BIODIVERSITAS
Volume 21, Number 4, April 2020
Pages: 1483-1487

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210428

Expression of *Mx* exon-13 SNPs in *Kampong*-Laying Type (*Kamper*) chicken crossbreeds of female Lohmann brown-classic and male *Pelung*

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Manuscript received: 11 December 2019. Revision accepted: 19 March 2020.

Abstract. *Afifah D, Lesmana I, Poerwanto SH, Joko T, Mahardhika IWS, Daryono BS. 2020. Expression of Mx exon-13 SNPs in Kampong-Laying Type (Kamper) chicken crossbreeds of female Lohmann brown-classic and male Pelung. Biodiversitas 21: 1483-1487.* The Gama Ayam Research Team has implemented marker-assisted selection (MAS) in selective breeding to provide a faster, more accurate, and more reliable selection of chicken. *Mx* gene expression has a vital role in chicken disease resistance. This research aimed to investigate the expression of *Mx* exon-13 single nucleotide polymorphisms (SNPs) in the population of female Layer Lohmann Brown-Classic, male *Pelung*, and its progenies *Kampong*-Laying Type (*Kamper*) chicken. The G1892A mutation in *Mx* exon-13 resulted in a change in the amino acid 631 of *Mx*. The substitution of serine to asparagine favored the ability of chickens to acquire immunity against viral diseases, including avian influenza. Asparagine (A allele) at position 631 is specific to Mx^+ (resistant), whereas serine (G allele) is specific to Mx^- (susceptible). DNA was amplified using the forward primer 5′-GCACTGTCACCTCTTAATAGA-3′ and the reverse primer 5′-GTATTGGTAGGCTTTGTTGA-3′ and then sequenced using the Sanger sequencing method. Four SNPs were obtained through *Mx* sequence alignment. They consisted of four substitutions (A20734T, C20737T, A20766G, and A20893G) with one haplotype. *Mx* exon-13 SNPs were detected in *Pelung* and *Kamper*. Therefore, *Kamper* chicken inherited the disease resistance gene of *Pelung* and could be a strong candidate for parental generation in the further selective breeding program.

Keywords: AI, *Kamper*, *Mx* gene, resistance, SNPs

INTRODUCTION

Since 2008, the Gama Ayam Research Team has conducted several selective breeding programs with a well-constructed breeding structure and a native chicken breed to produce meat-type and laying-type chicken breeds with disease resistance. Selective breeding between *Pelung Blirik Hitam* and Broiler Cobb 500 produced the hybrid chicken F_1 Broiler or *Kambro* (*Kampung*-Broiler Type; Mahardhika and Daryono 2019). In 2013-2014, this research team successfully bred the F_1 *Kamper*, a chicken breed that inherited the characteristics of female Lohmann Brown-Classic and male *Pelung* (Habibah et al. 2019, unpublished data). This research team also implemented marker-assisted selection (MAS) on numerous studies by using several genes. Tanjung et al. (2019) investigated the expression of the *Myostatin* gene in *Gama* chicken. Selective breeding in chicken is conducted based on egg productivity, meat productivity, and disease resistance. Diseases infecting chickens in tropical regions, such as Indonesia, include avian influenza (AI), Newcastle disease (ND), and chronic respiratory disease. The spread of the avian influenza A (H5N1) virus started when an outbreak occurred in poultry in August 2003 (Pracoyo 2009). Pracoyo (2009) also stated that the first human case was first recorded in July 2005 in Tangerang Municipality and then in eight provinces. In December 2008, the total cases increased by up to 139 people, and 113 of them died (Pracoyo 2009). To overcome the spread of AI in chickens, some researchers investigated the assembly, especially breeding, of chickens; when compared the resistance to AI of 63% of the local chicken population in Indonesia is relatively higher than that of imported broiler and layer chickens (Maeda 2005). Therefore, the native chicken breed can be further improved for selective breeding.

