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Research Article
Polymorphism of Mx|*Hpy*81 Genes in Native Chickens Observed using the PCR-RFLP Technique

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Abstract

Background: The Mx gene plays a crucial role in the antiviral responses of chicken. The Mx gene codes for Mx protein, which possesses antiviral traits. The non-synonymous G/A single nucleotide polymorphism (SNP) at position 2032 of chicken Mx cDNA results in a change at amino acid 631 of the Mx protein. This mutation affects the antiviral activity of the Mx molecule. **Objective:** The aim of the study was to evaluate the genetic polymorphism of the Mx gene in the native chickens of South East Sulawesi, Indonesia, using PCR-RFLP. **Materials and Methods:** The Mx gene was genotyped in 25 Tolaki chickens from each of Konawe Regency and South Konawe Regency and in 21 Kampong chickens from Kendari City. Tolaki chicken is traditionally used as a medium for medicinal treatments in the Tolaki culture. PCR was used to amplify genomic DNA for the Mx gene (299 bp). The amplifier was cut using the *Hpy*81 enzyme. **Results:** The genotyping of the Mx gene of native chicken produced three genotypes, AA, AG and GG and two alleles, A (299 bp) and G (200 bp and 99 bp); the frequency of A was higher than that of G. The value of x^2 showed that Mx|*Hpy*81 was in Hardy-Weinberg equilibrium. The values of He and PIC were 0.47-0.49 and 0.36-0.37, respectively. **Conclusion:** These results indicated that Mx|*Hpy*81 gene was polymorphic in all strains of chicken that were genotyped. The Mx|*Hpy*81 gene demonstrated a high potential for use as a genetic marker for resistance to *Avian influenza* and *Newcastle* disease infection in Indonesian native chickens.

Key words: Native chicken, Mx gene, Antiviral, SNP, PCR-RFLP

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Local chicken or Kampong chicken are a local germplasm with an existence that is closely related to the social and cultural values of rural communities in Indonesia. In addition, local chickens have higher economic value because they have several advantages, such as high adaptability and durability to the environment, an ability to utilize lower feed quality, easy maintenance and high economic value of both their meat and egg. Nevertheless, local chicken development faces some constraints in genetic quality and low productivity that result in difficulties in producing high-quality and available breeds. By utilizing the advantages of local chicken, its genetic quality and productivity can be optimized through breeding programs that include sustainable targeted selection.

Local chickens in Indonesia are known to have high genetic diversity, including an identified strain possessing the Mx gene. The Mx gene has been linked to chicken immunity levels against viral diseases such as *Avian Influenza* and *Newcastle* disease[1-3]. Tolaki chicken is one of the Indonesian local chickens originating from Southeast Sulawesi and is known to have antiviral genes with high contents of allele A[3,4].

The targeted selection method that was used for this case was molecular selection. This selection optimized some of the key genes that are responsible for the efficient expression of traits such as growth rapidity and viral disease resistance. The advantages of molecular selection include requiring less time to achieve an effective and low-cost procedure. Selection using marker genes, known as the MAS method (Marker Assisted Selection) was applied effectively[5]. The MAS method application requires initial information about genes that could become marker gene candidates. Based on the description above, this study was conducted to analyze the polymorphism of the Mx gene of Indonesian native chickens.

MATERIALS AND METHODS

Animal experiments and rearing: A total of 71 samples of Tolaki and Kampong chicken DNA were used, with 25 samples of Tolaki chicken taken from the Konawe Regency, 25 samples of Tolaki chicken taken from Southern Konawe Regency and 21 other samples of Kampong chicken taken from Kendari City of Southeast Sulawesi Province, Indonesia.

The chickens (7-8 months of age and 1.8-2 kg in weight) were maintained intensively for four weeks. The native chickens were randomly placed in individual cages with cage dimensions of $60\times60\times40$ cm^3. All cages were placed in a 5×15 m pen. The feed used was a commercial feed mix for the grower phase containing 18-19% crude protein and 2.850 kcal kg^{-1} metabolic energy. Feed and water were given *ad libitum*.

