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# Short Communication: Polymorphism of collagen type X (COLX) gene and their association with egg production traits and egg weight in Alabio and Mojosari ducks

**DEWI SARI KUMALAWATI[1,2], DYAH MAHARANI[1,♥], APRILIANA P. Z. N. L. SARI[1], GANJAR H. PRATOMO[1], HERU SASONGKO[1], AKHMAD FATHONI[1], TRIANA SUSANTI[2]**

[1]Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada. Jl. Fauna No.03, Karang Gayam, Caturtunggal, Depok, Sleman 55281, Daerah Istimewa Yogyakarta, Indonesia. Tel.: +62-274-513363, Fax.: +62-274-521578, ♥email: d.maharani@ugm.ac.id

[2]Indonesian Livestock Research Center. Jl. Banjarwaru, Ciawi, Bogor 16720, West Java, Indonesia

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**Abstract.** *Kumalawati DS, Maharani D, Sari APZNL, Pratomo GH, Sasongko H, Fathoni A, Susanti T. 2023. Short Communication: Polymorphism of collagen type X (COLX) gene and their association with egg production traits and egg weight in Alabio and Mojosari ducks. Biodiversitas 24: 1518-1523.* Type X collagen (COLX) is non-fibrillar collagen expressed in the white isthmus of the avian oviduct and plays a role in egg formation. This study aimed to identify COLX gene polymorphism and its association with egg production and egg weight in Alabio (*Anas platyrhynchos Borneo*) and Mojosari ducks (*Anas platyrhynchos Javanicus*). Blood samples of 100 female ducks were collected, and their deoxyribonucleic acid (DNA) was extracted. In addition, a 220 bp fragment of COLX gene was amplified using the polymerase chain reaction (PCR) method and sequenced for single nucleotide polymorphism (SNP) identification and genotyping. Data analysis was performed to calculate genotype and allele frequencies, and an independent sample t-test was used to examine the association between genotype and egg production and egg weight of each individual. Three genotypes (CC, TT, and CT) of SNP g.74C/T were detected in both breeds. Allele C (0.77) was dominant in Alabio population; otherwise, allele T (0.81) dominated Mojosari population. The frequency of CC (0.59) was highest in Alabio ducks, followed by CT (0.37) and TT (0.04), while TT (0.65) was highest in Mojosari ducks, followed by CT (0.33) and CC (0.02). Meanwhile, association analysis showed that ducks with CC genotype had significantly higher egg production at 28 and 34 weeks in Alabio than those with CT genotype ($p<0.05$). These results indicate that COLX is an effective genetic marker for a breeding program to increase egg production in Alabio ducks.

**Keywords:** Collagen type X, egg production, egg weight, local duck, single nucleotide polymorphism

## INTRODUCTION

Alabio (*Anas platyrhynchos Borneo*) and Mojosari ducks (*Anas platyrhynchos Javanicus*) are two Indonesian local ducks that are widely developed in South Kalimantan and East Java (Turnip et al. 2018; Rohaeni et al. 2021). As genetic resources that are known to have high productivity, both reached the average age of first laying at 24 weeks and could produce 55-67 eggs in the first three months of production (Prasetyo and Susanti 2000; Damayanti et al. 2019). Furthermore, crossbreeding of selected male Mojosari ducks (Mojomaster-1 Agrinak) with selected female Alabio ducks (Alabimaster-1 Agrinak) had been proven to produce hybrid ducks called Master ducks (MA), which could produce more eggs than their parents. The average age at the first laying of Master was 22 weeks and produced 74 eggs in the first three months of production (Prasetyo and Susanti 2000). As ducks intended to become superior commercial breeds for egg production, Master had high feed conversion (3.55) (Ketaren and Prasetyo 2002) and was still considered costly for some breeders. To obtain local ducks with superior characteristics, genetic improvement is needed through selection, especially in terms of production consistency and efficiency. During the last few years, selection activities based on phenotypic traits to increase livestock productivity have shown rapid development in cattle (*Bos indicus*) (Mustefa et al. 2021); goats (*Capra hircus*) (Sheriff et al. 2021); sheep (*Ovis aries*) (Tortereau et al. 2020); pig (*Sus scrofa domesticus*) (Nguyen et al. 2021); chicken (*Gallus gallus*) (Jambui et al. 2017); duck (*Anas platyrhynchos domesticus*) (Li et al. 2020); turkey (*Meleagris gallopavo*) (Hiscock et al. 2022); and quail (*Coturnix coturnix*) (Narinç et al. 2016). An intensive selection of Alabio and Mojosari was still being carried out to increase egg production and finally reduce the feed conversion ratio of Master ducks. However, this selection method still has some limitations, such as the duration of one-generation interval observations, difficulty in measuring phenotypic traits with low heritability, and high cost (Ibtisham et al. 2017).

