IJAS Iranian Journal of Applied Animal Science

Association between Ovoinhibitor (OIH) Gene Polymorphism and Egg Quality Traits in Golden Kamper Hybrid Chickens (*Gallus gallus domesticus*)

Research Article

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Received on: 10 Jan 2022
Revised on: 12 Apr 2022
Accepted on: 29 Apr 2022
Online Published on: Sep 2022

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Online version is available on: www.ijas.ir

ABSTRACT

The ovoinhibitor (OIH) has been identified in all egg compartments including egg white, egg yolk, and eggshell. The objective of this study was to analyze the association between OIH gene polymorphism and egg quality in the Golden Kamper Hybrid chickens. The chickens used in this study were BC Golden Kamper and F_4 Golden Kamper. Chickens were maintained in the individual cage to observe egg quality. Chicken blood samples were taken for DNA isolation by Chelex 5% method followed by OIH gene amplification and polymorphism detection with sanger sequencing. Significant differences in egg quality were analyzed using one-way ANOVA. The relationship between genotype and haplotype with egg quality was analyzed using Pearson correlation. Average egg weight, egg length, shell weight, and shell thickness of BC Golden Kamper were 47.06 ± 2.91 g, 5.13 ± 0.3 mm, 4.58 ± 0.52 g, 0.30 ± 0.03 mm and F_4 Golden Kamper was 47.42 ± 3.74 g, 5.16 ± 0.2 mm, 5.18 ± 0.33 g and 0.315 ± 00 mm. The mean albumen height, haugh unit, and yolk color of BC Golden Kamper was 5.4 ± 1.14 mm, 85.86 ± 8.71, 8.80 ± 0.45 and F_4 Golden Kamper was 6.13 ± 0.33 mm, 79.42 ± 2.21, 8.00 ± 0. There was a single nucleotide polymorphisms (SNP) G4363T in the OIH gene intron 7. Genotype (GG and TT) and haplotype (H1 and H2) were significantly negatively correlated with albumen weight ($P<0.05$), shell weight ($P<0.05$), and shell thickness ($P<0.05$).

KEY WORDS chicken, egg quality, ovoinhibitor gene, polymorphism.

INTRODUCTION

Increasing the productivity of native chickens has been carried out in several ways, including improving genetic quality, feed, and controlling the disease. One way to improve genetic quality is through the cross-breeding of chickens. In some literature, it has been proven that crossbreeding program is effectively able to improve qualitative and quantitative characteristics such as egg quality and egg production in chickens. So that the cross-breeding program is one of the breeding systems that play an important role in improving chicken performance (Sayed *et al.* 2017). Based on Saragih and Daryono (2012), cross-breeding between local chickens and broilers can improve the quality of local chickens. The quality of poultry eggs is very important to both farmers and consumers. The quality of an egg depends on internal and external quality. The overall quality of eggs, including weight, shell, Haugh units, albumen, and egg yolk content, is an important indicator for consumers and producers. Eggs with good external quality conditions will create a positive impression of internal quality, which will encourage sellers and consumers to buy eggs (Bhattacharya *et al.* 2019). The ovoinhibitor (OIH) is the major proteinase inhibitor in albumen (Kinoshita *et al.* 2004). OIH protein has the function of stabilizing the structural environment of the epididymis and vas deferens to improve the viability of spermatozoa and has the function of degrading protein in egg yolk and is related to the reproductive performance of

poultry (Słowińska *et al*. 2014; Gao *et al.* 2017). This type of inhibitor has several functions including binding to IgE from patients who are allergic to eggs (Martos *et al.* 2013), playing an important role as an anti-bacterial defense of eggs against *Bacillus* spp. acting as a protective embryo, and preventing contamination of unfertilized eggs (Bourin *et al.* 2011).

The OIH gene was also most abundantly expressed from the greatest to the least in the liver, magnum, and uterine tissues as well as in egg yolk and eggshell precursors of chickens. OIH gene expression in the liver is relatively higher during sexual maturation than in the post-adult period (Bourin *et al.* 2011).

Previous researchers have shown that chicken OIHs in eggs have an important function in protecting egg quality. To date, very few studies have been performed on the genetic correlation of the OIH gene with the quality of chicken eggs. Therefore, Huang *et al.* (2019) evaluated the effect of OIH on egg quality to determine the presence of SNPs in the OIH gene and its relationship with egg quality characteristics in chickens. In the study by Huang *et al.* (2019), it was found that the analysis of expression in tissues showed that OIH has an important role in egg production. The results of this study indicate that OIH can act as a potential candidate gene for improving chicken egg quality. Alleles and diplotypes of the OIH gene also function as genetic markers for improving chicken egg quality in the future using marker-assisted selection (Huang *et al*. 2019).

