



Original Article

## Genotype of Javanese backyard waterfowls based on antiviral myxovirus gene

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### Abstract

Myxovirus (Mx) proteins are essential antiviral protein components induced by interferon in many species. This study aims to determine the genotype of 72 Javanese backyard waterfowls based on the Mx gene. Mx gene was amplified using PCR-RFLP technique by primers NE-F2/F and NE-R2/R. The expected 100bp PCR product was visualized by 1.2% high-resolution MetaPhor™ Agarose. To determine the genotype of Mx gene, the PCR fragment was cut by *RsaI*. The results showed that all samples generated 73bp and 27bp fragments, indicating that all waterfowl species were Mx<sup>+</sup> (GG genotype) and showed susceptibility to the avian influenza virus infection, which is difficult to be identified based on waterfowl morphology since they do not show any symptoms of illness. Therefore, Mx gene genotyping has the potential for waterfowl breed selection to obtain healthy and resistant waterfowls. Therefore, targeting the Mx gene is a potential approach for development of avian influenza-resistant poultry.

**Keywords:** domestic waterfowl, genotyping, Mx gene, PCR-RFLP

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### 1. Introduction

According to World Health Organization (2014), data on the highly pathogenic avian influenza (HPAI) incidence and mortality reported 386 deaths in 650 cases worldwide. Since August 2003, the infection of HPAI virus H5N1 subtype in Indonesia was recorded as the highest number of cases and deaths in the world. In Indonesia, the outbreaks of avian influenza were associated with poor management of poultry and waterfowl farms. All findings related to avian influenza virus infection in waterfowls raises a presumption that domestic waterfowl has a unique immune system. For instance, waterfowls are potential hosts of avian influenza virus H5N1 subtype (Susanti *et al.*, 2007, 2008a).

Even when virus infections were proven molecularly and biologically pathogenic in waterfowls, they did not show any paralytic symptoms or death (Susanti *et al.*, 2008b). Susanti *et al.* (2008c) reported that waterfowls can be hosts in the avian influenza virus evolution while the specificity of the receptor (avian  $\alpha$ -2,3NeuAcGal) remained typical. A study by Susanti (2016) indicated that healthy waterfowls were susceptible as a reservoir for avian influenza virus H5N1 subtype. These findings were in line with the evidence that Anseriformes and Charadriiformes waterfowl species are reservoirs for the Eurasian main natural influenza viruses (Fouchier *et al.*, 2007; Olsen *et al.*, 2006; Webster *et al.*, 2007). The potency of waterfowls as the reservoir of avian influenza virus H5N1 is related to the limitation of its immune system due to IgYFc dominance (Feare & Yasue, 2006; Lundqvist *et al.*, 2006). Exclusively, waterfowls own a mechanism to control adverse effects on the environment (Pape *et al.*, 1998; Kapezynski & Pantin-Jackwood, 2007).

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The capacity of waterfowls as reservoirs of avian influenza virus of H5N1 subtype without showing any paralysis and death symptoms is regulated by myxovirus (Mx) genes. The Mx gene is a member of the guanine-3 phosphokinase gene family while its expression is induced by interferons (Haller *et al.*, 2007). In 1980, the Mx gene was first identified in birds, and it is involved in defence mechanisms against influenza virus infection (Livant *et al.*, 2007). At the cellular level, Mx genes are expressed into many Mx proteins which contribute to resistance of waterfowls to different diseases. Single base mutations at the Mx gene can trigger the resistance potency in the animal. In chickens, the Mx gene is located on chromosome 1, and the resistance level of avian influenza virus presents in exon 13, at codon 631 that codes asparagine (AAC/AAU) or the Mx<sup>++</sup> gene. Resistance to infection promotes an active immune system to produce anti-viral proteins and neutralize the virus. If a point mutation (the transition from adenine to guanine) occurs at nucleotide number 1892 with changes in codon 631, it will alter the amino acid production from asparagine into serine (AGC/AGU) (Li *et al.*, 2007). This condition promotes the polymorphism of the Mx gene causing the susceptibility of the host to be infected by a deadly virus and death of the host (Li *et al.*, 2004, 2007). The available data on the Mx gene of infected animals demonstrated that chickens have a weak level of resistance to avian influenza virus, whereas high resistance was found in domestic waterfowls (Susanti *et al.*, 2007). This was in accordance with previous data which revealed that in waterfowls the virus was present in equilibrium amounts and the waterfowls showed no clinical symptoms (Hulse-Post *et al.*, 2005; Lipatov *et al.*, 2004; Liu, 2007; Sturm-Ramirez *et al.*, 2005; Webster *et al.*, 2007). The immune system could not destroy the avian influenza virus in waterfowls. Therefore, it is assumed that the Mx gene regulates the resistance mechanism by the immune system. According to these findings, it seems promising that molecular methods could identify the polymorphism of the Mx gene.

