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Genetic Polymorphisms of The Chicken Antiviral Mx Gene in A Variety of Indonesian Indigenous Chicken Breeds

Sri Sulandari[1]✉, Moch Syamsul Arifin Zein[1], Dwi Astuti[1], Tike Sartika[2]

[1]Research Center for Biology, the Indonesian Institute of Sciences (LIPI), Widyasatwaloka Building, Cibinong Science Center, Jalan Raya Jakarta Bogor KM. 46, Cibinong 16911, E-mail: ssulanda@yahoo.co.id , Phone 021-8765056, Fax 021 8765068
[2] Indonesian Research Institute for Animal Production, PO Box 221, Bogor

ABSTRACT

It has previously been demonstrated that a G/A Single Nucleotide Polymorphism (SNP) at nucleotide position 1,892 of coding sequence of chicken Mx gene confers susceptibility/resistance to avian viral diseases. The aim of this study was to assess the geographical distribution of G/A alleles in relation to different genetic backgrounds of a wide range of chicken populations. Using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) methods, 492 samples from 15 breeds of indigenous chicken populations from Java, Sumatera, Kalimantan and Sulawesi islands were genotyped. Allele and genotype frequencies of each population were calculated. Deviations from Hardy-Weinberg equilibrium were tested and inbreeding coefficient F_{IS} estimated. Overall, the susceptible allele G had a frequency of 37.27% while the resistant allele A had a corresponding frequency of 62.73%. No clear relation of the geographical distribution of the G/A alleles to genetic backgrounds was found. The distribution of this SNP across populations seems to be affected by genetic drift rather than selection.

Key words: Mx gene, allele frequency, genetic background, geographical distribution

INTRODUCTION

Indonesian native chicken apparently have species physical characteristic are grouped into at least 31 breeds or distinct groups of local chicken namely: *Kampung*, *Pelung*, *Sentul*, *Wareng*, *Lamda*, *Ciparage*, *Banten*, *Nagrak*, *Walik*, *Siem*, *Kedu*, *Kedu Putih*, *Cemani*, *Sedayu*, *Olagan*, *Nusa Penida*, *Merawang*, *Sumatera*, *Balenggek*, *Melayu*, *Nunukan*, *Tolaki*, *Maleo*, *Jepun*, *Ayunai*, *Tukung*, *Bangkok*, *Bekisar*, *Cukir/Alas*, and *Kasintu* (Nataamijaya, 2000). Most native chicken in Indonesia are raised under extensive traditional system where they are free to scavenge around farmers house during the day. The keepers spend almost no input for raising the chickens mainly because of ability to feed all kind of sources available on the ground such as kitchen waste, insects, worms, grasses and vegetables. Previous study (Sulandari *et al*., 2006 and 2007) reported that global patterns of mitochondrial DNA variations of Indonesian indigenous chicken breeds support Indonesia as one of the major centers of chicken domestication. However, in recent times, Indonesia has experienced heavy mortalities in its poultry flocks due to recurrent outbreaks of Avian Inluenza (AI). Naturally, chicken has the ability to resist the virus attack. Each chicken's ability to resist the virus differs from one chicken to another, and the anti-viral gene control it.

Mx protein are key components of the innate antiviral state induced in many species such as human, mouse and chicken among other organisms (Haller *et al*., 2007) and belongs to the dynamin superfamily of GTPases (Praefcke and McMahon, 2004). After the 1918 influenza pandemic, several studies have been carried out in human and mouse, and these have shown the Mx gene to be a candidate gene for resistance to influenza due to SNPs that are present in the gene (Haller and Kochs, 2002). Ko *et al*., (2002) carried out studies of SNPs present in the Mx gene and also *in vitro* cell line association studies towards natural resistance to avian influenza in commercial populations as well as indigenous populations in Japan. Results from this study indicate that 50% of the chicken populations have natural resistance against viral diseases of the

orthomyxoviridae family, including avian influenza virus (AIV). This resistance is yet to be confirmed *in vivo* in AIV challenged chickens.