Therefore, in this study, genetic traits were also selected using the OIH gene as a genetic marker to determine the relationship between Single Nucleotide Polymorphisms (SNPs) and egg quality characters of Golden Kamper hybrid chickens. To provide genetic molecular markers that have the potential as a facility in improving egg quality traits in chicken breeding.

MATERIALS AND METHODS

Animals and data collection

This study was performed on three BC Golden Kamper chickens, eight F_4 Golden Kamper chickens, and five layer chickens as a control. The entire type of chicken was maintained in individual cages under standard management conditions and provided *ad libitum* access to water and feed. The experiment was conducted at the Pusat Inovasi Agro Teknologi, Universitas Gadjah Mada. 1 mL blood sample was taken from a wing vein of each 18-week-old hen for Genomic DNA (gDNA) extraction (Singh *et al*. 2018). Egg quality traits were analyzed for internal quality and external quality using an egg quality measuring instrument (EMT 5200, Robotmation Co. Ltd., Tokyo, Japan). Analysis of internal quality characters consisted of albumen height (AH), albumen length (AL), albumen width (AWD), albumen weight (AWG), yolk height (YH), yolk diameter (YD), yolk color (YC), and Haugh unit (HU). Analysis of external quality characters consisted of egg shape, shell weight, shell thickness, egg weight, egg length, egg width, and egg volume. Eggs were collected at 2-4 weeks after the first egg. The egg content analysis was carried out at the Milk and Egg Technology Laboratory, Faculty of Animal Husbandry, Universitas Gadjah Mada.

DNA isolation

Genomic DNA was isolated using the Chelex method 5%. 10 µL of blood samples were inserted into a 1 mL TE 10 mM pH 8 in a 1.5 mL Eppendorf tube. The centrifugation was carried out at 13000 rpm for 3 min. The supernatant from centrifugation results was discarded and then 200 µL Chelex 5%, 18 µL DTT 0.01 M, and 2 µL Proteinase K 10 mg/mL were added. Then the solution was homogenized for 30 seconds using a vortex and incubated at 56 ˚C for 2 hours with vortexed every 15 minutes to ensure it was well mixed. After that, incubation was carried out at 100 for 8 minutes and centrifuged at 14000 rpm for 3 minutes. The supernatant containing DNA isolate (±150 µL) was transferred to a new 1.5 mL Eppendorf tube for storage at -20 ˚C until used as a polymerase chain reaction (PCR) template (Singh *et al.* 2018).

Polymerase chain reaction (PCR)

Target was amplified using a thermocycler (BioRad, US). The reaction composition consisted of 12.5 L of master mix MyTaq HS Red Mix of Bioline, 1.25 L of forward primer, 1.25 L of reverse primer, 5 L of DNA template, and 5 L of ddH_2O so that a total volume of 25 L was obtained. A specific primer was used to amplify the OIH gene is shown in Table 1. PCR reaction consisted of initial denaturation 95 ˚C for 1 min, followed by 35 cycles of denaturation 95 ˚C for 15 sec, annealing 56 ˚C for 15 sec, an extension 72 ˚C for 10 sec, and the final extension of 72 ˚C for 2 min. Then the PCR product was visualized on 2% agarose gel electrophoresis. The 0.8 g agarose was diluted with 40 mL TBE 1X and then dissolved by heating in the microwave for 1 min. The agarose gel was added with 3 µL FloroSafe DNA Stain 1st base. The gel was put into the electroporator tank then TBE was poured until the gel was submerged. Electrophoresis was conducted with a Mupid-exU™ electroporator. Then 4 µL PCR sample and a 4 µL 100 bp ladder were added to each well. DNA migration was done by turning on the electrophorator with a 100 volt voltage for 20 minutes. Furthermore, the DNA bands were visualized using UV Geldoc.

Sequencing

The PCR product was sequenced using the Sanger method at the 1st base company, Selangor, Malaysia.