Along with the development of molecular technology, a new selection method emerged called genomic selection, where we could select livestock using single nucleotide polymorphism (SNP) markers (Collins et al. 2019). Genomic selection has been widely used in cattle (de las Heras-Saldana et al. 2020; Li et al. 2020); goat (Massender et al. 2022); sheep (Lillehammer et al. 2020); pig (Hidalgo et al. 2020); chicken (Dou et al. 2022); duck (Zhang et al.

2022); turkey (Abdalla et al. 2022); quail (Lee et al. 2021). The basic concept of this method was to use deoxyribonucleic acid (DNA) sequence changes in single nucleotides that differed in each individual, then associated with specific phenotypic traits and utilized for selection purposes. Utilization of this genomic selection would cover the lack of conventional selection to design more efficient breeding programs.

Egg production is highly dependent on the reproductive performance of poultry, while reproduction is a trait with low heritability (Collins et al. 2019). Several candidate genes had been explored in ducks and identified as having polymorphic properties that could be used as gene markers for duck productivity, such as collagen type X (COLX). Collagen type X or COL10A1 gene is located on chromosome 3 and has three exons based on the assessment number nc_051774.1, gene id. 101802636 (in ducks). It is a network-forming collagen and has a short-chain structure (Chang et al. 2012). COLX was mainly found in hypertrophic chondrocytes in cartilage and some calcified matrix (Gudmann and Karsdal 2016; Taylor et al. 2019), so it was thought to be involved in the mineralization process (Chang et al. 2012). This protein was also expressed in tubular gland cells in the white isthmus of the avian oviduct and related to egg formation (Wang et al. 2002: Yin et al. 2020).

The role of COLX in egg formation was evidenced by its presence in shell membrane (Du et al. 2015; Rose-Martel et al. 2015; Ahmed et al. 2017), eggshell matrix (Mann and Mann 2015; Marie et al. 2015a), and uterine fluid in early stages of eggshell mineralization (Marie et al. 2015b). Eggshell membrane is a double-layered fibrous meshwork consisting of collagens (type I, V, X), glycoproteins, keratin and calcium carbonate (Sah and Rath 2016). It plays an important role in preventing microorganisms contamination (Kaweewong et al. 2013) and supporting enzyme immobilization (Jiang et al. 2017; Morrovat et al. 2022). Collagen type X on eggshell membrane facilitates inhibiting mineralization of egg white, yolk, and on shell membrane fibers itself (Arias et al. 1997a). However, several studies have also reported the role of COLX in stimulating the early stages of mineralization (Marie et al. 2015a, b). COLX was highly cross-linked, insoluble, and tended to self-aggregate (Arias et al. 1997a, b), thus supporting the integrity of eggshell membrane structure and allowing it to play a role in eggshell formation and structure. Furthermore, the presence of this collagen in the eggshell membrane indicated its involvement in vascularization and transfer of calcium from eggshell to chicken embryo.

Indication of the contribution of COLX gene to the egg weight of Tsaiya ducks was reported by Chang et al. (2012), as one SNP and three genotypes (TT, TC, CC) were detected in the coding region (T74C:Val24Ala), with the first two genotypes contributing to the trait. Another study reported similar results, where T allele reached 84.25% in Central Java local ducks (Susanti and Yuniastuti 2020). However, no study had been conducted on this gene in Alabio or Mojosari ducks. Therefore, it was necessary as a preliminary study to identify polymorphisms in COLX gene and its association with production traits and egg weight of Alabio and Mojosari ducks.

## MATERIALS AND METHODS

### Animal handling and blood collection

One hundred female ducks aged 46 weeks consisting of 49 Alabio ducks and 51 Mojosari ducks from the Indonesian Livestock Research Center, Ciawi, were used in the study. Each duck was kept in an individual cage under the same environmental and maintenance condition. The ducks fed with a limited diet (160 g/day) containing 16-18% protein and provided with ad libitum drinking water during laying. As for data were collected when the ducks started laying until they were 40 weeks old. The data included: age at the first egg (AFE), body weight at first egg (BWFE), the weight of first egg (EWFE), egg weight at 28, 34, and 40 weeks (EW28, EW30, EW40), and the number of eggs produced to 28, 34 and 40 weeks (EN28, EN30, EN40). Blood was drawn from each duck through pectoralis vein, and transferred into a vacutainer containing tripotassium ethylenediaminetetraacetic acid (K3EDTA) and then stored at -18◦C.

