Hatchability genotype of Central Javanese local ducks based on ovomucoid gene

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Abstract. Ovomucoid genes are related to hatchability of duck eggs. The study would analyze the genotype of local duck hatchability of Central Java based on ovomucoid genes by the PCR-RFLP. This research is an exploratory study using 35 samples of local Central Java duck. Central Java local duck hatching genotypes were detected by ovomucoid genes fragment cutting as a result of PCR amplification using Apol restriction enzymes. DNA isolated from duck feathers using the gSYNC™ DNA Extraction Kit. Amplification ovomucoid gene was done by PCR using TovF2 and TovR2 primers. The results showed that entire samples were genotyped +/+ (high hatchability), because they were not cut off by the Apol restriction enzyme. The results of nucleotide sequences showed that Central Java local ducks had no cutting site for the Apol enzyme (5'-AAATTC-3'), because there were mutations in the nucleotides to A577G and A579C and no nucleotide deletions to 576-578. It was concluded that based on ovomucoid genes, all Central Java local duck samples were genotypes with high hatchability (genotype +/+). This previous research, strengthens previous argument that local Central Java ducks need to be maintained or preserved as laying ducks with high hatchability.

1. Introduction
Ducks are a poultry species that has high commercial value because of its ability to survive in various climatic conditions and the availability of nutrients [1]. It was also known that ducks are an important and essential genetic reservoir to face the challenges of disease resistance [2]. Ducks belong to a group of waterfowl that requires water habitat for their lives. The characteristic of waterfowl is its genetic superiority. It is shown by its better endurance compared to land birds. Data on waterfowl deaths due to infectious disease agents are very rarely reported.

Ducks also play an important role economically, as a source of eggs, meat and feathers [1]. Besides that, duck farming management has a critical role in providing animal protein sources in Indonesia. Duck meat has a distinctive sensation. Nevertheless, the main purpose and focus of local duck farming is egg production in local duck business. Demand for duck meat and egg production in Asian countries has increased every year. In Indonesia, data from the Directorate General of Animal Husbandry and Health (2018) [3] explained that duck egg production was 0.3 ton per year or 14% of all egg production in 2017. Duck eggs contain three main components namely egg shells and eggshell membranes (11-13%), egg white (45-58%), and egg yolk (28-35%) [4]. While, egg whites are composed of water (88%), protein (11%) and the rest are lipids, ash, and carbohydrates (1%) [5]. Egg whites contain many important functional proteins, including ovalbumin (40%), ovomucoid (10%), ovomucin (3%), ovotransferrin (2%), lysozyme (1.2%) and conalbumin are the main proteins with high potential for industrial applications, while minor proteins found in egg whites include avidin, cystatin, ovomacroglubulin, ovalflavoprotein, ovoglycoprotein, and ovoinhibitor [6].

Along with the large demand for duck eggs and meat, increasing the need for ducklings. The types of ducks are relatively extensive and disperse almost in utmost parts of Indonesia, nevertheless, their productiveness is still depressed, notably the local ducks with traditional ranch. One effort to overcome this is the selection of superior seeds. To gain high quality of ducks, ducklings selection process is needed. The selection process approach through with genetic methods, therefore it has a permanent impact because it will be inherited from the offspring. Duck genetic information is very useful for the
preparation of further duck breeding programs. In genetic selection, marker assisted selection (MAS) is used as a marker for controlling a trait gene [7-8], especially important traits that have economic value. Reproductive efficiency traits have economic value because they are related to the quantity and quality of superior seeds. Determination of duck genotypes based on genes that control reproductive efficiency, is more accurate, fast and efficient because it does not need to wait for cattle to reproduce and grow to maturity first. One of the genes associated with duck reproductive efficiency, specifically egg hatchability, is the ovomucoid gene [9].

The avian egg is a decisive well of diets, accommodating whole of the lipids, proteins, minerals, vitamins, and growth factors compelled by the evolving embryo. Egg contents affect hatchability [10]. This is because poultry embryos obtain all nutritional needs during incubation from albumen and yolk. Minerals are important for embryonic growth and development and mineral deficiency can cause a decrease in hatchability [11]. Previous study shown that the hatchability of Peking duck eggs is not influenced by breeder age [10]. In the alumen, proteases are possible to play roles in antimicrobial shield and embryogenesis. Ovomucoid activity as a protein serine protease inhibitor, probably related to hatchability.