Furthermore, high losses in stock farming arising from avian influenza virus infection require urgent prevention through high biosecurity techniques and vaccination. However, a prevention method is hard to apply at local farms that implement conventional farming. Therefore, building a breeding center to raise healthy poultry and waterfowl breeds is highly important. Genetic resistance profiling using molecular techniques is potentially an easy way for an assay. The Mx gene may be the target for genetic breeding or development of avian influenza-resistant breeds or both. Therefore, determination of the Mx gene and its diversity in chickens and ducks available in the country is significant. However, there is only a limited number of published reports on the study of the Mx gene in Indonesia. The objective of this study is to identify the genotype of Javanese backyard waterfowl species based on the Mx gene as a molecular marker of its resistance to avian influenza virus. The results of this study are expected to be used together as a part of material development in immunology study and to be compiled with the results of waterfowl immune system research to formulate comprehensive prevention and control models. Moreover, highly accurate and rapid selection using resistant gene methods will be applied to increase the selected healthy and highly resistant breeds.

## 2. Materials and Methods

### 2.1 Materials

Blood samples were collected from domesticated backyard ducks, Muscovy duck, and geese in Semarang, Brebes, and Magelang in the Central Java area of Indonesia. Various chemical reagents for genomic DNA extraction were purchased from Merck (Germany), Vivantis (Malaysia), and Roche (Switzerland). Agarose used for DNA quantification and PCR-RFLP analysis were purchased from Merck (Germany) and MetaPhor™ Fisher Scientific (USA). Primer pairs for PCR reaction were obtained from AlphaDNA (Canada). PCR reagents and restriction enzyme *RsaI* were obtained from Thermo Fisher Scientific (USA).

### 2.2 Sampling method

Backyard waterfowls were taken from local husbandries in three different locations in Central Java, Indonesia (Table 1). These places are considered the most productive waterfowl breeding and farming areas in Indonesia. There are many local husbandries for waterfowls in the backyards of residents. Egg-layer, broilers, and backyard chickens were also collected for comparative data. Blood samples were collected from the brachial veins of waterfowls and were preserved in 96% ethanol.

Table 1. Waterfowl and chicken samples from local husbandries in Central Java.

Area	Waterfowl Species			Number of samples
	Duck	Muscovy	Goose	
Semarang	10	8	5	23
Brebes	13	9	6	28
Magelang	9	7	5	21
Total	32	24	16	72
Area	Chicken Species			Number of samples
	Laying hens	Broilers	Indigenous chicken	
Semarang	4	4	6	14
Brebes	3	3	7	13
Magelang	3	3	6	12
Total	10	10	19	39

### 2.3 Genomic DNA extraction and Mx gene amplification

Genomic DNA was extracted from whole blood using the salting-out method (Gaaib *et al.*, 2011). DNA purification was performed using the NucleoSpin Blood kit (Macherey-Nagel, Germany). The DNA quality and quantity were measured using 1% agarose gel electrophoresis and spectrophotometry analysis, respectively. The PCR-RFLP method was used to analyse the genotype of nucleotide position 1892 in exon 13 of Mx gene coding sequence.

The primer sequences that amplified approximately 100bp fragments were the forward primer NE-F2/F (5'CC TTCAGCCTGTTTTCTCCTTTTAGGAA3') and reverse primer NE-R2/R (5'CAGAGGAATCTGATTGCTCAGGCG TGTA3') or reverse primer NE-R2/S (5'CAGAGGAATCT GATTGCTCAGGCGAATA3') (Seyama *et al.*, 2006). The PCR mix was comprised of 0.1  $\mu$ M forward primer, 0.1  $\mu$ M reverse primer, 50 ngDNA template, PCR master mix, and ultrapure water. The established following PCR condition was initial denaturation at 94 °C for 5 min, followed by 35 cycles of 60 sec at 94 °C, annealing temperature for 60 sec at 60 °C, and 72 °C for 60 sec, and the final extension at 72 °C for 5 min. (PeqSTAR thermocycler, Peqlab, GmbH, Germany). The PCR product was analysed by electrophoresis on 2% agarose gel in 1x TBE buffer and stained with EtBr.