### DNA extraction and amplification

The DNA was extracted by gSYNCTMDNA Extraction Kit (Geneaid, Taiwan) according to the manufacturer's instructions from 100 individual blood samples. While amplification was conducted using Primus 25 Advanced thermocycler (PEQLAB Biotechnologie, Fareham, UK), with primers based on a reference by Chang et al. (2012). The total reaction volume was 25 µL, consisting of 2 µL genomic DNA, 0.5 µL of each primer (F:5'-CTGGCAGTGCTGTCATCGAT-3' and R:5'-GCGTGACCTCCTAAAGGACATC-3'), 12.5 µL Taq DNA Polymerase (with 10x combination buffer), and 9.5 µl dd$H_2O$. PCR conditions were as follows: pre-denaturation at 94◦C for 5 minutes, 35 cycles of denaturation at 94◦C for 1 minute, annealing at 55◦C for 1 minute, extension at 72◦C for 1.5 minutes, and final extension at 72◦C for 10 minutes.

### Sequencing

The DNA sequencing was conducted by 1st BASE DNA Sequencing Division of PT. Genetics Science, and then analyzed using Bioedit 7.0 software. SNP and genotype were determined based on the sequence alignment results and confirmed by looking at the peaks at the exact location of each individual. SNP was named based on the sequence of the duck COLX gene by Chang et al. (2012).

### Data analysis

Genotype, allele frequencies, heterozygosity, and Hardy-Weinberg equilibrium of each breed were analyzed using PopGene32 software, with Hardy-Weinberg equilibrium indicated by the analysis's chi-square value. All data was performed by SAS software version 9.4. Except for EW40 in Alabio and AFE in Mojosari ducks, all trait data had been verified to have a normal distribution

using the Shapiro-Wilk test. The verified traits were utilized to perform genotype-trait association analysis with an independent sample t-test, while the two unverified traits were tested using the Wilcoxon Mann-Whitney U-test. The values are presented as LSM±SE and were considered significant at $p<0.05$.

## RESULTS AND DISCUSSION

### SNP identification and genotyping

The use of genetic markers using SNPs takes into consideration most of the factors that may affect the breeding program because those SNP in different quantitative traits might affect economic traits in animals. As a result, one SNP g.74C/T was identified in both populations, which consists of three genotypes (CC, TT, and CT) (Table 1). It was indicated by a clear peak in the chromatogram (Figure 1). These results confirmed previous studies that the same SNP with CC, TT, and CT genotypes had been found at the same location in Brown Tsaiya ducks (Chang et al. 2012); and local ducks in Central Java (Susanti and Yuniastuti 2020).

Based on analysis using Expasy.org software, SNP g.74C/T of COLX gene was a missense mutation with a change in the amino acid from valine to aline. Richards and Hawley (2011) reported that several missense mutations altered protein structure, protein folding, and the ability of proteins to interact with other molecules, allowing changes in protein function and ultimately changing the phenotype. However, sometimes these mutations had little or no change in the protein's function when the original amino acid and its replacement had the same size, shape, charge, and polarity (Richards and Hawley 2011). These mutations were called conservative mutations, including mutation in the present study, where valine and aline were amino acids that were non-polar and had a neutral charge (Zhang 2000).

### Genetic diversity

The variation in genes and genotypes within a species is referred to as genetic diversity. It is critical for the survival of a species. Genetic diversity gives individuals different physical characteristics and allows them to adapt better to stress, diseases, and unfavorable environmental conditions. The present study showed that at SNP g.74C/T, C allele (0.77) dominated the Alabio population, whereas T allele (0.81) was dominant in the Mojosari population. Similar allele frequencies with Mojosari were founded in Brown Tsaiya ducks (0.78-0.86) as well as ducks in Central Java (0.84) (Chang et al. 2012; Susanti and Yuniastuti 2020). A locus was considered polymorphic when the dominating allele did not have a frequency above 0.95 (95%) (Hanzawa et al. 2012). Furthermore, the heterozygous genotype frequency of both populations had similar values, while the homozygous genotype showed distinct values. The frequency of CC (0.59) was highest in Alabio ducks, followed by CT (0.37) and TT (0.04), while TT (0.65) was highest in Mojosari ducks, followed by CT (0.33) and CC (0.02). These differences might be due to the genetic distance between the two breeds, which was quite far, according to Gunawan (1998) report based on the analysis of polymorphisms of both blood proteins. This difference supported the high probability of heterosis in the crosses, as indicated by the superior performance of production traits of Master ducks compared to both parents (Prasetyo and Susanti 2000).