TAT deletions (g.576_578del) in the ovomucoid gene Tsaiya ducks were recorded in all ducks with low hatchability, and conversely those without deletions of 576-578 were recorded in all high-hatching ducks [12]. This study would analyze the genotype of local duck hatchability established on ovomucoid genes employing the PCR-RFLP approach.

2. Methods

This research design is an exploratory study that determine the genotype of Central Java local duck hatchability build on ovomucoid genes using the PCR-RFLP method. The 35 samples Central Java local ducks based on seven breeds of Central Java local duck breeds which are distinguished from the color of the feather covers, namely Tegal Branjangan (ITB), Peking (IK), Pengging (IG), Pengging (IG), Tegal Jarakan (ITJ), Tegal Blorong (ITL), Tegal Lemah (ITP), and Magelang (IM) where each type of duck breed was taken 5 samples. DNA samples were isolated, amplified by PCR technique using a specific primer pair. The amplification product was then digested with the ApoI enzyme. The results of digestion are visualized with gel electrophoresis to see the genotype that appears.

A 5 samples feather of Central Java local duck feather samples with follicles taken on the innards of the left and right wing. Feather samples used during isolation were only in the rachis calamus section of around 4-5 cm, which were cut into small pieces with a size of 1 mm. These parts are replete with marrow and has loads of tissue. DNA isolation process done using the gSYNC ™ DNA Extraction Kit. The final product is 100 μL local duck DNA isolates.

Ovomucoid gene amplification performed with the composition of PCR (total volume 25 μl) was a forward primer 1.2 μl, reverse primer 1.2 μl, DNA template (50 ng) 2 μl, PCR mix 12.5 μl and ddH2O 8.1 μl. The primary sequences used are TovF2 5-TTCGTTACAGTTC CCCTATACT-3 primers and TovR2 5-CCCTGTGTGCTGTAATCTGTTCTT-3. PCR was performed with the GeneAmpR PCR system thermocycler 2400 (Perkin Elmer). The PCR program is carried out with the program following (1) pre-danaturation of 95 °C for 2 minutes, (2) 30 cycles dwelling of denaturation of 94 °C for 30 seconds, annealing 59.3 °C for 30 seconds and extension 72 °C for 45 seconds, (3) final extension at 72 °C for 7 minutes.

Central Java local duck hatching genotypes were detected by ovomukoid genes fragments cutting as a result of PCR amplification using ApoI restriction enzymes. The PCR-RFLP component is mixed with a total volume of 15 μL with a composition of 1 μl restriction enzyme (Apol), 2 μl buffer, 6 μl ddH2O free nuclease, and 6 μl PCR product. The mixture was incubated for 16 hours (overnight) at 37°C. Gene fragments resulting from restriction enzyme digestion, envisioned through electrophoresis with 3% agarose gel stained by ethidium bromide.

The genotype result of PCR-RFLP process were analyzed based on the bands appeared. Genotype +/+ involved only a 168-bp fragment, with no cut by Apol, then conferred as a 168-bp band in electrophoresis analysis result; genotype +/- contained only a 165-bp fragment, which cut by Apol,  

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generating one 124-bp and one 41-bp fragment after digestion. Furthermore, for heterozygous ducks (+/-), three fragments of 168, 124 and 41 bp were examined [12].

Genotype that emerged confirmed by sequencing. Nucleotide sequencing aims to confirm the existence of the nucleotide sequence which is recognized by the ApoI restriction enzyme. The PCR product was then sequenced employing dideoxy termination approach, with Applied Biosystems (ABI) automatic sequencer using reverse and forward primers from 1st BASE Pte Ltd, Singapore via Genetics Science Indonesia, Ltd. The nucleotide sequences of ovomucoid gene from central Javanese local ducks in this study along with other Anas isolates from Genbank were then aligned by Clustal W of MEGA (Molecular Evolutionary Genetics Analysis) version 7.0.