DNA extraction and amplification by PCR: DNA was extracted from the samples using the Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The extraction procedure was performed following the manufacturer's instructions: $\pm$0.5 cm from the initial (root/kalamus) feather was transferred into a 1.5 mL tube and cut into several tiny parts. To the 1.5 mL tube was added 20 µL of *Dilution Buffer* and 0.5 µL of DNA Release™ Additive (Fisher Scientific Company, Wilmington, DE, USA). The mixture was processed with a vortex and centrifuged, followed by incubation for 2-5 min at room temperature and 2 min at 98°C. The DNA samples were then ready for use and stored at -20°C. DNA samples were amplified using a PCR (Polymerase Chain Reaction) machine. The specific primers used to amplify Mx[6] genes were the forward primer (5'-GCA TCA CCT CTG CTT AAT AGA-3') and reverse primer (5'-GTA GTA GTT GTT GGC TTT GA-3'). The DNA amplification was carried out in a total volume of 25 µL, consisting of 2 µL of 10-100 ng of DNA; 15.75 mL of sterile deionized water; 2.5 mL of $10\times$buffer without Mg^{2+}; 2 µL of $MgCl_2$; 0.5 µL of 10 mM dNTP; 0.25 µL of Taq Polymerase and 2 µL (25 pmol) of primer. The first phase was conducted for one cycle, including an initial denaturation process at 94°C for 4 min. The second phase was conducted for 30 cycles, including denaturation at 94°C for 10 sec, primer annealing at 60°C for one minute and DNA molecule elongation at 72°C for 2 min. The third phase was completed in one cycle and included the elongation of the end of the DNA molecule at 72°C for 7 min. Samples were incubated at 4°C until used for further analysis.

Mx gene genotyping: The Mx gene diversity was identified using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The enzyme used was *Hpy*8I, which recognizes the cutting site GTN|NAC. The RFLP process was performed by adding three units of *Hpy*8I enzyme (10 U μL^{-1}) and 0.7 mL of $10\times$Buffer Tango (Fermentas, Finland) to 5 µL of DNA produced by PCR and continuing with 16 h incubation at 37°C. DNA fragments became PCR-RFLP products through electrophoresis, using electrophoresis devices on 2% *agarose* gel (0.5 g/25 mL $0.5\times$TBE). The device was run using $0.5\times$TBE buffer at 100 volts voltage for 30 min. Electrophoresis gel visualization was performed on an *Alpha Imager* gel documentation device.

Data analysis: The counting of allele frequency, genotype frequency and Mx gene heterozygote was based on genotyping results[7], the Hardy-Weinberg balance value[8] and the Polymorphic Informative Content (PIC)[9], using the following formulas:

Allele frequency[7]:

$$xi = \frac{(2nii + \sum_{j \neq 1} nij)}{2N}$$

x_i = i-allele frequency
n_{ii} = Individual number of ii-genotype
n_{ij} = Individual number of ij-genotype
N = Total sample

Genotype frequency[7]:

$$xii = \frac{\sum_{i=1}^{n} ni}{N}$$

xii = ii-genotype frequency
ni = Individual number of ii-genotype
N = Total sample

Hardy-Weinberg (H-W) balance[8]:

$$x^2 = \sum \frac{(O-E)^2}{E}$$

x^2 = Chi-square value
O = Genotype observation number
E = Genotype expectation number

Heterozygosity[7]:

$$H_o = \sum \frac{N_{1ij}}{N}$$

$$H_e = 1 - \sum P_{1i^2}$$

H_o = Heterozygosity observation
N_{1ij} = No. of heterozygous individuals in the 1st locus
N = No. of individuals observed
H_e = Expectation heterozygosity
P_{1i} = Frequency of i-allele at locus-1

Polymorphic Informative Content (PIC)[9]:

$$PIC = 1 - \sum_{i-1}^{n} P_i^2 - \sum_{i-1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

PIC = Polymorphic Informative Content
p_i = Allele frequency-i
p_j = Allele frequency-j
n = Total allele

RESULTS AND DISCUSSION

Figure 1 shows the result of the PCR-RFLP treatment of a Mx gene fragment (299 bp) that was cut by restriction *Hyp*81 enzymes at the 2032nd site in *exon* 13 (GTN|NAC). Cutting with *Hpy*81 produces two alleles (A and G) and three genotypes (AA, AG and GG). A alleles cannot be cut by *Hpy*81 and thus produce one DNA fragment (299 bp), whereas the G allele, which can be cut by *Hpy*81, produces two DNA fragments (200 bp and 99 bp). The cutting results for cDNA 2032 of the Mx gene showed any transition base mutations (single mutation) and there were mutations in base pairs from GC into AT.

Genetic frequency and allele frequency of Mx|*Hpy81* Gene: The genetic diversity of the Mx gene in Tolaki chickens was determined from the genotype and allele frequencies and is presented in Table 1.