Table 1 GenBank accession number, location of polymorphic site, annealing temperature, lenght (bp), and pimer sequences OIH gene

Gene	GenBank accession number	Site	Primer	Length (bp)
OIH/ *SPINK5*	NC_006100.5	Intron 7 (A4363G)	F: 5'-AGGCTGAAGCCCTACTTTGT-3' R:5'-GGGTCCTCTGCTCTCTGAAA-3'	132
		Intron 14 (C8937G)	F:5'-GTGAGTGTGAAAGGATGGGC-3' R:5'-ACTGTTCCTGTTCACTGCCA-3'	178

Table 2 External egg quality traits for Golden Kamper hybrid chickens and Layer chicken

Parameter	Breed types and their quality traits		
	BC Golden Kamper	F_4 Golden Kamper	Layer
Egg weight (g)	47.06±2.91[a]	47.42±3.74[a]	51.95±5.37[b]
Egg length (mm)	5.13±0.31[a]	5.16±0.2[a]	5.33±0.25[b]
Egg width (mm)	5.3±7.02[a]	4.63±4.8[a]	4.17±0.20[a]
Shell weight (g)	4.58±0.52[a]	5.18±0.33[a]	6.42±0.61[b]
Shell thickness (mm)	0.30±0.03[a]	0.315±00[a]	0.343±0.01[b]

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Data analysis

Internal and external quality traits of eggs were analyzed with a significance level of P<0.05 using SPSS 20.0 one-way ANOVA software to determine the significant difference between groups of chickens (SPSS, 2011). Chromatograms from the sequencing results were analyzed using GeneStudio software. Consensus sequences were aligned and the location of the SNPs was tracked using the Clustal Omega Program. Association of genotype data with egg quality characters was tested using Pearson correlation analysis SPSS 20.0 program.

RESULTS AND DISCUSSION

The external characteristics of egg quality such as egg weight, egg width, egg length, shell weight, and shell thickness are shown in Table 2. Based on Table 2, it was known that the average egg weight of BC Golden Kamper chicken was 47.06 ± 2.91 g, F_4 Golden Kamper chicken was 47.42 ± 3.74 g, and layer chicken was 51.95 ± 5.37 g. Based on statistical analysis, the average weight of BC Golden Kamper and F_4 Golden Kamper chickens eggs with the average egg weight of Layer chicken had a significant difference.

The average length of BC Golden Kamper eggs was 5.13 ± 0.3 mm, F_4 Golden Kamper was 5.16 ± 0.2 mm, and layer was 5.33 ± 0.25 mm. F_4 Golden Kamper chickens (5.16±0.2 mm) had the highest length compared to the other two types of chickens, BC Golden Kamper chickens (5.13±0.31 mm) and layer chickens (5.33±0.25 mm). The average egg length also showed a significant difference between BC Golden Kamper and F_4 Golden Kamper chickens eggs with the average egg length of layer chicken.

BC Golden Kamper chickens had an average egg width of 5.3 ± 7.02 mm, F_4 Golden Kamper chickens had an average egg width of 4.63 ± 4.8 mm, and layer chickens had an average egg width of 4.17 ± 0.20 mm. BC Golden Kamper chickens had the largest egg width (5.3±7.02 mm), while layer chickens had the smallest mean egg width (4.17±0.20 mm). However, the mean egg width of each type of chicken showed no significant difference in statistical test results.

The significant differences in egg weight and length can be interpreted that the variety of chicken affecting the weight and length of eggs. Meanwhile, the width of the eggs was not affected by the variety of chicken, because there was no significant difference in the width of the eggs between the different chicken varieties. Different egg weights were caused by differences in the metabolic ability of each type of chicken. This is in accordance with previous studies that the weight of chicken eggs is influenced by the age of the chickens (Padhi *et al.* 2013), temperature, environment, strain or breed (Hanusová *et al.* 2015), nutritional content in feed, and chicken body weight (Isidahomen *et al.* 2013).

The average shell weight of BC Golden Kamper, F_4 Golden Kamper chickens, and Layer chicken, was 4.58 ± 0.52 g, 5.18 ± 0.33 g, 6.42 ± 0.61 g. Based on the results of statistical analysis, the average weight of the shells of the three types of chickens was not significantly different.