3. Result and Discussion

The results confirmed that all samples were well extracted their DNA and amplified its ovomucoid genes. Isolation of genomic DNA process obtained with gSYNC™ DNA Extraction Kit that was ± 100 μl per sample. The ovomucoid genes of all samples were successfully amplified using primers TovF2 and TovR2, with a product of 168 bp. It suggests that the ovomucoid gene can be well amplified using primers TovF2 and TovR2 at an annealing temperature of 59,3°C. The present study indicate that annealing temperature optimization is carried out using a temperature gradient at temperatures of 49.0 °C, 50.7 °C, 52.4 °C, 54.1 °C, 55.9 °C, 57,6 oC, 59.3 °C and 61.0 °C. The annealing temperature is too low and too high to cause the primer cannot be attached to a specific place so that the target DNA amplification is not obtained.

Further analysis of polymorphism of the ovomucoid gene was performed by cleaving the ovomucoid gene PCR product using restriction enzyme. The PCR products of 35 duck samples were digested with the Apol restriction enzyme. All RFLP products of duck samples showed one band with 168bp length (+/+ homozygous high-hatching). Results from the 35 duck samples by specific PCR-RFLP indicated a monomorphic at the ovomucoid gene (Figure 1), which is associated with the high hatchability. This shows that ovomucoid gene fragments do not have a cutting site for ApoI/XapI enzymes (5'-AAATTC-3'), that means, all samples are of the +/+ type (high-hatching).

Figure 1. PCR-RFLP products of central Javanese ducks ovomucoid gene cleaved by Apol in 3% agarose analysis. Well M: DNA ladder 100bp.

Genotyping is important way to reveal the molecular identity of a species. It is crucial as the morphological characteristics that in a time do not represent molecular expressions. Our present study was successfully identified the genotyping of Central Java local ducks stand on their hatchability employing the PCR-RFLP technique. RFLP markers are markers for the identification of an organism based on analysis of the pattern of cutting DNA by restriction enzymes. RFLP can be applied in biodiversity and phylogenetic studies of individuals in populations and species. The PCR-RFLP technique can be developed as an alternative method for analyzing genetic diversity and genetic uniformity of individuals and diversity in native duck populations. The PCR-RFLP is impressive as a
breed selection of waterfowl and chicken based on the antiviral myxovirus gene [13-14]. Indonesian domestic water flow genetic diversity analysis could be examined by PCR-RFLP analysis of mtDNA D-loop region [15]. RFLP has served as a verification tool for food products from various animals [16].

Ovomucoid (OVM) is one of the proteins in albumen which bears unique characteristics. It is stable against heating and digestion enzymes and strong allergenic (strong allergenicity) compared to other albumen’s components. According to Caubet et al. [17] ovomucoid contains nine disulphide bridges which has high content of helical and beta-pleated sheets structure. So that it is a very stable structure which resistant to denaturation. Ovomucoid is known as the main allergen in albumen [18]. Ovomucoid allergy needs to be treated differently from allergy to other egg proteins that have denaturation more easily when exposed to heat and digestion [19]. Moreover, ovomucoid character is resistant to heat and digestion that causes a person whom allergic to this protein not to be apt to accept it either in brewed or original form [20-22]. On the other hand, the character of ovomucoid resistant to digestive enzymes such as trypsin, chymotrypsin and elastase, therefore ovomucoid can be used as an oral delivery of protein/peptide therapeutics. Co-administration of ovomucoid with calcitonin, is generally used in the osteoporosis management. Other study revealed that ovomucoid has been used to boost the insulin oral delivery insulin [23].

Ovomucoid showed IgE binding activity, possessed Angiotensin I convert the enzyme inhibitory and was able to maintain trypsin inhibitory activity [24]. Ovomucoid also shows immunomodulation activity against T-cells and has potential as a pharmaceutical candidate protein because it can prevent tumor growth and anticancer agents [5]. Other biological works related to albumen involve immunomodulatory, antioxidant, anticancer and antihypertensive properties.

