PRL* F: TTTAATATTGGTGGGTGAAGAGACA and R: ATGCCACTGATCCTCGAAACTC were designed to amplify a 130-154 PCR product with or without the 24-bp indel (Ahmadi *et al.*, 2019). On the other hand, the *PRLR* primer pair of F: TTGTCTGCTTTGATTCATTTC and R: TGCATTTCATTCTTCCCTTTTT was used to amplify a 250 bp product (Rashidi *et al.*, 2012). Final concentration of the components of the PCR reaction mix was optimized in a 20 µL total volume per sample. All PCR reactions of *PRL* consisted of 1X of PCR buffer, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 0.5 µM each of reverse and forward primers, 0.5 U of *Taq* polymerase, 20 ng of DNA template, and nuclease-free water. For *PRLR* reactions, 1.7 mM of MgCl₂, 0.7 µM each of forward and reverse primer, 0.4 U of *Taq* polymerase, and nuclease-free water and the same concentrations of PCR buffer, dNTPs, and DNA template were used. For *PRL*, PCR amplification was conducted with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes (Ahmadi *et al.*, 2019). As for the *PRLR* gene, a similar approach was adopted, but only 30 cycles with annealing at 58°C for 30 seconds were used (Ahmadi *et al.*, 2019; Rashidi *et al.*, 2012). PCR products underwent electrophoresis on a 1.5% agarose gel using Mupid® One Electrophoresis System Complete Apparatus (Eurogentec, Belgium). It was visualized using ENDURO™ GDS Gel Documentation System (Labnet International, New Jersey, USA). Polymorphism in *PRLR* was detected via restriction enzyme (RE) digestion (20 µL per sample) using 1U *Bam*HI at 37°C for 1 hour, followed by inactivation at 65°C for 20 minutes. Digested products (10 µL) were electrophoresed on a 3% agarose gel. *PRL* was genotyped through indel analysis.

Genotypic and allelic frequencies were calculated. The Hardy-Weinberg equilibrium for each gene was checked using a chi-square test. Prior to association, tests for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests were performed, respectively. The association between polymorphisms, egg production, and egg quality was analyzed using the GLM procedure in SAS® Studio (SAS Institute, Inc., Cary, NC). The model included genotype (G_i) and batch (H_j) as fixed effects; $Y_{ijk} = \mu + G_i + H_j + e_{ijkl}$. The batch effect was retained in the model if found significant. Results were considered significant if $P < 0.05$ and were reported as least squares mean $\pm$ standard error. For *PRL*, since II genotype had only two samples and a high standard error of the mean, only DD and ID genotypes were compared.

RESULTS AND DISCUSSION

Figure 1 shows three *PRL* genotypes observed: II (*Insertion-Insertion*) with 154 bp; ID (*Insertion-Deletion*) with 154 bp and 130 bp; DD (*Deletion-Deletion*) with 130 bp, similar to the expected product size presented by Ahmadi *et al.* (2019) and Cui *et al.* (2006). This study showed that genotype DD (0.59) exhibited the highest frequency, while genotype

II (0.05) was rarely observed. The frequency of allele D (*Deletion*, 0.77) was higher than allele I (*Insertion*, 0.23). Results of the chi-square test showed that the study population conforms to Hardy-Weinberg equilibrium ($P=0.96$) (Table 1).

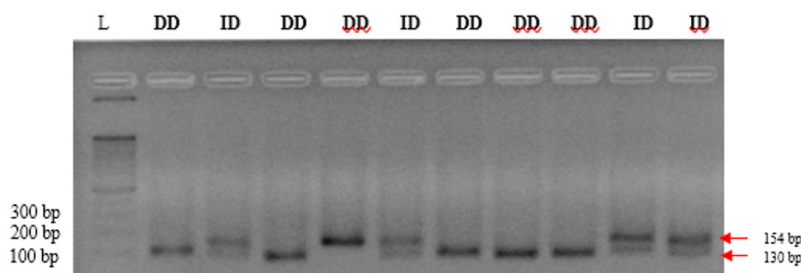


Figure 1. Visualization of *PRL* using indel genotyping in 3% agarose gel. Three genotypes were identified namely II, ID, DD. L represents the 100 bp ladder.

Table 1. Genotypic and Allelic frequency of *PRL* of *Darag* chickens.

N (41)	Genotypic Frequencies			Allele Frequency		χ^2	p-value
	f(DD)	f(II)	f(ID)	f(D)	f(I)		
Observed values	0.59 (24)	0.05 (2)	0.37 (15)	0.77	0.23	0.071	0.96
Expected Values	0.59 (24)	0.05 (2)	0.36 (14)				

p-value > 0.05 = not significantly different, N = total number of chickens in the population

Similar to previous reports, the I allele is less frequent in local breeds compared to commercial breeds. In local Papua chickens, the frequencies of I and D were 0.31 and 0.69, respectively (Lumatauw and Mu'in, 2016); while in different Vietnamese indigenous chicken breeds, the I allele frequency ranges from 0.12 in Mia chickens (Vinh *et al.*, 2021), followed by 0.13 in Lien Minh chickens (Nguyễn *et al.*, 2018), to 0.19 in Silkie (Tu *et al.*, 2023) and Ri chickens (Vinh *et al.*, 2021). Other native chicken breeds genotyped for PRL predominantly had D allele frequencies between 0.78 – 0.95; while on the contrary, only the I allele was present in White Leghorn (Cui *et al.*, 2006).