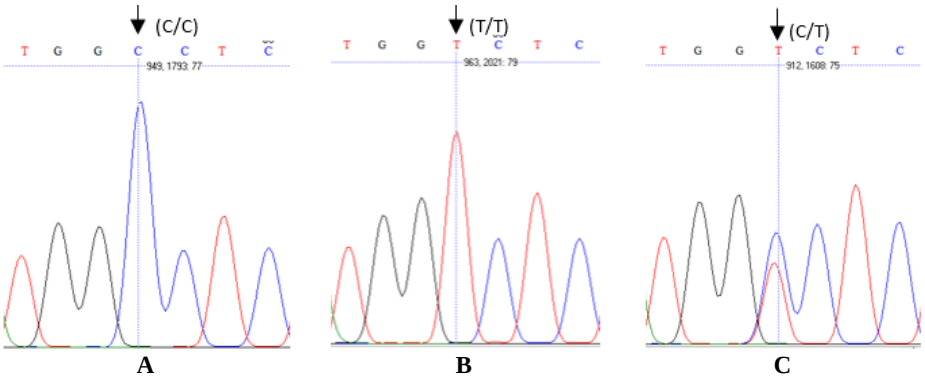


**Figure 1.** Visualization of the SNP g.74C/T of COLX gene in Mojosari duck: (A) CC genotype; (B) TT genotype; and (C) CT genotype

**Table 1.** Allele and genotype frequencies, heterozygosity, and chi-square test values on the SNP g.74C/T COLX gene in Alabio and Mojosari ducks

| Breed | Genotype | n | Genotype frequency | Allele | Allele frequency | Observed heterozygosity | Expected heterozygosity | $\chi 2$ |
|---|---|---|---|---|---|---|---|---|
| Alabio | CC | 29 | 0.59 | C | 0.77 | 0.37 | 0.35 | 0.10 |
| | TT | 2 | 0.04 | T | 0.23 | | | |
| | CT | 18 | 0.37 | | | | | |
| Mojosari | CC | 1 | 0.02 | C | 0.19 | 0.33 | 0.30 | 0.42 |
| | TT | 33 | 0.65 | T | 0.81 | | | |
| | CT | 17 | 0.33 | | | | | |

Note: $\chi 2$, chi-square test value

Alabio and Mojosari populations had similar heterozygosity values, with slightly higher observed heterozygosity (0.37 and 0.33) than expected (0.35 and 0.30). As one of the parameters to measure genetic diversity, heterozygosity values in the present study indicated that the populations of the two breeds had low genetic diversity. It might be due to a specific selection purposely applied at the Indonesian Livestock Research Center to both populations of ducks. In agreement with this, the Chi-Square test resulted that the chi-square of the Alabio and Mojosari ducks was smaller than the chi-square critical values ($\chi 2_{0,05}(1) = 3.84$), indicating the population was in a state of genetic balance (in Hardy-Weinberg equilibrium). It means that allele and genotype frequencies in populations remained constant from generation to generation as long as there was no selection, mutation, migration, genetic drift caused by evolutionary force and assortative mating (non-random mating) (Gupta 2022: Xu 2022).

**Association with production traits and egg weight**

Genetic association studies are used to find candidate genes or genome regions that contribute to a specific trait by testing for a correlation between the trait and genetic variation which are represented by genotype of animals. In this study, an analysis of the association between genotypes with production traits and egg weight of ducks was conducted to discover the COLX role in Alabio and Mojosari ducks. As TT genotypes in Alabio ducks and CC genotypes in Mojosari ducks were only found in one and two samples, association tests were only carried out between two main genotypes and their phenotypic traits. The association of SNP g.74C/T on production traits and egg weight in Alabio and Mojosari ducks showed in Tables 2 and 3. Significant associations were found in egg production at 28 and 34 weeks in Alabio ducks ($p<0.05$) but not in Mojosari and other traits in both breeds. The CC genotype significantly had higher egg production when compared to the CT genotype. This information could be used as a reference to determine selection or/and mating programs for obtaining a population with a higher CC genotype frequency. Chang et al. (2012) found the same results in Tsaiya ducks, where there was no significant association between the SNPs on egg production at 40 weeks of age. Nevertheless, a significant association was also found for egg weight at 40 weeks, which differed from the results of the present study.