Based on a genotype analysis of the 71 tested samples of local chicken feathers, the average genotype frequency values obtained are, from high to low, AA (0.42), AG (0.32) and GG (0.26). The A allele frequency average value (0.58) was slightly higher than that of the G allele (0.42). The genotyping results suggest that the Mx gene in *Hpy*81 loci was polymorphic (various). This is consistent with the allele frequency theory: If the relative frequency of two or more alleles in the population is greater than 0.01 (1%), the gene is called polymorphic[10]. It was indicated that both Kampong and Tolaki chicken are resistant to virus attacks (AI and ND). This resistance is due to the flow of the A allele, which causes serine amino acid (AGT) changes to asparagine (AAT). The presence of asparagine amino acid (A) at *exon 13* indicates that the chickens are resistant to viral infections; in contrast, when a base mutation occurs in the serine amino acids (G), the chicken is vulnerable to virus attacks[1,2,11].

Genetic Mx gene index in population: The results of the genetic diversity analysis of the Mx gene, obtained through genetic index value calculations are presented in Table 2.

Based on the results (Table 2), the average total values of Ho, He and PIC on the Mx gene *Hpy*81 locus are, 0.32, 0.48 and 0.37, respectively. This indicates that the Mx gene's genetic diversity in each population was high and polymorphic, with moderate PIC values[9]. This is understandable in the case of a chicken sample obtained from free range chicken population, which implies a cause of naturally random mating with a low chance of inbreeding.

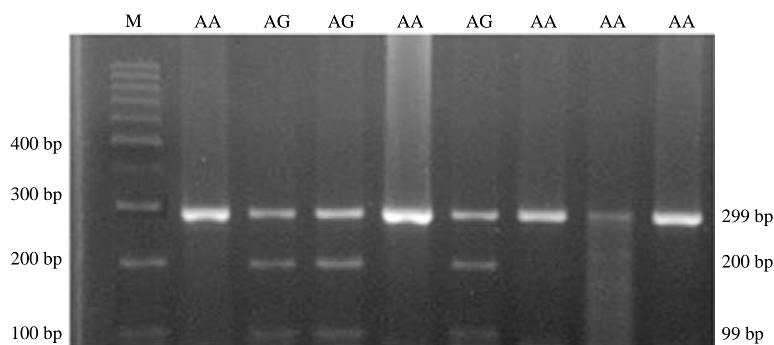

Fig. 1: Products PCR-RFLP Mx gene amplification Mx on *exonal 3* (cut by *Hpy81*). M: Marker 100 pb, N: Mx gene fragments (299 bp). AA, AG and GG: Mx genotype in local chicken analyzed

Table 1: Genetic and Allele frequency of MX|*Hpy81* gene in native chickens

		Genotype frequency (%)			Allele frequency (%)	
Sample sources	N	AA	AG	GG	A	G
Tolaki chicken (A)	25	0.40	0.44	0.16	0.62	0.38
Tolaki chicken (B)	25	0.44	0.32	0.24	0.60	0.40
Kampong chicken	21	0.43	0.19	0.38	0.52	0.48
Total	71	0.42	0.32	0.26	0.58	0.42

Table 2: Mx gene index value (value of heterozygosity, polymorphic informative content and chi-square) in native chickens

Generations	Ho (%)	He (%)	PIC (%)	XHWE
Tolaki chicken (A)	0.44	0.47	0.36	0.002^{ns}
Tolaki chicken (B)	0.32	0.48	0.36	0.054^{ns}
Kampong chicken	0.19	0.49	0.37	0.184^{ns}
Total	0.32	0.48	0.37	0.080^{ns}

ns: Not significant, x^2 count <x^2 table (0.01,2)

The results of chi-square test $(x)^2$ in all populations were not significantly different, which means that the Mx genes in the population were in Hardy-Weinberg balance: The allele and genotype frequencies were still hereditary from one generation to the next due to the random gamete diffusion within the population[12].

However, more variations and samples are required in different local chicken strains in different regions of Indonesia to obtain more accurate results, considering candidate Mx genes that may have different genetic expression levels that are influenced by many factors.

CONCLUSION

These results showed that the Mx|*Hpy*81 gene was polymorphic in all genotyped strains of chickens. The allele A frequency of Mx|*Hpy*81 gene in Tolaki chicken was relatively higher than that of Kampong chicken. The Mx|*Hpy*81 gene has potential to serve as a genetic marker for resistance to *Avian influenza* and Newcastle Disease infection in Indonesian native chickens.

SIGNIFICANCE STATEMENTS

This study highlights the polymorphism of the Mx|*Hpy*81 gene in Tolaki chicken and Kampong chicken, which could be beneficial in enhancing the genetic selection programs for local chickens. This study will assist the researcher in revealing the high-level distribution of the frequency of allele A in the Mx|*Hpy*81 gene in Indonesian native chickens, which many researchers have been unable to explore. It was indicated that chickens with the relevant alleles possess heightened resistance to viral attacks (*Avian influenza* and *Newcastle* disease).

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