MAS can be applied in selective breeding to provide a faster, more accurate, and more reliable selection of chicken. *Mx* molecular marker can be used to identify the expression and probability of disease resistance in *Kamper* chicken. Pagala et al. (2017) found that *Mx*|*Hpy 81* is polymorphic in all genotyped chicken strains. *Mx*|*Hpy 81* may be used as a genetic marker of resistance to AI and ND in Indonesian native chickens (Pagala et al. 2017). Permatasari et al. (2015) stated that *Mx* (GenBank accession number: **DQ788615**) is located on chromosome 1 having a fragment length of 20,767 base pairs (bp) and consisting of 13 exons. The *G/A* polymorphism on exon-13 at nucleotide position 1,892 of coding the sequence of *Mx* results in changes in 631 amino acids of the *Mx* protein. Fourteen amino acid variants have been identified in the *Mx* protein from multiple chicken breeds, whose antiviral activity is seemingly linked to one amino acid variant at position 631 (S631N; Fulton et al. 2014). Fulton et al. (2014) also reported *Mx* polymorphisms in many chicken

breeds, including Australorp, Fayoumi, Japanese native chickens, Indonesian native chickens, White Leghorns, broilers, and inbred laboratory lines. Pagala et al. (2017) found that *Kampong* and *Tolaki* chickens are resistant to virus attacks (e.g., AI and ND) because of the flow of the A allele, which causes serine (AGT) to change into asparagine (AAT). The presence of asparagine (A) at exon 13 indicates that chickens are resistant to viral infections; by contrast, chickens are vulnerable to virus attacks when a base mutation occurs in serine (G) (Ko et al. 2002; Watanabe 2003; Ko et al. 2004; Pagala et al. 2017).

The parental generation of the breeding tree of *Kamper* chicken includes *Pelung* chicken as the male generation. The *Mx* gene expression is expected to reside in Indonesian native chicken breeds. The *Mx* expression and its correlation with growth performance in *Kamper* chicken have never been explored. In this research, the relation of *Mx* polymorphism associated with the growth of *Kamper* chicken was investigated.

MATERIALS AND METHODS

The research was conducted in January 2017-June 2018 at Pusat Inovasi Agroteknologi (PIAT) UGM, Berbah, Sleman, Yogyakarta, and at the Laboratory of Genetics and Breeding, Faculty of Biology UGM. Whole blood samples used in this research are female Layer Lohmann Brown-Classic, male *Pelung*, its progenies generation *Kampong*-Laying Type (*Kamper*) chicken and Broiler Cobb 500 chicken. Feeds and equipments used in this research are chicken feeds (BR-I and AD-II), supplements, 1 mL syringe, ethylenediaminetetraacetic acid (EDTA), TE buffer, collection tube, master mix PCR (MyTaq HS Red Bioline), agarose, 70% alcohol, ddH_2O, FloroSafe DNA stain (BIO-5170, 1st BASE, Malaysia), 100 bp DNA ladder (*Bioron*), chelex 5% solution, 18 µL of 0.05 M DTT, 10 mg/mL proteinase K, 1× TAE, 10× TAE, PCR thermocycler (*Bio-Rad*), PCR tube (*Biologix*), waterbath, centrifuge, pipette tips (blue, yellow, and white), 1.5 mL microtube, and UV transilluminator (*Geldoc*). This study was performed under the Animal Welfare Act of Indonesia and all procedures involving the handling of animals were approved by the local office of occupational and technical safety (Ethical Clearance Commission of Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada, Yogyakarta No: 00038/04/LPPT/VI/2018).

Procedures

Chicken maintenance, body weight data records, and whole blood sampling

The chicken population groups were randomly sampled as follows: a) layer Lohmann Brown-Classic (n: 5), b) *Pelung* (n: 5), c) Broiler Cobb 500 (n: 5), and d) *Kampong*-Laying Type (*Kamper*) (n: 5). Each chicken population group was reared starting from day-old-chick (DOC) until 7-weeks-old in a semi-intensive rearing system at PIAT UGM. *Ad libitum* standard feed diets of BR-I (0-4 weeks old) and AD-II (4-7 weeks old) were supplied by PT., Japfa Comfeed. The body weight in each week was recorded with a semi-analytical scale. Chicken blood samples were obtained using a syringe (1 mL), placed in EDTA + 1.5 mL microtube, and stored in a freezer at the temperature of −20 °C.