Furthermore, the average thickness of the shells of the three types of chicken from the highest to the lowest was layer, F_4 Golden Kamper, and BC Golden Kamper. Layer chickens had the highest average shell thickness 0.343 ± 0.01 mm compared to BC Golden Kamper chickens 0.30 ± 0.03 mm and F_4 Golden Kamper 0.315 ± 00 mm. The statistical test results show that there were significant differences in the average shells thickness of the three lines. Aberra *et al.* (2012) reported that the variation in shell thickness could be attributed to the quality, quantity, and nutritional composition of the feed available to the poultry, which of course would vary by location. The trait is heritable and also affected by the genotype of the bird, and calcium and phosphorus metabolism which varies with the age of the bird as well as the bioavailability of the two nutrients mentioned. This shell trait is of commercial importance

because eggs with thick, solid shells are usually the easiest to sell (Aberra *et al.* 2012). The thickness of the eggshell quality is affected by many different factors, including the type of chicken, the age of the chicken, nutrition including a protein source, molting status, water quality, heat stress, disease, housing farms, production systems, and environmental pollutants. Age affects eggshell formation. As chickens get older, they produce thinner eggshells because the reproductive function of these birds declines with age. Characteristic of the most important qualities of an eggshell is strength and thickness (Hanusová *et al.* 2015).

The internal egg quality traits (Table 3) yolk height, albumen height, yolk width, yolk color, Haugh unit were calculated. Significant differences in several internal egg quality characters ($P<0.05$) were analyzed in two types of Golden Kamper chickens and the layer population. The results of the analysis showed that there were significant differences in yolk height, albumen height, and yolk color. Meanwhile, the yolk diameter and Haugh Unit did not show any difference.

The average yolk height of F_4 Golden Kamper chicken (16.34±0.58 mm) was higher than BC Golden Kamper chicken (15.15±0.80 mm). Yolk height of F_4 Golden Kamper chickens had a significant difference from the yolk height of BC Golden Kamper and Layer chickens.

In addition, F_4 Golden Kamper chickens had higher albumen height (6.13±0.33 mm) compared to the BC Golden Kamper, but still lower than layer chickens. Albumen height measurement aims to determine the value of its viscosity. Albumin that has a low viscosity level is considered to be of poor quality (Eddin *et al.* 2019). The observed variation in albumin height is correlated with the freshness of the eggs.

In yolk diameter, there was no difference between the two types of Golden Kamper chickens. BC Golden Kamper chickens had the highest average yolk diameter of 3.93 ± 0.25 cm, the average value was higher than F_4 Golden Kamper (3.88±0.17 cm) and layer (3.74±1.87 cm).

In the egg yolk color, there was a significant difference between BC Golden Kamper chickens, F_4 Golden Kamper chickens, and layer chickens. BC Golden Kamper chickens (8.80±0.45) compared to F_4 Golden Kamper chickens (8.00±0.00) and layer chickens (7.00±0.00).

One of the indicators that can be used to determine the quality of an egg is the color of the yolk. The color of egg yolk varies from light yellow to dark orange with a score of 1-15. The darker the yellow color, the better the egg quality. Variations in egg yolk color are determined by the presence or absence of xanthophyll, which are precursors of vitamin A.

If the food contains a lot of the yellow plant pigment called xanthophyll, it will be stored in the egg yolk, making the yolk's color more concentrated. Xanthophyll is a carotenoid pigment found in chicken feed. The pigment is transferred into the bloodstream and egg yolk. As a result, more pigment is deposited in the egg yolk. Egg yolk color is influenced more by environmental factors than by genetics. The influence of genes is not obvious to score yolk color (Kostaman and Sopiyana, 2016).

One of the things that can be used as a reference for the condition of egg freshness is the Haugh unit. In this study, there was no difference in the Haugh unit between the two strains of Golden Kamper chicken. F_4 Golden Kamper chickens had the highest Haugh unit 79.42 ± 2.21 compared to BC Golden Kamper chickens (75.96 ± 11.53). However, it was still lower than the control chicken, which was 85.86 ± 8.71. Aberra *et al.* (2012) reported that the value of the HU vary due to factors such as management, the quality and quantity of the feed, and the production environment in which the animals are raised.

The results of the electrophoresis visualization above used a 100 bp marker with a band size of 100-3000 bp. Based on Figure 1, it was known that the results of the amplification of the OIH intron 7 produced a fragment of 132 bp. While in Figure 2, it was known that the amplification of the OIH intron 14 resulted in a fragment of 178 bp.

In this study, SNP analysis was performed on the OIH gene. In this study, SNPs were observed at two target locations, namely in the OIH gene intron 7 and intron 14. The results obtained (Table 4) were that only one SNP was found located at intron 7 (G4363T), while in intron 14 (C8937C) no SNP was found.

This is different from the research of Huang *et al.* (2019), from this study, 2 SNPs are identified in the Xinhua E-strain population, the SNPs are located in intron 7 (A4363G), and intron 14 (C8937G).

