The polymorphism analysis of gene resistance to avian influenza virus (*Mx* gene) in KU-Phuphan black-bone chicken

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ABSTRACT

The *Mx* gene is an antiviral gene used to determine resistance or susceptibility to different types of viruses, including the Avian Influenza virus. Three populations of KU–Phuphan black–bone chickens (KP), crossbreed broiler (BR) and layer (LA) chicken were examined resistant and sensitive genotypes of the *Mx* gene using PCR–RFLP. DNA amplification results showed that the *Mx* gene was found in a 100 bp fragment. The *Mx* resistant genotype AA was found in both KP and BR, but not the LA population. The frequencies of allele A were 0.319, 0.443, and 0.550 for KP, BR and LA populations, respectively. The frequencies of allele G were 0.681, 0.557 and 0.450 for KP, BR and LA populations, respectively. While KP was in the Hardy-Weinberg equilibrium, BR and LA were not (P<0.01). Sequence analysis of homozygous resistant and sensitive genotype confirmed the single nucleotide substitution (SNP) causes a single amino acid substitution from Serine (Ser) to Asparagine (Asn) at position 631. It is important for the poultry industry to protect their birds from pathogenic infection, which correlated to antiviral performance. Therefore, the polymorphism in the *Mx* gene is important for the KP population to protect their chickens from pathogenic infection. Further studies are needed with regard to the genetic resistance to avian influenza in different populations with various domestication background and selection history.

Keywords: antiviral gene; *Mx* gene; PCR–RFLP; SNP

INTRODUCTION

Living organisms have ability to defend themselves against attacks from microorganisms. The self-defense mechanism is capable of causing them to be resistant or sensitive to invasive microorganisms. There are many genes encoding the natural resistance proteins and interferons (IFN) such as the major histocompatibility complex genes and gene resistant to viruses, which can produce a variety of board spectrum antiviral proteins (Schusser *et al*., 2011).

The *Myxo* virus resistant gene (*Mx* gene) exists in many species, from yeast to vertebrates (Ko *et al*., 2002). The *Mx* gene produced the MX protein against the avian influenza virus AIV (Li *et al*., 2006). The MX protein is an interferon-induced guanosine triphosphate enzyme and predominantly presents in the cytoplasm, and also showed an extraordinary high degree of resistance against infection with influenza A viruses (Haller *et al*., 2007). In chickens, the *Mx* gene is located on chromosome 1 and contains 14 exons. The translational initiation codon is in the second exon (Schumacher *et al*., 1994). Bernasconi *et al*. (1995) reported the first study of *Mx* gene in chicken that they got the full-length chicken *Mx* gene from the IFN induced Leghorn fibroblast by RT-PCR. The resistance against the AIV was found in exon 13 where it undergoes alkaline transition mutation (Ko *et al*., 2002). The chicken *Mx* alleles are highly polymorphic with a single-nucleotide substitution (Seyama *et al*., 2006). The natural variation of chicken *Mx* gene was found only at the amino acid position 631 which changes the MX antiviral protein activity. The antiviral MX protein possesses activity only when a single nucleotide substitution G2032A and the 631 amino acid changes from serine to asparagines (S613N) (Ko *et al*., 2002; Watanabe, 2007; Elfidasari *et al*., 2013). The presence of the amino acid asparagine indicates that chickens conferred resistance to AIV including subtype H5N1, whereas the mutation that changes the amino acid to serine results in AIV susceptibility (Li *et al*., 2006). In chickens, the effect of the *Mx* gene on antiviral activity is unclear, which is likely due to variable experimental setups and different AIV strains (Abdelwhab and Hafez, 2012). Sironi *et al*. (2008) found no association between the *Mx* genotype and the survival of chicken experimentally infected *in vivo*

with a highly pathogenic H7N1 avian influenza virus. In addition, Benfield *et al*. (2008) showed chicken containing the Asn631 allele of *Mx* gene is unable to inhibit *in vitro* replication of 5 influenza H5N1 strains. On the contrary, many reports showed the benefit of the *Mx* gene. Davison *et al*. (2008) found that a single nucleotide substitution of the *Mx* gene showed genetic variation among the Iowa State University chicken lines, with some lines having the resistant allele while other lines had the susceptible allele. Yin *et al*. (2010) found that tissues and chicken embryo fibroblast (CEF) cells with different genotypes (A/A or G/G) expressed different expression levels of *Mx* and that CEF cells with different genotypes had different antiviral activities. Ewald *et al*. (2011) conduced an *in vivo* study using commercial meat-type (broiler) chicks and suggested that those with the Asn631 allele were more resistant to viral challenge than those with the Ser631 allele, and Wang *et al*. (2012) found the *Mx* heterozygote chickens had significantly greater *Mx* mRNA expression with AIV infection than non-infected birds. However, to elucidate the role of *Mx* gene in the resistance of poultry to AIV more in-depth investigations using several chicken breeds from different country are highly required (Ewald *et al*., 2011).

