Genotyping of Mx Gene Related to Avian Influenza (AI)
Using PCR-RFLP Analysis on KUB Chicken

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Abstract. Mx gene is antiviral molecules which is part of the innate immune response. In chicken, antiviral activity against vesicular stomatitis virus and the H5N1 virus has been reported to be dependent on the presence of asparagine (A) at position 631 of the Mx protein. The objective of this study was to detect the genotype of Mx gen on KUB chicken as a basis to do molecular selection on natural antibody trait. A total of 120 KUB chicken were used in this study. DNA fragment of Mx gene was amplified and analyzed using PCR-RFLP method. Three types of restriction enzymes (RsaI, SspI and HpaI) were used to determine Mx gene genotype. The results showed that the frequency of the Mx gene genotype on KUB chicken was polymorphic. The AA genotype frequencies resulted from RsaI, SspI and HpaI restriction enzymes were 0.50, 0.425 and 0.283, respectively. While the AG genotype frequencies were 0.40, 0.492 and 0.634; and GG genotypes were 0.10, 0.083 and 0.083, respectively. The frequency of A and G alleles were 0.657 and 0.343. KUB chicken population in this study remained at Hardy-Weinberg equilibrium and three types of restriction enzyme could be used to detect Mx gene.

1. Introduction
The Avian Influenza (AI) was a zoonosis disease which required serious treatment. This disease has become an international concern because it has become a global problem. The main focus of AI treatment attention was on the H5N1 virus spreading, so the most effective treatment so far was the application of vaccination and biosecurity. These steps were already well-implemented on a huge commercial farm, so the AI cases on huge commercial farm have been overcome. However, AI cases still occur in several places, especially in partnership with several small farmers. The application of biosecurity and vaccination still become an obstacle in traditional farmer which rear their local chicken with backyard farming patterns. Therefore, the genetic of AI resistance in the chicken as a host needs to be studied, considering that local chicken has higher frequencies of resistance allele of AI compare to commercial breed [1-5]. In other research, Mx gene were also identified as a specific marker for New castle disease (ND) [6,7]. Currently, The Indonesian Research Institute for Animal Production (IRIAP) has been developing Kampung Unggul Balitbangtan (KUB) chickens which have been spread to 18 provinces in 2019 [8]. By Mx gene marker as AI resistance in chicken [1,9-12] selection of KUB chicken which are resistance to AI disease can be done. The objective of this study was to investigate the Mx...
gene genetic diversity of KUB chicken using the PCR-RFLP method which can be used as basic MAS (marker-assisted selection) to produce local chicken breed resistance to AI disease.

2. Method
Blood samples of 120 KUB chicken which were consisted of 100 hens and 20 cocks were used in this research. The samples were taken from the brachial vein in wing area. All procedures have fulfilled the animal welfare of the Indonesian Agency for Agricultural Research and Development or Badan Penelitian dan Pengembangan Pertanian (Balitbangtan) ethics code with registration number: Balitbangtan/Balitnab/A/03/2020. All the samples were extracted using a DNA extraction kit for the blood sample. DNA fragment amplification of the Mx gene was done using the thermal cycle Polymerase Chain Reaction (PCR) machine. Three specific primers according to Seyama et al. [2] were used to amplified Mx gene, (1) Forward primer NE-F2 (5’-CCTTCAGCCTGTTTTTCTCCTTTTAGGAA-3’), (2) Reverse primer NE-R2/R (5’-CAGAGGAATCTGATTGC-3’), with restriction enzyme Rsal; (3) Forward primer NE-F2 (5’-CCTTCAGCCTGTTTTTCTCCTTTTAGGAA-3’) and Reverse primer NE-R2/S (5’-CAGAGGAATCTGATCGCTAGGCCGAATA-3’), with restriction enzyme SspI; and (3) according to Sulandari et al [8], Forward primer : 5’-GAGTACCTTCAGCCTGTTCCTTTTTTTTCAAG-3’ and Reverse primer: 5’-ATCTGATTGCTCAGGCCCTAA-3’ restricted using Hpal restriction enzyme.