Table 3 Internal egg quality for Golden Kamper Chicken anda layer chicken

Parameter	Breeds types and their quality traits		
	BC_Golden Kamper	F_4 Golden Kamper	Layer
Yolk height (mm)	15.15±0.80[a]	16.34±0.58[b]	15.18±0.25[a]
Albumen height (mm)	5.4±1.14[a]	6.13±0.33[a]	7.3±1.43[b]
Yolk diameter (cm)	3.93±0.25[a]	3.88±0.17[a]	3.74±1.87[a]
Yolk colour	8.80±0.45[a]	8.00±0.00[b]	7.00±0.00[c]
Haugh unit	75.96±11.53[a]	79.42±2.21[a]	85.86±8.71[a]

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).

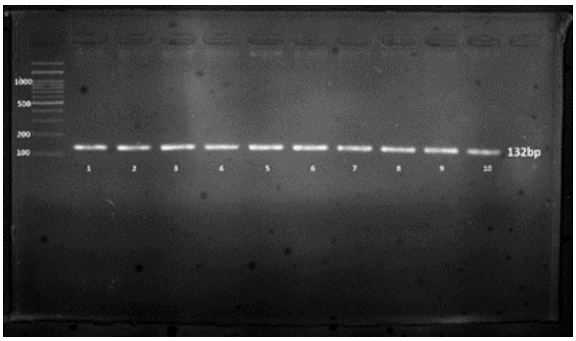

Figure 1 OIH gene visualization results exon 7 (132 bp)
1-7: Golden Kamper Hybrid Chicken; 8-9: layer chicken and 10: Pelung Chicken

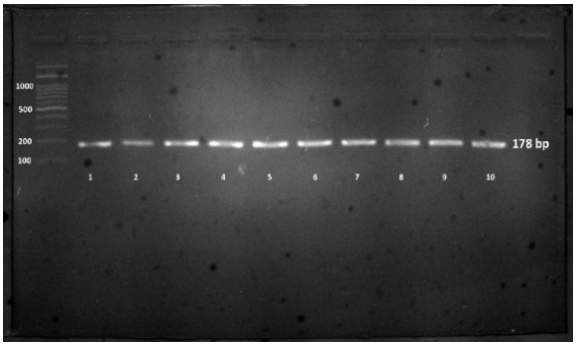

Figure 2 OIH gene visualization results exon 14 (178 bp)
1-7: Golden Kamper Hybrid Chicken; 8-9: layer chicken, 10: Pelung Chicken

Table 4 Single nucleotide polymorphisms (SNP) of ovoinhibitor (OIH) gene

Sample	Position in the OIH Gene	
	Intron 7 (G4363T)	Intron 14 (C8937C)
BC Golden Kamper	T	C
BC Golden Kamper	T	C
BC Golden Kamper	T	C
F_4 Golden Kamper	G	C
F_4 Golden Kamper	G	C
F_4 Golden Kamper	G	C
F_4 Golden Kamper	G	C
F_4 Golden Kamper	G	C

The relationship between genotype and egg quality characters was estimated and the results were summarized in Table 5. The G43636T genotype was significantly correlated with albumen weight (P<0.05), shell weight (P<0.05), and shell thickness (P<0.05). Chickens with the GG genotype had higher albumen weight (AWG), sheel weight (SWG), and ST compared to the TT genotype (P<0.05). The results of statistical test analysis showed a significant negative correlation between genotype and egg quality. This can be interpreted that the mutation causing variations in the sequences of nucleotide bases, causing the egg quality value to decrease. The group of chickens with the GG genotype had an albumen weight value of 31.29 g, while the chickens with the TT genotype had an albumen weight value of 26.60 g.

The shell weight of the chicken group with the GG genotype was 5.30 g, while the shell weight of the chicken group with the TT genotype was 4.58 g. The chicken group with the GG genotype had a shell thickness of 0.33 mm, while the chicken group with the TT genotype had a shell thickness of 0.30 mm.

The location of C8973C did not find any SNP, so the relationship between genotype characters and egg quality could not be analyzed.

In contrast to Huang *et al.* (2019), in this study, the genotypes of A4363G and C8937G are significantly affected HU (P<0.05) and AH (P<0.05) while HU and AH in birds heterozygous for AG and CT are significantly higher than in birds homozygous for AA and TT, respectively (P<0.05).