DNA isolation and sequencing

Whole blood DNA was isolated using the Chelex method. In this procedure, 10 µL of the blood sample was added to 1 mL of TE buffer and then centrifuged at 13000 rpm for 13 min. The supernatant was removed, the pellet was extracted, and a 5% chelex solution, 18 µL of 0.05 M DTT, 2 µL of 10 mg/mL proteinase K were added. The solutions were incubated at 56 °C for 2 h, vortexed for homogenization for 15 min, further incubated for 8 min, and centrifuged for 3 min, The supernatant was transferred to a microtube and stored in a freezer at the temperature of −20 °C. The DNA fragment was amplified with 25 µL of PCR solution consisting of 12.5 µL of Mastermix PCR (MyTaq HS Red Bioline), 1.25 µL of forwarding primer (`5′-GCACTGTCACCTCTTAATAGA-3′`), and 1.25 µL of reverse primer (`5′-GTATTGGTAGGCTTTGTTGA-3′`), 5 µL of DNA samples, and 5 µL of ddH_2O. PCR amplification was performed using a thermal cycler PCR machine with a predenaturation condition at 95 °C for 3 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 25 s, and post extension at 72 °C for 5 min (Sironi et al. 2010). The PCR products were detected through electrophoresis on 2% agarose gel in a submarine electrophoresis system (Mupid-EXU) device. Agarose gel was initially prepared by dissolving 0.8 g of agarose powder in 40 mL of 1× TAE. Then, 4 µL of FloroSafe was added to this solution and inserted into a mold equipped with a comb. The solidified gel was placed in an electrophoresis chamber and soaked with 0.5× TAE. Next, 5 µL of the ladder and PCR samples were inserted into gel wells. The electrophoresis system was set at 100 V for 20 min. Lastly, observations were performed under ultraviolet light (λ = 260 nm). All the experimental animals used in this study were cared for and maintained throughout the experiments in strict accordance with the ethics and biosecurity guidelines approved by the Institutional Animal Care and Use Committee of UGM, Yogyakarta, Indonesia. DNA sequencing was carried out by PT. Genetika Science Indonesia (Ruko Puri Mansion, Blok A, Jalan Lingkar Luar No. 19, Kembangan Selatan, Kembangan, West Jakarta City, Special Capital Region of Jakarta).

Data analysis

The body weights of *Pelung*, Broiler Cobb 500, and *Kampong*-Laying Type (*Kamper*) chickens were then analyzed with ANOVA analysis. DNA was sequenced through the Sanger sequencing method and analyzed with Gene Studio software. The correlation between body weight and *Mx* expression was analyzed using Pearson's correlation with IBM© SPSS© version 21.

RESULTS AND DISCUSSION

Crossbreeds between female Lohmann Brown-Classic and male *Pelung* produced the progenies generation *Kampong*-Laying Type (*Kamper*). Ernanto (2017) concluded that the egg productivity of *Kamper* is higher (HDP: 140 eggs/300 days production) and its growth rate is relatively faster than those of *Pelung* chicken. Visual observation has shown that *Kamper* has several distinguishing traits, including white shank, golden and brown-barred feather, and single comb (Figure 1).

Bodyweight

Based on the 7 weeks of bodyweight measurement of each chicken population group under semi-intensive rearing system with standard feed diet the results are as follows, *Pelung* (289.4 g), *Kampong*-Laying Type (*Kamper*) (441.2 g), and Broiler Cobb 500 (2897 g; Figure 2). Fisher's LSD *post hoc* analysis revealed that the growth rate of *Kamper* was significantly faster than that of *Pelung* ($p<0.05$).

Mx gene polymorphisms

Mx exon-13 was subjected to image analysis by using Gene Studio software, and the results revealed that the size of the DNA fragment from each sample (n=16) was 300 bp (Fig. 3). Pagala et al. (2017) reported a similar DNA fragment size of the DNA band of *Mx* in *Tolaki* chicken (299 bp). *Mx* exon-13 discovered in White Leghorn and New Hampshire chickens have a similar size of 300 bp (Pagala et al. 2017). Afterimage analysis, *Mx* amplicons were further analyzed with the Sanger sequencing method. The obtained sequence results were assembled using Gene Studio and then aligned with Clustal Omega to identify the polymorphic site.

Table 1 shows four SNPs of *Mx* were detected in *Kamper* and *Pelung* chickens and identified as a substitution mutation specified in several gene sites, namely, A20734T, C20737T, A20766G, and A20893G. They formed one haplotype in *Kamper* chicken. Three *Kampong*-Laying Type (*Kamper*) chickens formed one haplotype, and one *Kamper* chicken had the same nucleotide composition as the reference in GenBank (*Mx* accession number: DQ788616.1).

Table 2 indicates that *Mx* polymorphisms were not significantly correlated with body weight but were positively and weakly correlated in the four SNPs sites of A20734T, C20737T, A20766G, and A20893G. *Mx* SNPs had no significant effect on the growth and bodyweight performance of *Kamper* chicken.