Chickens are natural host for the influenza virus (Easterday, 1975), which replicates fulminantly in the bird, causing fatal disease. The chicken Mx gene (Accession no. Z23168) is predominantly present in cytoplasmic and consists of 705 amino acids encoded by 13 exons. A G/A polymorphism at nucleotide position 1,892 in the 13th exon of coding sequence (Livant *et al*., 2007; previously referred to as nucleotide position 2,032 of Mx cDNA sequence by Ko *et al*., 2002) of the Mx gene was demonstrated to influence resistance against vesicular somatitis virus (VSV) and avian influenza virus (AIV) (Ko *et al*., 2004; Watanabe, 2007). The allele A encodes asparagine at the 631st amino acid of the chicken Mx protein and is supposed to be resistant against both recombinant VSV and AIV on cell cultures. The susceptible allele G encodes serine at this amino acid position. Livant *et al.,* (2007) found that the A allele is favorably associated with body weight at 40 days of age in low hygiene environment and higher infectious bursal disease (IBD) antibody titers in high hygiene environment but unfavorably associated with leg defects in line Z, and unfavorably associated with a higher early mortality in both low and high hygiene environments in line X of commercial broilers, indicating a trade off and a line specific effect of this SNP.

The distribution of the alleles A and G in chicken populations has received limited attention. Ko *et al*., (2002) investigated the nucleotide and amino acid variations in a total of 24 individuals of six Japanese and one Egyptian native breeds (1-2 birds per breed or strain) and four commercial breeds including Australop, Black Minorca, Rhode Island Red and three lines of White Leghorn (1-3 birds per breed or strain). Seyama *et al*., (2006) detected the allele frequencies of the A/G SNP in eight commercial breeds of White Leghorn (ten strains), Barred Plymouth Rock, White Plymouth Rock (four strains), Rhode Island Red (six strains), Light Sussex, Black Minorca, Australorp and White Cornish (3-10 birds per breed or strain), seven Japanese (including two strains of each Shamo and Nagoya breeds), one Egyptian and one South American native breeds (3-6 birds per breed or strain), four red jungle fowls from Laos and Indonesia, four green jungle fowls from Laos and one grey jungle fowl from Indonesia. Li *et al*., (2006) assessed the distribution of the alleles A and G in a large number of sample sizes of fifteen indigenous chicken populations from China (32-72 birds per breed), four commercial breeds of White Plymouth Rock, White Leghorn, Rhode Island Red and Dwarf White Plymouth Rock (64 birds per breed) and one population of 32 red jungle fowls. Balkissoon *et al*., (2007) analyzed the G/A SNP in a number of commercial all-purpose breeds, layer and broiler lines and ancestral stocks.

In Indonesia, although issues concerning host genetic responses to AI infections have not been adequately addressed, in this study an attempt was made to characterize Indonesian 15-breeds of indigenous chickens to evaluate their genetic background and explore potential differences in genetic resistance/susceptibility to AI viruses. The objective of this study was to assess the distribution pattern of the alleles G and A in 15 breeds of Indonesian indigenous chicken populations undergone different domestication processes and selection pressures from wide geographic areas in Indonesia. If these polymorphism of the Mx gene could be detected in domestic animals, it would be possible to produce breeds that show resistance to infectious diseases.

RESEARCH METHODS

Chicken Populations

A total of 492 samples from 15 breeds of indigenous Indonesian chickens were used in this study (Table 1).

DNA Extraction

Venous blood from chickens was collected and preserved in 96% absolute alcohol. Genomic DNA was extracted from whole blood using the phenol-chloroform method (Sambrook and Russell , 2001).