The direct effect of the COLX gene on egg production was still unclear and needed further study. However, the correlation between egg production, eggshell quality, and eggshell membrane might temporarily explain the results of this study. It was well known that almost 10% of shell membrane protein fibers consist of cross-linked collagens (types I, V, X) (Arias et al. 1997a; Wang et al. 2002; Zhao and Chi 2009), with type X having the largest percentage compared to other types (Arias et al. 1991). Arias et al. (1997b) reported that the disruption of cross-linked collagen in the shell membrane would result in shell quality and strength decreasing. It was related to the role of COLX in stimulating the initial stages of shell mineralization (Kirsch et al. 2000; Marie et al. 2015a; Marie et al. 2015b), as evidenced by the discovery of peptides from COLX in the eggshell matrix, which was associated with mammillary cones (Marie et al. 2015a; Rose-Martel et al. 2015) and in uterine fluid during early stages of mineralization (Marie et al. 2015b). Mineralization started from the outer membrane surfaces, where the nucleation sites developed into calcified mammillary cones that led to the mineralization of the eggshell inner layer (Du et al. 2015). This initial process affects the texture of the shell and mammillary cones and the attachment to membrane fibers and is substantial for shell strength (Hincke et al. 2012). Meanwhile, a genetic correlation between the number of eggs and eggshell strength in poultry had been reported in ducks (-0.33) at 40 weeks of age (Cheng et al. 1995) and quail (-0.62) (Lotfi et al. 2012).

**Table 2.** Association between genotype and production traits in Alabio and Mojosari ducks

| Breed | Genotype | n | AFE | BWE | EN28 | EN34 | EN40 |
|---|---|---|---|---|---|---|---|
| Alabio | CC | 22-27 | 147.55±09.24 | 1768.23±69.15 | 44.65±09.03[a] | 80.17±10.59[a] | 113.91±14.53 |
| | CT | 15-18 | 152.89±11.75 | 1778.80±36.88 | 37.41±08.79[b] | 72.76±10.74[b] | 109.68±13.74 |
| Mojosari | TT | 22-25 | 168.92±17.72 | 1536.88±113.14 | 23.68±14.27 | 59.80±16.89 | 96.44±18.36 |
| | CT | 13-14 | 167.57±19.41 | 1568.50±143.85 | 22.69±18.37 | 55.85±25.43 | 88.38±32.52 |

Note: AFE: age at the first egg; BWFE: body weight at first egg; EN28: number of eggs produced to 28 weeks; EN34: number of eggs produced to 34 weeks; EN40: number of eggs produced to 40 weeks; [a,b] different uppercase letters represent a significant difference at the $p<0.05$ level for ducks with different genotypes within a trait

**Table 3.** Association between genotype and egg weight in Alabio and Mojosari ducks

| Breed | Genotype | n | EWFE | EW28 | EW34 | EW40 |
|---|---|---|---|---|---|---|
| Alabio | CC | 22-27 | 53.70±05.87 | 53.93±06.44 | 59.10±06.00 | 60.28±03.99 |
| | CT | 15-16 | 53.56±05.60 | 52.46±03.62 | 57.74±03.95 | 60.91±04.98 |
| Mojosari | TT | 19-25 | 51.64±03.64 | 61.47±05.20 | 63.03±05.03 | 67.52±04.24 |
| | CT | 9-14 | 54.38±06.41 | 63.35±04.12 | 63.18±05.93 | 68.83±06.82 |

Note: EWFE: weight of first egg; EN28: egg weight at 28 weeks; EN34: egg weight at 34 weeks; EN40: egg weight at 40 weeks

In conclusion, one SNP g.74C/T of COLX gene had been identified in both Alabio and Mojosari duck breeds and had been shown to affect egg production in Alabio ducks. Therefore, it indicated that COLX gene could be used as a DNA marker in a breeding program to increase egg production in Alabio ducks without affecting their egg weight.

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