Based on nucleotide alignment as sequencing process Central Java local duck ovomucoid gene fragments (ITB1 and IM1) with GenBank data (accession: HM776315) known that TovF2 and TovR2 primers apply nucleotides at position 455-622 (168bp). Ovomucoid gene fragments in all Central Java local duck samples did not have ApoI / XapI restriction sites (5’-AAATTC-3’), so they were not cut off by the enzyme when RFLP was analyzed (Figure 2). The alignment results also showed the presence of three SNPs in the nucleotides G502A, A577G, A579C. The presence of single nucleotide polymorphism (SNP) in nucleotides to A577G and A579C and no nucleotide deletions to 576-578 is the cause of the absence of restriction sites for ApoI. According to other study explained that Apol (XapI) restriction sites in ovomucoid gene fragments occur due to TAT deletions in nucleotides 576-578 (g.576_578del) (GenBank HM776315) [12]. Ovomucoid gene fragments with g.576_578del can be cut off by the Apol endonuclease restriction enzyme (5’-AAATTC-3’), producing 124bp and 41bp bands (genotype +/-). TAT deletions (g.576_578del) in the ovomucoid Tsaiya duck gene were recorded in all ducks with low hatchability. While intact gene fragments, without g.576_578del, cannot be cut by Apol, producing 1 band of 168bp in size, recorded in all high-hatchling ducks [12].

The research result describe that all local duck samples in this study were genotypes with high hatchability (genotype +/+).. Although further studies are needed regarding the phenotype of local duck egg hatchability in this study, this finding further strengthens the argument that the Central Java local duck needs to be maintained or preserved as laying ducks with high hatchability. Previous studies [25] showed that 22 (62.86%) of the local ducks were genetically superior respect to the hatchability (TT and CC genotype) based on ovalbumin genes.
Figure 2. Nucleotide base alignment sequencing results of Central Java local duck ovomucoid gene fragments (ITB1 and IM1) with GenBank data (accession: HM776315)

Nucleotide deletions 576-578 (g.576_578del) led to the formation of a protein POU (pronounced 'pow') domain binding site, the new 'TAAAT'. POU domain protein binding sites are conserved (conservative) in most eukaryotes as a transcription factors binding sites that broadly regulate cellular differentiation and organogenesis [26]. The site is a binding site for transcription factors, so that ovomucoid genes with deletions of 576-578 show over-expression of ovomucoid genes. That means, egg hatchability is low. High expression levels of ovomucoid might not be helpful to hatchability, because ovomucoid is a serine protease inhibitor. Salman et al (2013) revealed that protease is needed for the development of embryogenesis and antimicrobial defense [23]. Over expression of ovomucoid (serine protease inhibitors) causes protease activity to be inhibited, therefore the development of embryogenesis is impaired and its hatchability is low. Ovomucoid overexpression causes an overabundance of ovomucoid numbers, exceeding the amount needed for normal physiological conditions, resulting in failure of hatchability. Although ovomucoid overexpression is not beneficial for the development of embryogenesis, but the character of ovomukoid which is resistant to heat and digestive enzymes, allows ovomucoid has been found to be appropriate for protein/peptide therapeutics oral delivery [27].

Moreover, four protease inhibitors have been examined in albumen, ovomucoid, cystatin, ovoinhibitor and ovomacroglobulin (also known as ovostatin). Ovomucoid duck has a molecular weight of 29,300 Dalton, with carbohydrates of 22% [28]. Avian albumen are a great resource of protein inhibitors of proteinases belonging to all four mechanistic classes. Ovoinhibitors and ovomucoid are multidomain Kazal-type inhibitors with particular domain involving a putative or actual reactive site for a serine proteinase.

4. Conclusion
In summing up, all of the Central Java local duck ovomucoid genes samples has genotypes with high hatchability (genotype +/+). This finding strengthens the argument that local Central Java ducks need to be maintained or preserved as laying ducks that has high hatchability.
Acknowledgements
The author would like to thank the Institution of Research and Community Service, Universitas Negeri Semarang which fully supports this research through Fundamental Research Fund 2019.

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