Genotyping of Mx gene

PCR-RFLP method was employed to genotype the G/A SNP at nucleotide position 1,892 in the 13th exon of coding sequence of the Mx gene using PCR-RFLP mismatched primers. The Hpa I enzyme (Fermentas) was used with a recognition sequence of 5' GTT|AAC 3' or 3' CAA|TTG 5' to cut the fragment at the position of interest when there is an allele G. The mismatch primer sequences (Ommeh *et al*.,

Table 1. Blood sample collection of indonesian indigenous chicken breeds

No	Chicken Breeds	ID Name	Site Collection	Total Samples
1	Cemani	CMP	BPTU Ayam, Sembawa, South Sumatera	2
		CM	Kedu, Temanggung, Central Java	35
2	Kapas	KPS	Kedu, Temanggung, Central Java	30
3	Pelung	PL	BPTU Ayam, Sembawa, South Sumatera	20
		PLC	Cianjur, West Java	30
4	Arab Golden	ARG	Kedu, Temanggung, Central Java	30
5	Merawang	MR	BPTU Ayam, Sembawa, South Sumatera	30
6	Arab Silver	ARS	BPTU Ayam, Sembawa, South Sumatera	30
7	Kedu	KD	Kedu, Temanggung, Central Java	30
		KDH	Kedu, Temanggung, Central Java	15
8	Kedu Putih	KDP	Kedu, Temanggung, Central Java	18
		KDPJ	Jatiwangi, West Java	9
9	Kate	KT	Yogyakarta, DIY	32
10	Gaok	GA	Bangkalan, Madura Island	10
11	Sentul	STJ	Jatiwangi, West Java	31
		STC	Ciamis, West Java	17
12	Wareng	T	Tangerang, Banten	17
13	Tolaki	KTO	Konawe, Southeast Sulawesi	21
14	Kalosi	KAL	Gowa, South Sulawesi	30
15	Nunukan	NT	Tarakan, East Kalimantan	30
		NN	Nunukan, East Kalimantan	13
		NS	Sebatik, East Kalimantan	12
TOTAL SAMPLES				492

2009) which amplify approximate 101 bp long fragment (due to the poly Ts) were as follows: the forward primer: 5' GAG TAC CTT CAG CCT GTT TT 3' and the reverse primer: 5' ATC TGA TTG CTC AGG CGT TAA 3'. The reverse primer produced a mismatch at the 3' end by introducing an A nucleotide at its second last position. As a consequence, a recognition site for the Hpa I is resulted in the presence of a nucleotide G. The polymorphic G/A site is located at position 80 bp (based on nine poly Ts) of the amplified fragment. The enzyme cuts at position 82 bp when an allele G is present. The mismatch RFLPs yielded one visible fragment of either 101 bp for allele A without a recognition site or 82 bp for allele G (Figure 1).

PCR was done using an initial denaturation at 92°C for 5 min, followed by 30 cycles at 92°C for 30 s, 56°C for 30 s and 72°C for 30 s, and completed by a final extension at 72°C for 1 min. PCR products were analysed by electrophoresis through 2% agarose gel in 1XTAE buffer, and stained with ethidium bromide. Amplicons were cleaved with the restriction enzyme the *Hpa I* (1 U/μg) for 6-8 hours at 37°C following the manufacturer's instructions. The digested fragments were analyzed on a netic us g a high hygiene environments environment iene environment and and leg defects in another line of commercial broile12% polyacrylamide gel for 12 h at 160 V. The gel was stained with silver nitrate (Sulandari and Zein, 2003) and scanned for an image using Adobe Photoshop.

Statistical Analysis

Allele frequencies were calculated per breed population, within the fifteen (15) breeds described above, and over all populations from allele counts using FSTAT (Goudet, 2001). F_{IS} statistics (Cockerham and Weir, 1984) per population were also calculated using FSTAT (Goudet, 2001). Genotype frequencies were obtained by counts, and was performed to test departure of observed from expected genotypes using Popgen32 version 1.32 (Yeh, 2004).

RESULTS AND DISCUSSION

Since chickens are one group of birds, their erythrocytes contain nuclei. Therefore, 492 samples of chicken genomic DNA belonging 15 breeds (Table 1) were extracted successfully from a little quantity of blood. With mismatch primer sequences (See material and method), not all the

extracted DNAs were amplified successfully. Seven out of the 492 samples (5 samples from Pelung, Kedu, Kedu Putih, Merawang, and Wareng, and 2 samples from Cemani) were failed to be amplified. The genomic DNA of 485 amplicons were successfully amplified. A simple method for identification of resistant and sensitive chicken Mx genes was established by developing PCR-Restriction Fragment Length Polymorphisms (RFLP) with genomic DNA.