### 2.4 PCR-RFLP for genotype identification

The restriction enzyme *RsaI* with a recognition sequence of 5'GT↓AC3' was employed to cut the fragment where an allele G was located in the coding sequence (Ko *et al.*, 2002; Maeda, 2007). For the purpose of identifying the genotype of the *Mx* gene, 10  $\mu$ L amplicons were cut by *RsaI* restriction enzyme (1 U/ $\mu$ g DNA) for 6–8 hours at 37 °C following the manufacturer's instructions. The digested fragments were visualised by 3% superfine resolution MetaPhor™ agarose gel in 1x TBE buffer (Sartika *et al.*, 2010).

### 2.5 Data analysis

The results of PCR-RFLP were analyzed according to the clue of avian influenza virus resistance found in amino acid 631 at exon 13, which was triggered by a mutation of transition base (single/point mutation) GC into AT. The mutation caused transformation of serine to asparagine that resulted in the resistance of waterfowls to avian influenza virus ( $Mx^{++}$ ), whereas serine amino acid represented the susceptibility of waterfowls to avian influenza virus ( $Mx^{-}$ ). A/A genotype with 100 bp product of enzyme restriction showed a homozygous resistant  $Mx^{++}$  allelic gene, whereas two bands with 100 bp and 73 bp in length showed A/G heterozygous  $Mx^{+}$  allelic gene and one band with 73 bp showed G/G homozygous sensitive  $Mx^{-}$  allelic gene (Jahangir *et al.*, 2015; Sartika *et al.*, 2010).

## 3. Results and Discussion

### 3.1 DNA extraction

The salting-out DNA extraction method followed by purification was adequate to obtain good quality and sufficient quantity of genomic DNA from the blood samples. The results of gel analysis showed clear bands of DNA which indicated that the DNA was suitable for PCR amplification (Figures 1A and 1B). The salting-out method is a useful, simple, cheap, and easy DNA extraction technique for blood source (Gaaib *et al.*, 2011).

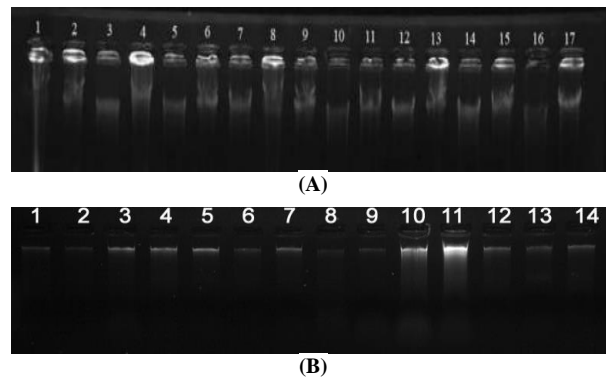


Figure 1. Genomic DNA of waterfowls (A) and chicken (B) extracted from blood samples using salting out method in 1% agarose gel.

### 3.2 *Mx* gene genotype identification

The genomic DNAs of 72 waterfowls were successfully amplified with 100bp products (Figure 2A). In addition, the results of chicken DNA amplification showed 100bp products in all chicken species (Figure 2B). The products of amplification were in line with previous studies (Elfidasari *et al.*, 2013; Maeda, 2005; Sartika *et al.*, 2010; Seyama *et al.*, 2006; Sulandari *et al.*, 2009). All findings showed that 100bp DNA fragment could trace the *Mx* gene location. Further analysis of polymorphism of the *Mx* gene was performed by cleaving the *Mx* gene PCR product using restriction enzyme (Sartika *et al.*, 2010; Sironi *et al.*, 2010).