The SNPs identified in the introns (A363G and C8937G) of OIH are significantly associated with HU and AH, an important clue to egg freshness and egg white quality (Huang *et al.* 2019). Previous studies have shown that transcription factors can bind a few specific sites located in the promoter and intron regions of genes to influence gene expression (Myers *et al.* 2007; Nagpal *et al.* 2014; Yang *et al.* 2015) and protein translation (Karve *et al.* 2011; Petibon *et al.* 2016). May alter the expression of the OIH gene, although further investigation is required in this regard. It has been reported that certain synonymous mutations can affect the splicing, stability, structure, or folding of messenger RNA, which significantly affects protein function (Li *et al.* 2015).

Based on Table 6, the Golden Kamper chicken had one SNP on the OIH intron 7 gene (G4363T). Furthermore, the SNPs were grouped to form 2 haplotypes. The results of grouping individuals based on haplotype can be seen in Table 4. Golden Kamper chickens consist of 2 haplotypes. One haplotype had a population percentage of 57.14%, while the other haplotype had a population percentage of 42.86%.

Based on Table 7, the statistical test analysis showed a significant negative correlation between haplotype and egg quality. Individuals with haplotype H1 had higher albumen weight, shell weight, and shell thickness values compared to individuals with haplotype H2. In this study, superior haplotypes could be selected and maintained in specific lines to improve egg quality in the chicken breeding program. In the previous study, a total of four haplotypes are obtained, with H1, H2, and H4 being the major haplotypes. Association analysis of haplotype combinations reveals a significant association with HU and AH. Nine diplotypes are obtained based on four haplotypes.

Table 5 Association analysis between genotypes and egg quality traits

Site	Genotype	Egg quality traits									
		AH (mm)	HU	YD (cm)	YWG (g)	YH (mm)	AL (cm)	AWD (cm)	AWG (g)	SWG (g)	ST (mm)
G4363T	GG	6.12	79.79	3.84	14.99	16.04	8.58	6.43	**31.29**	**5.30**	**0.33**
	TT	5.44	75.96	3.92	14.36	15.31	7.99	6.45	**26.60**	**4.58**	**0.30**
	GT	-	-	-	-	-	-	-	-	-	-

AH: albumen height; HU: Haugh unit; YD: yolk diameter; YWG: yolk weight; YH: yolk height; AL: albumen length; AWD: albumen width; AWG: albumen weight and SWG: sheel weight.
The bolded values indicate a significant relationship in the statistical test.

Table 6 The haplotypes based on OIH gene single nucleotide polymorphism

Haplotype	G4363T	C8937C	Population percentage (%)
H1	G	C	57.14
H2	T	C	42.86

Table 7 Association analysis between haplotype and egg quality traits

Haplotype	AH (mm)	HU	YD (cm)	YWG (g)	YH (mm)	AL (cm)	AWD (cm)	AWG (g)	SWG (g)	ST (mm)
H1	6.12	79.79	3.84	14.99	16.04	8.58	6.43	**31.29**	**5.30**	**0.33**
H2	5.44	75.96	3.92	14.36	15.31	7.99	6.45	**26.60**	**4.58**	**0.30**

AH: albumen height; HU: Haugh unit; YD: yolk diameter; YWG: yolk weight; YH: yolk height; AL: albumen length; AWD: albumen width; AWG: albumen weight and SWG: sheel weight.
The bolded values indicate a significant relationship in the statistical test.

Diplotypes are found to be significantly associated with HU ($P<0.05$) and AH ($P<0.05$). In the previous investigation into layer population, the frequency of H1H4 individuals is 24.89%, so this advantageous diplotype can be selected and kept in a specialized line to improve egg quality in layer breeding programs (Huang *et al.* 2019).

CONCLUSION

In summary, this is the research to report the influence of the OIH gene on egg quality traits in chicken. There was SNP (G363T) in the OIH gene intron 7. The OIH gene intron 14 did not find any SNP at the C8937C. Genotype (GG and TT) and haplotype (H1 and H2) were significantly negatively associated with albumen weight, shell weight, and shell thickness egg quality.

ACKNOWLEDGEMENT

This study was funded by the Ministry of Education, Culture, Research, and Technology Republic of Indonesia (Kementerian Pendidikan Kebudayaan, Riset dan Teknologi) through the Applied Research Grant Program (Penelitian Terapan Unggulan Perguruan Tinggi) PTUPT 2021: 7260/UN1/DITLIT/DIT-LIT/PT/2021. The authors would also like to express their gratitude to the Gama Ayam research team and Pusat Inovasi Agroteknologi (PIAT) UGM for their assistance during this research.

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