PCR conditions used for the first and second primers were: pre denaturation temperature 94°C for 5 minutes, denaturation 94°C for 60 seconds, annealing 60°C for 60 seconds and elongation 72°C for 60 seconds, on 35 cycle, and final extension temperature on 72°C for 5 minutes. Condition for the third primer was pre denaturation at 94°C for 5 minutes, on 35 cycles; denaturation 94°C for 60 seconds, annealing 56°C for 60 seconds and elongation 72°C for 60 seconds, and final extension temperature on 72°C for 5 minutes.

The PCR products were segregated to 2% gel agarose electrophoresis apparatus in 40 mM Tris Acetate EDTA/TAE (pH 8.0) buffer for 60 seconds on 100-volt constant voltage, staining using Gel Red and visualized on UV instrument. Documentation was done by imaging using electrophoresis Gel-doc tool. The AA, AG and GG genotype determined by using PCR RFLP, the PCR results of each Mx gene fragment of three primers pairs which were restricted using Rsal, SspI and Hpal restriction enzyme. Polymorphism as a results of restriction enzymes can be known by the migration of the DNA, using 8% acrylamide gel electrophoresis with silver nitrate staining.

The calculation of genotype and allele frequencies were using a formula according to Nei and Kumar [13], genotype frequency as follows:

\[
x_{ij} = \frac{n_{ij}}{N}, \text{ and allele frequency as follows: } x_i = \frac{(2n_{ij} + \sum_{i,j} n_{ij})}{2N}
\]

Where:
- \(x_{ij}\) = Allele frequency number-i
- \(x_i\) = Genotype frequency number-i
- \(n_{ij}\) = Total amount of the individual with ii genotype
- \(n_{ij}\) = Total amount of the individual with ij genotype
- \(N\) = Total amount of sample

The heterozygosity was calculated based on observed heterozygosity and expected heterozygosity. The calculated formula according to the Weir [14] as follows:

\[
H_o = \sum_{i\neq j} \frac{n_{ij}}{N} \quad ; \quad H_e = 1 - \sum_{i\neq j} X_i^2
\]
Where,

$H_0$ = heterozygosity observations (population);

$H_e$ = value of expected heterozygosity;

$n_{ij}$ = number of heterozygous individuals;

$N$ = number of individuals observed;

$X_i$ = allele frequency; and

$q$ = number of alleles.

Chi-square test was conducted to calculate genotype frequency of 3 types restriction enzyme according to Nei and Kumar [13] formula:

$$X^2 = \sum_{i=1}^{n} \frac{(O-E)^2}{E}$$

Where,

$X^2$ = chi-square test;

$O$ = Frequency of observed sample genotypes; and

$E$ = frequency of expected genotypes.

### 3. Results and Discussion

#### 3.1. Mx Gene Genotyping

The PCR-RFLP mismatch primers were used to determine the Mx gene genotype using the restriction enzymes Rsal, SspI dan HpaI separately. Based on the results by Seyama et al [2] nucleotide mutations occur at the position of nucleotide number 2,032 or position of amino acids 631. The position of the nucleotide mutations was in the intron 13 and at the beginning of exon 14 regions of the GED (GTPase Effector Domain) Mx gene genome. Therefore, design of the primers to amplifying Mx gene DNA fragments was carried out in that area with DNA fragment length of 100 bp as shown in Figure 1.

![PCR product of Mx gene DNA fragment](image)

**Figure 1.** PCR product of Mx gene DNA fragment

The PCR results of all samples showed that it can be amplified at 100 bp and it were appropriate to targeted DNA fragments. Then using restriction enzymes Rsal, SspI dan HpaI which were digested separately, it could be determined whether the chicken reflected to resistant or sensitive to avian influenza. If Rsal restriction enzyme with a restriction site GT $|$ AC did not match with asparagine/Asn amino acid codon (AAT), it reflected undigested or resistant to avian influenza. If A nucleotide mutated into G, recognized and match to Rsal restriction enzyme GT $|$ AC, then the DNA fragments are cut and recognized serine/ser codons (AGT or TCA), it reflect to sensitive to avian influenza.
The AA genotype of 100 bp DNA fragment showed the resistant genotype. AG genotype with 100 bp and 73 bp DNA fragments showed resistant and sensitive genotype. While GG genotype with 73 bp of DNA fragment showed the sensitive genotype against avian influenza [2] [4]. The results of this study, polymorphism of the Mx gene restricted by restriction enzymes can be determined by using 8% polyacrylamide gel electrophoresis, and silver staining. The results of the KUB chicken Mx gene fragments using forward primer NE-F2 and reverse primer NE-F2 restricted by Rsa I enzyme on polyacrylamide gel were shown in figure 2.