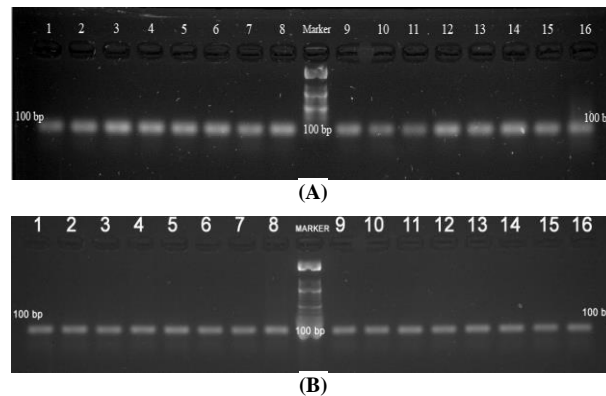


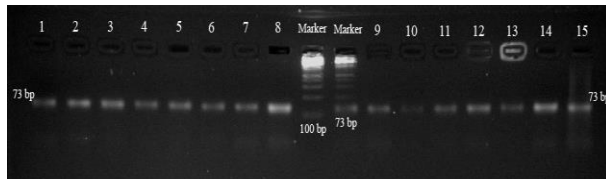
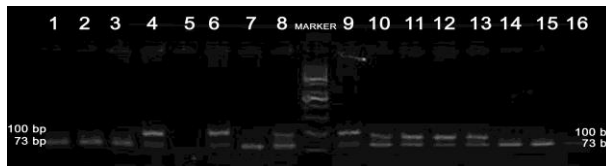
Figure 2. PCR amplicon of *Mx* gene on waterfowl (A) and chicken (B) DNA samples shows 100 bp in length product in 2% agarose gel.

The PCR products of 72 waterfowl samples and 39 chicken samples were digested with *RsaI* restriction enzyme and a summary of the sample genotypes is shown in Table 2. All RFLP products of waterfowl samples showed one band with 73bp length (G/G homozygous sensitive *Mx* allelic genes). Results from the 72 samples by specific PCR-RFLP

Table 2. Waterfowl and chicken genotypes based on RFLP results.

Species	Genotypes			Unknown
	GG (homo- zygote sensitive)	AG (hetero- zygote)	AA (homo- zygote resistance)	
Duck	32	-	-	-
Muscovy	24	-	-	-
Goose	16	-	-	-
Broiler	10	-	-	-
Laying hens	1	8	-	1
Indigenous chicken	13	6	-	-

indicated a monomorphic at the Mx gene, which is associated with the avian influenza virus susceptibility in waterfowls (Figure 3). Various results were found in the digestion products of various chicken samples. All samples of broilers produced 73bp DNA bands (GG genotype/sensitive) (Figure 4). Meanwhile, egg-layer hens suggested that 8 of them belonged to the AG genotype that resulted in 100bp and 73bp DNA band, and the other two belonged to the GG genotype (73 bp) and an unknown genotype (unable to digest). The backyard chicken samples indicated GG genotype and AG genotype in 13 and 6 samples, respectively.

Figure 3. PCR-RFLP products of indigenous waterfowls Mx genes cleaved by *RsaI* in 3% MetaPhor™ agarose analysis.Figure 4. PCR-RFLP products of chicken Mx genes cleaved by *RsaI* in 3% MetaPhor™ agarose analysis. Sample no 1-3: broilers, 4-8: laying hens, 9-16: indigenous chickens.

Genotyping is important to reveal the molecular identity of a species. It is crucial as the morphological characteristics sometimes do not represent molecular expressions. Previous research by Sartika *et al.* (2010) showed that local chickens from 12 Asian countries generally have Mx<sup>++</sup> and Mx<sup>-</sup> genes. In accordance with the differences in chicken genotypes, the phenotypes of the chicken population also differed with regard to resistance to avian influenza virus, i.e.

in terms of sensitivity and resistance. Studies on local chicken genotyping in many countries, including Indonesia, reveal that these chicken species express a higher Mx<sup>++</sup> gene frequency (63%) than Mx<sup>-</sup> (37%). A study conducted by Sulandari *et al.* (2009) using the PCR-RFLP method on 485 chickens from 15 locations in Indonesia showed that Ayam Cemani (black chicken) is the most resistant among other chicken species.

In this study, the genotypes of all waterfowl samples were Mx<sup>-</sup> which represented the sensitivity of their bodies to avian influenza virus infection. In fact, asparagine in amino acid 631 represented animal resistance to avian influenza virus, whereas serine was related to the susceptibility to the virus. It indicated that PCR-RFLP could be applied to detect S613N mutation (Elfidasari *et al.*, 2013; Sartika *et al.*, 2010; Sironi *et al.*, 2010). Moreover, non-synonymous G/A polymorphism in Mx gene position 2032 could lead to the Mx protein transformation at amino acid 631 from serine to asparagine. One-point mutation S631N was reported to have a relation with antiviral capacity in coping with avian influenza virus in vitro (Ko *et al.*, 2006).