For *PRLR*, no mutation was detected, concluding that the population was monomorphic at this locus (Figure 2). All samples consistently exhibited fragment sizes of 195 bp and a faint 55 bp band, corresponding to the AA genotype. Similarly, Vinh *et al.* (2021) reported closely aligned results, with Vietnamese Ri chickens showing genotypic and allelic frequencies of 0.99 for the AA genotype (A allele) and 0.01 for the BB genotype (B allele). No heterozygous genotype was seen in all the hen samples because *PRLR* is sex-linked and can only be seen in the Z chromosome (Rashidi *et al.*, 2012). The results of the present study indicate that for this polymorphic site, the alleles are fixed and thus cannot be analyzed further for association.

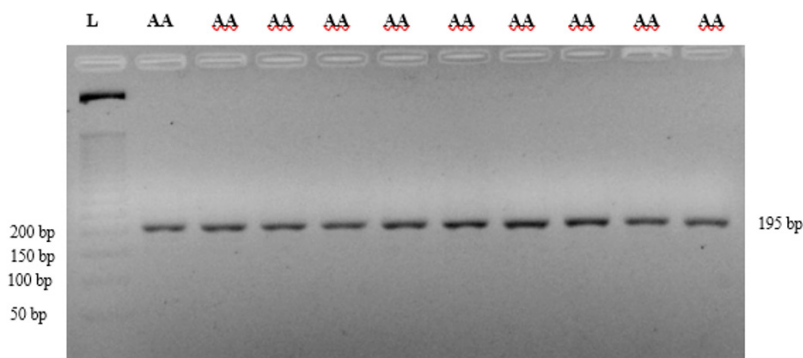


Figure 2. Visualization of *PRLR* using *Bam*HI genotyping in 3% agarose gel. Only one genotype was identified namely AA. L represents the 50 bp ladder.

Interestingly, for *PRL*, birds with ID genotype had 9.6% heavier egg weight, 11.2% heavier yolk weight, and 3.9% higher egg length at 40 weeks of age (on average), than those with DD genotype ($P < 0.05$). There was also a tendency ($P < 0.10$) for EN40 and shell weight to be different between genotypes (Table 2). The role of *PRL* in egg production is consistent with the findings of the study. Elevated PRL levels suppress the secretion of estrogen, which normally provides feedback to the pituitary gland to regulate luteinizing hormone (LH) release. In the absence of sufficient LH, ovulation does not happen (Prastiya *et al.*, 2022). Instead of follicular growth stopping before ovulation, the follicle sequesters more yolk material and continues to grow, resulting in eggs with larger yolks and higher concentrations of proteins, lipids, vitamins, and minerals, though at the cost of reduced laying frequency. Shell weight can also be affected since prolactin prolongs the time between laying (Reddy *et al.*, 2002), leading to longer intersequence pause and shell formation in the oviduct.

Table 2. Effect of *PRL* gene on egg production and egg quality traits of *Darag*.

Traits	<i>PRL</i> Genotype		p-value
	DD (24)	ID (15)	
EN40	75.75 $\pm$ 3.62	65.43 $\pm$ 4.74	0.0919
EN52	78.72 $\pm$ 6.27	67.31 $\pm$ 6.94	0.2021
Egg weight 40, g	38.83 $\pm$ 0.86	42.54 $\pm$ 1.13	0.0160
Egg length, cm	50.51 $\pm$ 0.54	52.47 $\pm$ 0.71	0.0379
Egg width, cm	38.09 $\pm$ 0.38	38.53 $\pm$ 0.47	0.4728
Albumen height, mm	4.01 $\pm$ 0.10	3.89 $\pm$ 0.14	0.5025
Yolk weight, g	13.03 $\pm$ 0.32	14.49 $\pm$ 0.38	0.0177
Shell weight, g	4.20 $\pm$ 0.14	4.58 $\pm$ 0.16	0.0900
Shell thickness, mm	0.29 $\pm$ 0.00	0.29 $\pm$ 0.01	0.7686

Values presented are in LSM $\pm$ SEM

EN 40 is the total number of eggs produced from the start of lay until 40 weeks of age.

EN 52 is the total number of eggs produced from the start of lay until 52 weeks of age.

In conclusion, the present study showed that there was an association between DNA polymorphisms of *PRL* and egg quality. Hence, *PRL* can be used as a potential genetic marker for marker-assisted selection (MAS) programs. The results indicate that the heterozygous genotype ID exhibited superior egg quality compared to the DD genotype. Birds with genotype ID can be selected for the genetic improvement of egg qualities of *Darag* layers. Further, it is recommended to conduct experimental validations such as biochemical analyses, gene expression studies, ligand-receptor binding/affinity assays to further substantiate the association established in the present study.

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