Conversely, If the SSpI restriction enzyme with the AAT | ATT cut site did not match to serine/ Ser (AGT) amino acid codon, it was not cut off. If A nucleotide was matched with the SSpI restriction enzyme AAT | ATT, the DNA fragments were cleaved and recognized Asn/asparagine (AAT) amino acid codon, it reflected resistant to avian influenza. The results of the SSpI restriction enzyme in this study were visualized by migrating Mx gene DNA fragments on acrylamide gels with silver nitrate staining as shown in figure 3.

HpaI was a restriction enzyme which isoschizomer to RsaI because it has the same recognition site. On other hand, the Mx gene molecules were restricted in different position. Therefore, it resulted different sizes of fragments. HpaI which cut on the GTT | AAC site and the restricted nucleotide variations at 2032 bp position was A nucleotide. It would become AAT (asparagine) which reflected resistant to avian influenza. If the restricted nucleotide was G, it would become serine (AGT) which reflected sensitive to avian influenza. In this study, the HpaI restriction enzymes results were visualized by migrating Mx gene DNA fragments on acrylamide gels with silver nitrate staining as shown in figure 4.
3.2. Genotype Frequency

Mx gene genotype was obtained by the PCR-RFLP technique using restriction enzymes RsaI, SspI and HpaI separately. The results of RsaI restriction enzymes showed that there were 45 hens and 15 cocks KUB chickens with AA genotype (resistant to avian influenza), 45 hens and 3 cocks with AG genotype (resistant or sensitive to avian influenza), and 10 hens and 2 cocks with GG genotype (sensitive to avian influenza). The results showed that the AA genotype frequency was 0.403 and 0.509, respectively.

AA genotypes restricted by SspI restriction enzymes were 38 hens and 13 cocks samples, AG genotype were 52 hen and 7 cocks samples, and GG genotypes were 10 hen and 0 cocks KUB chicken. While, the AA genotype restricted by the HpaI restriction enzyme were 22 hens and 12 cocks, AG genotype were 71 hen and 5 cocks, and GG genotype were 7 hens and 3 cocks KUB chicken.

Table 1. Genotype and allele frequencies of Mx Gene restricted by three restriction enzymes.

| Sample (n) | Restriction Enzyme | Genotype Amount | Genotype Frequency | Allele Frequency |
|------------|--------------------|-----------------|--------------------|-----------------|
| Hen        | RsaI               | AA 45, AG 45, GG 10 | AA 0.45, AG 0.45, GG 0.10 | A 0.675, G 0.325 |
| KUB Chickens (100) | SspI               | 38, 52, 10 | 0.38 AG 0.52, GG 0.10 | 0.64 A, 0.36 G |
| Cocks      | HpaI               | 22, 71, 7    | 0.22 AG 0.71, GG 0.07 | 0.575 A, 0.425 G |
| KUB Chickens (20) | Rsal           | 15, 3, 2      | 0.75 AG 0.15, GG 0.10 | 0.825 A, 0.175 G |
| SspI       | 13, 7, 0            | 0.65 AG 0.35, GG 0 | 0.825 A, 0.175 G |
| HpaI       | 12, 5, 3            | 0.60 AG 0.25, GG 0.15 | 0.725 A, 0.275 G |
| Total (120) | RsaI               | 60, 48, 12   | 0.50 AG 0.40, GG 0.10 | 0.70 A, 0.30 G |
| SspI       | 51, 59, 10          | 0.425 AG 0.492, GG 0.083 | 0.671 A, 0.329 G |
| HpaI       | 34, 76, 10         | 0.283 AG 0.634, GG 0.083 | 0.60 A, 0.40 G |
| Mean       | 0.403, 0.509, 0.088 | 0.657 | 0.343 |

The genotypes and the alleles frequencies of KUB chicken digested by three types of restriction enzymes were shown in table 1. The results showed that the AA genotype frequency was 0.403. It means that KUB chickens which were resistance to avian influenza was about 40.3%. Heterozygote genotypes showed the highest frequency (0.509). While genotypes that susceptible to avian influenza were only 0.088. The frequency of all A alleles was more than the G allele, which was 0.657 for A alleles and 0.343 for G alleles, respectively. In general, local chickens have higher A allele compared to commercial chickens, which means that probability of local chicken to resistance to the avian influenza virus is quite high [1,3,4,15-17].
3.3. Mx Gene Heterozygosity