Based on a study by Li *et al.* (2004), most of the wild-type waterfowls possessed Mx protein with serine (AGC/AGU) at its 631 position. The study of Elfidasari *et al.* (2013) also revealed that the GG genotype in the Mx gene dominated all Muscovy duck species in Cagar Alam Pulau Dua National Park. Uniquely, all waterfowls with this kind of genotype showed healthy conditions without any sickness, paralysis or death symptoms. It showed that all waterfowls had the distinctive regulation in its body. In general, chickens with Mx<sup>-</sup> genes were vulnerable to avian influenza virus attack (Seyama *et al.*, 2006). The Mx gene in waterfowls indicates different characteristics of the Mx protein, which does not express the antiviral response when the virus infects the body.

Recent research by Zeng *et al.* (2016) identified goose Mx (goMx) mRNA that shared highly conserved domains. In contrast with the amino acid position of chicken Mx protein, the goMx protein was serine (Ser) at position 629 aa. Based on the assay of transcription, it was revealed that goMx was mainly expressed in the immune, respiratory, and digestive systems. Further study on the effects of avian influenza virus on goose suggested that the virus affected the goMx expression, and significant changes were also observed in the trachea, lung, and small intestine. These findings indicated that goMx could change the amino acids at selected sites and express it in a different manner showing its broad antiviral activity.

### 3.3 Mechanism of waterfowls as the virus reservoir

According to the study results, Mx protein in waterfowls did not show any antiviral activity even when there was a chance of viral infection. In fact, the ultimate factors that affected the resistance mechanisms and disease sensitivity were the host and virus. As mentioned above, waterfowls are potentially natural reservoirs of the avian influenza virus. In the body of waterfowls, the avian influenza virus stayed in a steady-state condition without showing any

clinical symptoms with efficient viral replications (Hulse-Post *et al.*, 2005; Lipatov *et al.*, 2004; Liu, 2007; Sturm-Ramirez *et al.*, 2005; Webster *et al.*, 2007). Since the virus replicates itself in the gastrointestinal tract of waterfowls, transmission to other birds or mammals occurs via fecal-oral transmission due to virus shedding with the feces (Sturm-Ramirez *et al.*, 2005; Webster *et al.*, 2007). Waterfowl-rich intestinal receptor  $\alpha$ -2, 3NeuAcGal was a predilection organ of influenza virus replication (Glaser *et al.*, 2005; Kobasa *et al.*, 2001; Suzuki *et al.*, 2000; Vines *et al.*, 1998).

Characterization of the Mx gene in waterfowls is the most crucial stage to determine the role of the gene in terms of resistance and susceptibility to viral infection mechanisms. The Mx gene could be used as a viral ecology primary determiner in the environment. Regulation of the Mx gene in waterfowls is unique because the Mx protein is inactive and it is not involved in viral infection defence mechanisms. There is no antiviral response when the virus enters the body of a waterfowl. All findings that showed GG genotype of domestic waterfowls confirmed the unique immune system in waterfowls. It was found that chickens with GG genotype had small antiviral activities and had a high susceptibility to avian influenza virus of H5N1 subtype in the form of innate immune response in comparison with waterfowls (Dillon & Runstadler, 2011; Ewald *et al.*, 2011; Li *et al.*, 2006).

Moreover, the innate immune system of the waterfowl Mx protein is different from the chicken Mx protein, which also plays a major but different role in invading the avian influenza virus of H5N1 subtype (Dillon & Runstadler, 2011; Lee & Vidal, 2002). Feare and Yasue (2006) stated that the potency of waterfowls as reservoirs of the avian influenza virus of H5N1 subtype and Newcastle disease was related to the natural limitation of its immune system to eliminate the viral invasion. This limitation is caused by the dominance of IgY $\Delta$ Fc (Bando & Higgins, 1996; Humprey *et al.*, 2004), the inability of MHC-I to generate peptides (Moon *et al.*, 2005), and the sensitivity of waterfowls to mucosal pathogen caused by the late-response of IgA (Magor *et al.*, 1998). However, waterfowls could survive in the wet environment due to the limitation by controlling the adverse effects of the environmental pathogens (Pape *et al.*, 1998). The innate immunity response of waterfowls adequately protects them from infection. High expression of cytokines IFN $\alpha$ , IL2, and IL4 in waterfowls infected by avian influenza virus H5N1 was directly elaborated in the resistance ability of the virus (Kapezynski & Pantin-Jackwood, 2007).