Four hundreds and eighty five (485) indigenous chickens from 15 breeds were examined by mismatch PCR-RFLP (Table 2). The PCR product was cleaved with the restriction enzyme of the Hpa I and the digested showing one band with 101 bp in length (A/A, homozygous resistant Mx allelic genes); two bands with 101bp and 82 bp in length (A/G, heterozygous Mx allelic genes); and one band with 82bp in length (G/G, homozygous sensitive Mx allelic gene). An example of genotyping results is presented in Figure 1 and the distribution of resistant and sensitive Mx genes in 15 breeds of Indonesian indigenous chickens is presented in the Table 2.

This is the first study in Indonesia that analyzes this A/G SNP of the Mx genes in such a wide range of Indonesian indigenous chicken populations from different islands (Java, Kalimantan, Sulawesi and Sumatera) and breeding strategies. For selective breeding of strains with a resistant Mx gene, a PCR-RFLP technique was used for rapid determination of whether chickens carry the resistant or sensitive allelic gene used in this study. It is shown in Table 3, polymorphisms of the Mx gene (which putatively associated with AI resistance/ susceptibility in chickens), indicates that the frequency of a putative resistant allele (A nucleotide of G/A polymorphism at 1,892 bp of coding sequence) varied from 0.32 in Kapas (the lowest frequency) to 0.87 in Cemani chickens (the highest frequency). These values are within the upper range of those observed in a worldwide survey of around 100 commercial and indigenous chicken populations. As resulted in the examination of 485 samples from 15 breeds of indigenous chicken by a specific PCR-RFLP technique showed that the averaged frequency of resistant allele (A allele) was 62.73% and that of sensitive allele (G allele) was 37.27%. A representative Kedu Putih, Arab Gold, Sentul, Kate, Kedu, Pelung, Gaok, Kalosi, Tolaki, Merawang, and Cemani, tended to have a higher frequency of the resistant allele (0.58, 0.62, 0.63, 0.66, 0.68, 0.69, 0.70, 0.70, 0.74, 0.81, and 0.87, respectively) while Kapas, Wareng, Nunukan and Arab Silver had a higher frequency of the sensitive allele (0.32, 0.44, 0.45, and 0.47, respectively; Table 3). Investigation of distribution of the alleles A and G on chickens has also been reported by other researchers (Ko *et al*., 2002; Li *et al*., 2006; Seyama *et al*., 2006 and Balkissoon *et al*., 2007).