Heterozygosity values indicates the diversity of a population. Higher value of heterozygosity indicates more diverse the observed population. Heterozygosity was determined by calculated the allele frequency of a gene which in this study the calculation was based on the allele frequency of Mx gene. The heterozygosity in this study was calculated based on observed and expected heterozygosity. In a natural population, the value of heterozygosity often refers to the Hardy-Weinberg Equilibrium (HWE). If the value of observed heterozygosity is lower than expected heterozygosity, it means that inbreeding is occurred. Vice versa, if expected heterozygosity is lower than observed heterozygosity, it means that the effect of isolate separation of a population is suspected being occurred and still in Hardy-Weinberg Equilibrium. In this study the calculation of the expected heterozygosity and observed heterozygosity was presented in table 2. The results showed that overall of the expected heterozygosity (He) was lower than the observed heterozygosity (Ho). This result showed that the observed KUB chicken samples were still in Hardy-Weinberg Equilibrium (no inbreeding effect). Li et al [15] showed that local chickens were generally still in Hardy-Weinberg Equilibrium. However, especially for cocks KUB, the value of observed heterozygosity (Ho) was lower than expected heterozygosity (He). It was probably due to the limited number of samples and might lead high variation. Therefore, cocks KUB samples in this study were in the disequilibrium population caused by the systematic selection on closed populations, high selection intensity, genetic drift and non-random mating [13].

| Number of Sample | Restriction Enzyme | Mx Gene Heterozygosity Value | Coefficient of Variation (CV %) |
|------------------|-------------------|-----------------------------|-------------------------------|
|                  |                   | Observed Heterozygosity (Ho) | Expected Heterozygosity (He)  |
| Hen KUB Chickens | Rsal              | 0.45                        | 0.439                         | 3.182 |
| (100)            | SspI              | 0.52                        | 0.461                         | 2.407 |
|                  | HpaI              | 0.71                        | 0.489                         | 2.259 |
| Mean             |                   | 0.56 ± 0.134                | 0.463 ± 0.014                 | 3.02  |
| Cocks KUB Chickens | Rsal             | 0.15                        | 0.289                         | 20.88 |
| (20)             | SspI              | 0.35                        | 0.289                         | 20.88 |
|                  | HpaI              | 0.25                        | 0.399                         | 10.88 |
| Mean             |                   | 0.25 ± 0.100                | 0.325 ± 0.037                 | 11.38 |
| Total            | Rsal              | 0.40                        | 0.480                         | 5.83  |
| (120)            | SspI              | 0.49                        | 0.420                         | 3.49  |
|                  | HpaI              | 0.63                        | 0.442                         | 4.33  |
| Mean             |                   | 0.506 ± 0.116               | 0.447 ± 0.017                 | 3.8   |

The heterozygosity value can be as a reference for a more homogeneous selection. In this study, the selection was aimed of the traits that related to avian influenza virus resistances. The application of three types of enzymes to analyse the Mx gene genotypic polymorphism was carried out in order to find the most accurate molecular markers. To find out the differences results of three types restriction enzymes, chi-square tests were carried out (table 3). The results of the chi-square test showed no significant difference. Therefore, Rsal, SspI or HpaI restriction enzymes can be used to determine Mx Gene that related to immunity trait to avian influenza.
Table 3. Chi-square test calculations of three types of enzymes.

| Genotype | Observed (O) | Expected (E) | Rsal | SspI | HpaI | Genotype Total | Genotype Frequency |
|----------|--------------|--------------|------|------|------|----------------|--------------------|
| AA       | O            | E            | 60   | 51   | 34   | 145            | 40.28              |
| AG       | O            | E            | 48.3 | 48.3 | 48.3 | 183            | 50.83              |
| GG       | O            | E            | 12   | 10   | 10   | 32             | 8.89               |

Result: F value = 4.65
F distribution = 9.49, it means not significant

4. Conclusions
The AA, AG and GG genotype frequencies of Mx Gene in KUB chickens were 0.403, 0.509, and only 0.088, respectively. A allele frequency was 0.657 and G allele frequency was 0.343. The Mx gene heterozygosity value in KUB chicken population was still quite diverse and remained in Hardy-Weinberg Equilibrium. The results of three types of restriction enzymes in this study to determine the Mx gene genotype was not significantly different. Therefore, one of these restriction enzymes can be used to determine Mx Gene in KUB chicken.