The Mx protein accumulates predominantly in the cell nucleus. A significant portion of Mx protein was also found in the cytoplasm where it formed large granules (Bazzigher *et al.*, 1993). However, not all Mx proteins had an antiviral function. No antiviral function was detected in human Mx2 (Pavlovic *et al.*, 1993) and rat Mx3 (Meier *et al.*, 1990). It was found that some waterfowls, especially ducks, developed an innate immune response to the influenza virus. It was characterized by the expression of Mx, a type-I interferon (IFN)-induced gene transcript in the enterocytes, which are the main target cells of low pathogenic avian influenza viruses in vivo. Interestingly, Mx transcript levels were proportional to the viral load in the ileum. It suggested that a type-I IFN-mediated immune response in the enterocytes and the activation of IFN-c-secreting cells contributed to the influenza virus replication control in the duck intestine (Volmer *et al.*,

2011). Viruses, in turn, have evolved multiple strategies to escape from the IFN system. They tried to go undetected, suppressed IFN synthesis, bound and neutralised the secreted IFN molecules, blocked IFN signalling, or inhibited the action of IFN-induced antiviral proteins (Haller *et al.*, 2007).

### 3.4 Role of waterfowls as the source of avian influenza virus H5N1 transmission

The prevalence of avian influenza virus of H5N1 subtype in waterfowl species correlates with the breeding and farming systems. Ducks in a backyard cage demonstrated the highest prevalence of avian influenza virus of H5N1 subtype (47%), in comparison with ducks that were raised in rice fields (45.9%) and ducks that were kept in the open cages (23.5%) (Songserm *et al.*, 2006). A farming system with a low bio-security level was less likely to prevent the prevalence of avian influenza virus infection in Asia, including Indonesia (World Health Organization [WHO], 2014). The transmission of avian influenza virus of H5N1 subtype to other animals has the potential to be delivered by waterfowl and is the potential source of transmission. Avian influenza H5N1 virus isolated from healthy ducks in southern China was identified as biologically and molecularly pathogenic. When the virus was transferred to chickens and mice, some sickness and symptoms of paralysis were found in those animals. It was assumed that the hemagglutinin gene was involved in the transmission of the virus from avian to mammals (Chen *et al.*, 2006). Besides, waterfowls are the mixing vessels that enable gene reassortment among co-circulating viruses; thus, it can bring out new varieties of virus subtypes (Li *et al.*, 2007). Reassortment of viral genes was demonstrated by genotyping of avian influenza virus of H5N1 subtype isolated from healthy waterfowls in southern China. The virus was the result of hemagglutinin gene re-assortment between A/Gs/Gd/1/96 with Eurasian avian influenza virus that formed 9 different genotypes (Chen *et al.*, 2006). Further, G genotype of avian influenza virus of H5N1 subtypes isolated from duck meat in Vietnam (Dk/VNM/568/05) was re-assorted among avian influenza virus Z genotype with PB2 gene from another avian influenza virus (Smith *et al.*, 2006). An avian influenza virus outbreak in Hong Kong in 2001 originated from duck and goose reservoirs that re-assorted with other avian influenza viruses (Sturm-Ramirez *et al.*, 2005). Newly re-assorted genes have created a new pathogenic virus that can infect other animals and even humans. Various genotypes of avian influenza and Newcastle disease virus isolated from domestic waterfowls were involved in long-term endemicity of the virus in East Asia and South-East Asia (Liu, 2007).

### 4. Conclusions

According to the findings in this study, it can be concluded that the Mx<sup>-</sup> allele dominated the Indonesian backyard waterfowls, especially in Central Java Province. The frequency of this allele was relatively high, which demonstrated that waterfowls were resistant to avian influenza virus. Moreover, the PCR-RFLP technique is effective as a breed selection method. Furthermore, an in-depth study and analysis of the waterfowl immune system uniqueness are necessary to reveal what will happen in the body of waterfowls which can be a virus reservoir. In addition, the Mx gene, which can be

used for methods of waterfowl breed selection to obtain healthy and resistant waterfowls, is a promising approach to produce avian influenza-resistant poultry.

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