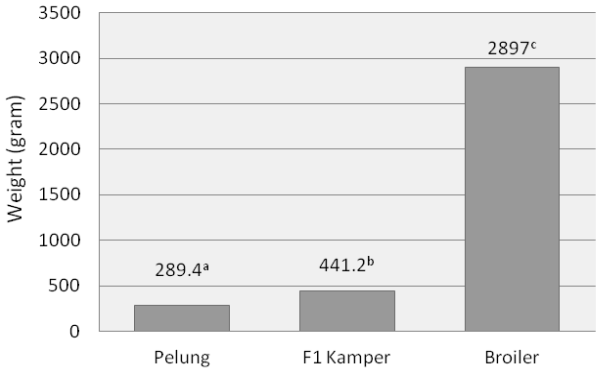

Figure 2. Bodyweight comparison of *Pelung* chicken, *Kamper* chicken, and Broiler Cobb 500 in 7 weeks. The averages with different superscripts differ significantly ($p<0.05$) as indicated by Fisher's LSD *post hoc* analysis.

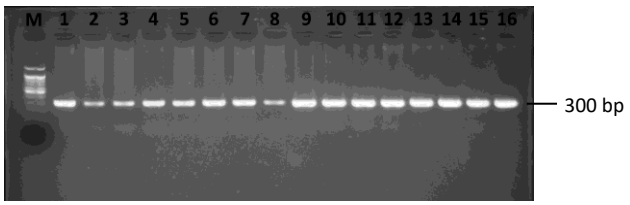

Figure 3. *Mx* gene visualization. M: 100 bp DNA ladder; 1-5: *Kampong*-Laying Type (*Kamper*); 6-10: *Pelung*; 11-16: Broiler Cobb 500.

Figure 1. Phenotypic traits of *Kamper* chicken (Gama Ayam Research Team 2019)

Table 1. Single nucleotide polymorphisms of *Mx* in *Kamper* and *Pelung* chickens

Sample codes	*Mx* polymorphism Exon-13				Haplotype	7-week-old chicken body weight (gram)
	Substitution A20734T	Substitution C20737T	Substitution A20766G	Substitution A20893G		
DQ788616.1	A	C	A	A	Reference*	-
2	T	T	G	G	1	297
3	T	T	G	G	1	488
4	T	T	G	G	1	452
5	A	C	A	A	Reference*	397
6	A	C	A	A	Reference*	336
7	A	C	A	A	Reference*	321
10	A	C	A	A	Reference*	231

Note: *Reference refers to the DQ788616.1 *Mx* GenBank accession number.

Table 2. Correlation of *Mx* polymorphism with the bodyweight of *Kamper* chicken in 7 weeks

Single nucleotide polymorphisms	Substitution A20734T			Substitution C20737T			Substitution A20766G			Substitution A20893G		
Genotype	AA	AT	TT	CC	CT	TT	AA	AG	GG	AA	AG	GG
Genotype frequency	0.25	0	0.75	0.25	0	0.75	0.25	0.75	0	0.25	0	0.75
Average body weight for 7 weeks	397	-	412.3	397		412.3	397	412.3	-	397	-	412.3
Correlation coefficient (r)	0.092*			0.092*			0.092*			0.092*		
Significance	0.908^{ns}			0.908^{ns}			0.908^{ns}			0.908^{ns}		

Note: ns: nonsignificant (p>0.05); *: positively weak correlation (Pearson's correlation)

Discussion

The image analysis of *Mx* exon-13 via Gene Studio revealed that the size of the DNA fragment from each sample (n=16) was 300 bp (Fig. 3). Pagala et al. (2017) reported a quite similar DNA fragment size of the DNA band of *Mx* in *Tolaki* chicken (299 bp). *Mx* exon-13 discovered in White Leghorn and New Hampshire chicken has a similar size of 300 bp (Pagala et al. 2017). Permatasari et al. (2015) stated that *Mx* is a specific genetic marker of disease resistance in avian species, including chicken (*Gallus gallus*). *Mx* is located in chromosome 1 (GenBank accession number: DQ788615) having a fragment length of 20,767 bp and consisting of 13 exons, 1115 bp coding regions, and 705 remaining amino acids (Permatasari et al. 2015). *Mx* has been linked to chicken immunity levels against viral diseases, such as AI and ND (Ko et al. 2002; Ko et al. 2004; Pagala et al. 2013; Pagala et al. 2017). *Mx* and the large GTPase protein it encodes are among the most studied interferon-stimulated antiviral effector molecules (Verhelst et al. 2013; Fulton et al. 2014). Their identity and names are based on their ability to inhibit the replication of viruses, specifically influenza virus (Verhelst et al. 2013; Fulton et al. 2014).