References
[1] Maeda 2005 Polymorphism of Mx Gene in Asian Indigenous chicken population Presented in Seminar Nasional Tentang Unggas Lokal III 25 Agustus 2005 (Semarang: Universitas Diponegoro)
[2] Seyama T, Ko J H, Ohe M, Sasaoka N, Okada A, Gomi H, Yoneda A, Ueda J, Nishibori M, Okamoto S, Maeda Y and Watanabe T 2006 Population research of genetic polymorphism at amino Acid position 631 in chicken Mx protein with differential antiviral activity Biochem. Genetics 44 432–43
[3] Sulandari S, Zein M S A, Astuti D and Sartika T 2009 Genetic polymorphisms of the chicken antiviral Mx Gene in a variety of Indonesian Indigenous chicken breeds Jurnal Veteriner 10 50-56
[4] Sartika T, Sulandari S and Zein M S A 2011 Selection of Mx gene genotype as genetic marker for Avian Influenza resistance in Indonesian native chicken On-line Journal Publishing of BMC Proceedings 2011 5(Suppl 4) S37
[5] Ramasamy K T, Reddy M R, Raja Ravindra K S, and Chatterjee R N 2017 Chicken Mx gene polymorphisms in Indian native chicken breeds and White Leghorn by real time multiplex allele specific PCR Indian J. Anim. Res. 52 649-51
[6] Pagala M A, Muladno, Sumantri C and Murtini S 2013 Association of Mx Gene Genotype with Antiviral and Production Traits in Tolaki Chicken Int. J. Poult. Sci. 12 735-39
[7] Mamutse J, Gunawan A, Sumantri C, Murtini S and Sartika T 2018 Association of the Toll-like Receptor 4 (TLR4) and Myxovirus (Mx) Genes With Resistance to Salmonella and Newcastle Disease in Selected Sentul Chickens Int. J. Poult. Sci. 17 591-99
[8] Zainal H, Sartika T and Komarudin 2020 Profil dan Potensi Akselerasi Distribusi Ayam KUB-1 dan SenSi-1 Agrinak untuk Menunjang Adopsi Inovasi Badan Litbang Pertanian Proceeding Semnas Peternakan dan Veteriner Oktober 2020 20 pp 525-35
[9] Watanabe T 2007 Polymorphisms of the chicken antiviral Mx gene Cytogenetics and Genome Research 117 370-75
[10] Ko J H, Takada A, Mitsuhashi T, Agui T and Watanabe T 2004 Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631 Animal Genetic 35 119-122

[11] Sartika T 2005 Gen Mx+ sebagai penyeleksi resistensi Flu Burung Short Communication Warta Penelitian dan Pengembangan Pertanian 27 6-7

[12] Ye X, Tan Z, Zhang Y and Li K 2010 Single nucleotide polymorphism in the chicken Mx gene at position 2032 by real time allele specific PCR melting curve analyses J. Poul. Sci. 47 133-38

[13] Nei M and Kumar S 2000 Molecular Evolution and Phylogenetics (New York (US): Oxford Univ Press)

[14] Weir B S 1996 Genetic data analysis II methods for discrete population genetic data Sunderland (UK: Sinauer Associates Inc.)

[15] Li X Y, Qu L J, Yao J F and Yang N 2006 Skewed allele frequency of an Mx Gene mutation with potential resistance to Avian Influenza virus in different chicken population Poul. Sci. 85 1327-29

[16] Balkisson D, Staines K, Cauley J M C, Wood J, Young J, Kaufman J and Butter C 2007 Low frequency of the Mx allele for viral resistance predates recent intensive selection in domestic chickens Immunogenetics 59 687-691

[17] Hassanane M S, Hassan A A M, Ahmed F M, El-Komy E M, Roushdy K M and Hassan N A. 2018 Identification of Mx gene nucleotide dimorphism (G/A) as genetic marker for antiviral activity in Egyptian chickens J. Genetic Eng. Biotechnol. 16 83–88