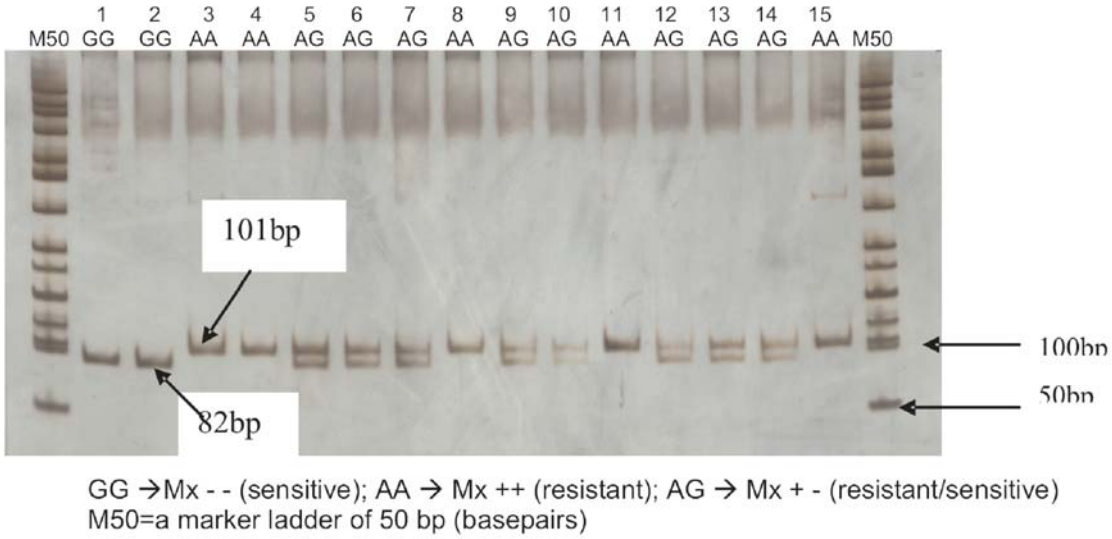

Figure 1. PCR-RFLPs of Mx gene using mismatched primers for the determination of chicken resistant and sensitive Mx genes.

Table 2. Distribution of resistant and sensitive Mx genes in various breeds of chicken

NO.	POPULATION GROUP OF CHICKENS	PCR-RFLP°			TOTAL
		AA	AG	GG	
1	Sentul	19	21	7	47
2	Pelung	23	22	4	49
3	Kedu	23	14	7	44
4	Gaok	4	6	0	10
5	Kedu Putih	7	16	3	26
6	Arab Silver	8	12	10	30
7	Arab Golden	11	15	4	30
8	Cemani	27	7	1	35
9	Merawang	20	7	2	29
10	Wareng	5	5	7	17
11	Kalosi	17	8	5	30
12	Tolaki	11	9	1	21
13	Nunukan	13	23	19	55
14	Kate	14	14	4	32
15	Kapas	3	17	10	30
TOTAL		205	196	84	485
(%)		42.3%	40.4%	17.3%	100%

° Identification was performed by PCR-RFLP. 'A' indicates the resistant Mx allelic genes, and 'G' indicates the sensitive allelic gene.

Those results can be explained that the antiviral activity phenotype depended on the amino acid difference at position 631; that is, the genotype coding Asn (AAT) at position corresponds to be positive antiviral phenotype and the genotype coding Ser (AGT) corresponds to the negative phenotype. The positive and negative antiviral activities of chicken Mx protein have been reported to be associated with not only VSV but also influenza virus (Ko *et al*., 2002). Thus, a single amino acid substitution of the chicken Mx protein was concluded to influence the antiviral potential. Ko *et al*. (2004) reported further, cell lines expressing chicken Mx protein with asparagines (Asn) at position 631 had higher antiviral activity than those with serine (Ser) and stated that the serine 631 allele as the susceptibility allele and the asparagines allele as the resistance allele.

All Mx proteins are known to have leucine repeats at the carboxy (C) terminus, named the leucine zipper, which are thought to be responsible for molecular oligomerization (Melen *et al*., 1992). Amino acid position 631 of the chicken Mx protein is very close to the region of the leucine zipper motif at the C terminus, indicating that this region is important for antiviral specificity. Artificial mutants with a single amino acid substitution in a region near the leucine zipper motif of human MxA and mouse Mx1 have been demonstrated to cause different antiviral activities from those of their original Mx proteins (Zurcher *et al*., 1992a, b). Ko *et al*. (2004) confirmed that the antiviral specificity of chicken Mx protein is determined by an amino acid substitution at carboxy terminus. Furthermore, Mx protein induced by type I IFN is known to inhibit the multiplication of various viruses, including influenza virus (Lee and Vidal, 2002).