The frequency of A/G alleles in chickens can be a genetic indicator of the level of chicken resistance to virus attacks (Ko et al. 2002). Sulandari et al. (2007) stated that the A/G allele distribution in the native chicken population (*Pelung*, *Sentul*, *Kedu*, *Kedu Hitam*, *Kedu Putih*, *Cemani*, *Wareng*, *Merawang*, *Gaok*, *Kate*, *Kapas*, *Arab Gold*, and *Arab Silver*) in Indonesia can be identified with an *Mx* genetic marker. The results of this research showed that nucleotide polymorphisms in *Mx* exon-13 were as follows: 1) G allele (GG genotype) is susceptible to AI virus; 2) A allele (AA genotype) is resistant to AI virus; 3) AG genotype can be resistant or susceptible to AI virus (Sulandari et al. 2007). Four *Mx* exon-13 SNPs were identified as follows: A20734T, C20737T, A20766G, and A20893G (Table 1). Pagala and Ulupi (2014) found a similar SNP A20766G substitution. *Mx* polymorphism at 20766 of the nucleotide site was identified as Single-Nucleotide Polymorphism (SNP) with the substitution of *GT* to *AT* (A20766G). Substitution mutation alters the translation of serine (*AGT*) into asparagine (*AAT*). Asparagine (*A*) in *Mx* exon-13 indicated chicken viral resistance known as Mx^+, whereas serine (*G*) translation indicated chicken susceptibility to viral infections known as Mx^- (Maeda 2005). Three *Kamper* chickens had the *AGT* genotype (Mx^-), whereas one *Kamper* chicken had the *AAT* genotype (Mx^+). This finding demonstrated that *Kamper* chicken had an acquired immunity against viral infection through selective breeding by using *Pelung*, which has been known as one of the native chicken breeds expressing *Mx* in Indonesia.

Table 2 indicates the correlation of *Mx* polymorphisms with bodyweight was not significant, and a positive weak correlation was observed in four SNPs sites of A20734T, C20737T, A20766G, and A20893G. *Mx* SNPs had a nonsignificant effect on the growth and bodyweight performance of *Kamper* chicken. Fulton et al. (2014) reported the correlation between *Mx* SNP and several performance traits, including the egg productivity (*MxCDS122*) and rate of lay (*MxCDS122*, *MxCDS351*, *MxCDS694*, and MxCDS62), but *Mx* SNPs were not correlated with the bodyweight of chickens. Livant et al.

(2007) found that *Mx1* exon-13 SNPs are significantly associated with the mortality rate and leg defects in broiler breeder chickens.

High body weight selection in broiler chickens and turkeys has resulted in a negative correlation in immune performance. The broilers selected for a high growth rate show lower antibody responses when they are challenged with sheep erythrocytes than those of a low BW line and a randomly bred control line (Knap and Bishop 2008). *Kamper* chicken has an inherited disease resistance allele of *Pelung* and grouped as a slow-growth broiler. The body weight gain and feed conversion of local chickens with AA and AG types are significantly higher than those of chickens with the GG type. This finding was consistent with several previous studies, which showed that genotypes containing A alleles are positively associated with the weight of 40-day-old chickens in a poorly hygienic environment. These results supported earlier assumptions that a strong link exists between the character of disease resistance and the ability of livestock to produce.

Chicken productivity depends on environmental conditions and disease resistance adaptation. Livestock that has a good fitness level and can resist infections tends to display a better production performance (Knap and Bishop 2008). However, Pearson's correlation revealed that the polymorphism point (A20766G) related to the weight of 7-week-old chickens was not significant and positively very weak. A decisive conclusion on the correlation between *MxA20766G* SNP and chicken body weight must be validated using a large population size. Genotyping results indicated that three *Kamper* chickens were susceptible to AI virus with AG genotype (Mx^{-}), and one *Kamper* chicken was resistant because of the AA genotype expression (Mx^{+}). MAS could be used as a precise and highly efficient tool in selective breeding. *Kamper* chicken generation could be selected based on these results to further improve this breed through continuous selective breeding.

ACKNOWLEDGEMENTS

In 2018, this research was funded by the STRANAS Grant (No. 1734/UN1/DITLIT/DIT-LIT/2018) from the Ministry of Research, Technology and Higher Education, Republic of Indonesia. In 2019, this research was further supported financially by the Applied Research Grant (Penelitian Terapan/PT No. 2830/UN1.DITLIT/DIT-LIT/LT/2019) from the Ministry of Research, Technology and Higher Education, Republic of Indonesia. The authors acknowledge the Gama Ayam Research Team, Gadjah Mada University, Yogyakarta, Indonesia, for helping maintain day-old-chick (DOC) and for conducting molecular analysis at the Laboratory of Genetics and Breeding.

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