It remains a question whether the allele with positive antiviral response also has the activity in vivo or not. If the answer is positive, then the high frequency of the favorable Mx allele could help native breeds better resist an outbreak of avian influenza. In existing disease-control strategies, selection of resistant animals is very effective. It would be great value for the poultry industry and also for public health to develop chicken breeds equipped with Mx protein resistant to RNA viruses. Further analysis of the chicken Mx gene should be performed in *in-vivo* experiments using influenza virus (H5N1) of Indonesian strain on Indonesian chicken populations to determine whether resistant or sensitive character of chicken Mx gene. In contrary, a recent result reported by Sironi. *et al*. (2008), indicated that the Mx genotype did not affect the clinical status or the time course of infection after viral inoculation (the virus

Table 3. Observed and expected (in bracket) genotype frequencies and allele A frequencies (*P*(A)) of the G/A SNP at nucleotide position 1,892 of coding sequence of the Mx gene 398 in different groups of Indonesian indigenous chicken breeds

NO.	POPULATION GROUP OF CHICKENS	CATEGORY	N	GENOTYPE FREQUENCY (%) AA	AG	GG	*P*(A)	*F*is
1	Ayam Sentul	Indigenous	46	40.4 (39.1)	44.7 (47.8)	14.9 (13.1)	0.6522	0.087
2	Ayam Pelung	Indigenous	49	46.9 (47.9)	45.0 (42.9)	8.2 (9.2)	0.6939	-0.047
3	Ayam Kedu	Indigenous	44	52.3 (46.2)	31.8 (43.9)	15.9 (9.9)	0.6818	0.277
4	Ayam Gaok	Indigenous	10	40 (47.9)	60.0 (44.2)	0.0 (7.9)	0.7000	-0.385
5	Ayam Kedu Putih	Indigenous	26	26.9 (32.8)	61.5 (49.8)	11.5 (17.4)	0.5769	-0.242
6	Ayam Arab Silver	Indigenous	30	26.7 (20.0)	40.0 (50.6)	33.3 (28.0)	0.4667	0.213
7	Ayam Arab Golden	Indigenous	30	36.7 (37.6)	50.0 (48.1)	13.3 (14.3)	0.6167	-0.041
8	Ayam Cemani	Indigenous	35	77.1 (75.8)	20.0 (22.7)	2.9 (1.5)	0.8714	0.128
9	Ayam Merawang	Indigenous	29	71.4 (65.4)	24.1 (31.3)	6.9 (3.3)	0.8103	0.231
10	Ayam Warene	Indigenous	17	29.4 (18.7)	29.4 (50.8)	41.1 (30.5)	0.4412	0.429
11	Ayam Kalosi	Indigenous	30	56.7 (43.9)	26.7 (42.7)	16.7 (13.4)	0.7000	0.380
12	Ayam Tolaki	Indigenous	21	52.4 (54.0)	42.9 (39.6)	4.8 (6.4)	0.7381	-0.088
13	Ayam Nunukan	Indigenous	55	23.6 (19.6)	41.8 (49.9)	34.5 (30.5)	0.4455	0.163
14	Ayam Kate	Fancy	32	43.75 (42.7)	43.8 (45.8)	12.5 (11.5)	0.6562	0.046
15	Ayam Kapas	Fancy	30	10 (14.3)	56.7 (48.1)	33.3 (37.6)	0.3833	-0.182

Note: P(A)=frequency resistant allele

strain used was H7N1 HPAI A/chicken/Italy/13474/99).

Discussing on *F*is values as presented in Table 3, *F*is is known as the Wright's inbreeding coefficient and it is a measure of heterozygosity deficiency or excess. It is calculated as the difference between the observed and expected heterozygosity divided by the expected heterozygosity. Negative *F*is values indicate a heterozygosity excess which would translate to outbreeding while positive *F*is values would indicate a heterozygosity deficiency or inbreeding within population (Tevfik, 2006). *F*is values would imply that in the indigenous populations this is as a result of a heterozygote deficiency or population subdivision possibly due to natural selection.

Considering the great effects of the avian influenza outbreaks on worldwide economy and public health, the chicken Mx gene should be a focus for future research in the context of both pathogen and disease resistance of avian influenza. Therefore *in vitro* and *in vivo* studies on Mx gene need to be conducted to test for associations, and it is recommended to continue the same research on examination polymorphism present at Mx gene in a larger sample and more population size, in *Kampung* chickens (collected from outbreak and un-infected area), commercial chickens (broiler and layer) and jungle fowls.

CONCLUSION

The averaged frequency of resistant allele on Indonesian indigenous chicken breeds are high.

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