Studies of the *Mx* gene have been reported in variety breeds of Asian chickens such as Chinese native chickens, Japanese native chickens, Indonesian native chickens, Red jungle fowl, and commercial chickens with two alleles of the *Mx* gene. Surveys of various chickens have reported that the native breeds have a higher frequency of the resistant allele than commercial production chickens (Li *et al*., 2006; Seyama *et al*., 2006; Sartika *et al*., 2011). In 2006, Li *et al*. detected allele frequencies of the *Mx* mutation in 15 Chinese native chicken breeds, 4 highly selected commercial lines, and the Red Jungle Fowl. They found that allele frequencies of the Red Jungle Fowl were between those of native breeds and commercial populations. The possible explanation for the distinct differences of the advantageous allele frequencies between native chickens and commercial populations are positive selection on this position in the chicken genome. In addition, Yin *et al*. (2010) revealed that the local chicken breeds have adapted to the local environment gradually and survived long-term natural and artificial selection resulted in an abundant resource for genetic improvement, more candidate genes for diseases control. The frequency of the resistance allele (A) was higher than the frequency of the susceptible allele (G), white leghorns and broilers (Wang *et al*., 2012) which developed the *Mx1* gene and study the association between the *Mx1* genotypes and low pathogenesis AIV infection both *in ovo* and *in vivo* in the chicken (Balkissoon *et al*., 2007; Livant *et al*., 2007).

In Thailand, the demand of black-bone chicken has increased. The health benefits provided by the black-bone chicken is to have carnosine antioxidant properties with protect the body against inflammation (Tian *et al*., 2007). KU–Phuphan black–bone chicken (KP) was developed from the local Mongolia black chicken the Advanced Studies for Livestock and Poultry Production Research Unit of Kasetsart University, Chalermphrakiat Sakonnakhon Province Campus, Thailand. At present, KU–Phuphan black–bone chickens were in the second generation containing 27 males and 45 females which used as the parent stock. They were selected to improve meat and egg to use them as an alternative commercial chicken. Recently, live weight and carcass traits of black-bone chickens were studied by Bausap *et al*. (2017), they found that KU–Phuphan black–bone chicken showed better growth than other meat-black chickens and also their carcasses were improved, thus, the KP chickens could be produced for market value. However, the weak correlations between production traits and the resistance of disease has been reported (Zekarias *et al*., 2002; Livant *et al*., 2007; Sartika *et al*., 2011; Molee *et al*., 2016). Chickens with high productivity are susceptible to disease. Therefore, the correlation of disease-resistance traits and production traits should be considered. However, study regarding disease resistant trait especially for virus–borne diseases have to be under the supervision of virologist and its required the special tools, but our work could not proceed because we lack of virologist and tools for virology study. Due to this limitation, thus, the genetic marker is suitable in our study to detect the polymorphism of gene resistant to virus. The aim of this study was to assess the allele frequencies and genotypes frequencies of the *Mx* gene in the KU-Phuphan black-bone chickens parent stock compared with crossbreed broiler and layer breeds which represented for native and commercial breed, respectively. The polymorphism of the *Mx* gene detected in the selected KU-Phuphan black-bone chickens, especially for resistant allele, would be beneficial to evaluate the efficient of our selective breeding program.

MATERIALS AND METHODS

Chickens and samples collection

A total of 137 blood samples were collected from three groups of chickens (*Gallus domesticus*). Of these 72 samples were obtained from KU–Phuphan black–bone chickens (KP) parent stocks, and 35 samples from crossbreed native broiler (BR) and 30 samples from commercial layer chicken (LA). Blood samples (0.3 μL from each chicken) were collected from the wing veins in syringes containing100 μL EDTA (0.5 M, pH 8.0), then transferred to the laboratory for genomic DNA extraction. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University (ID: ACKU60-ETC-007).

Genomic DNA extraction

Genomic DNA was obtained from 0.1 μL of whole blood using Genomic DNA isolation kit (Invitrogen, USA). The quality of genomic DNA was determined using 1% agarose gel and the DNA concentration were calculated using 100-bp ladder (Vivantis, Malaysia).

Polymerase chain reaction (PCR) amplification of the chicken *Mx* gene

The reactions were assembled in a 25μl reaction volume containing: 100 ng genomic DNA, 0.2μM of each primer, 1x buffer (including 2mM $MgCl_2$), 100μM dNTPs (RBC Bioscience, Taiwan) and 1 unit of *Taq* DNA polymerase (RBC Bioscience, Taiwan). PCR amplification was performed using mismatched primers described in Seyama *et al*. (2006). The PCR primers of the *Mx* gene were: forward primer NE–F2 (5′CCTTCAGCCTGTTTTT CTCCTTTTAGGAA3′, intron 13) and mismatched reverse primer NE–R2/R (5′CAGAGGAATCTGATT GCTCAGGCGTGTA3′, 2060–2033) or reverse primer NE–R2/S (5′CAGAGGAATCTGATTGCTCAGGCG AATA3′, 2060–2033). The cycling protocol was 5 min at 94ºC, 35 cycles of denaturing at 94ºC for 60 sec, annealing at 60ºC for 60 sec, extending at 72ºC for 60 sec, with a final extension at 72ºC for 5 min. The amplified products were displayed in 2% agarose gel.

Restriction with *Rsa*I and *Ssp*I

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method was used to genotype the G/A SNP as described by Sartika *et al*. (2011). The pools amplicons containing 1 μg PCR product obtained from the NE–F2 and NE–R2/R primers were digested with *Rsa*I (1U/μg) restriction enzyme (New England Biolab, USA) for 1 hour at 37°C following the manufacture's instruction to completely digest. *Rsa*I has a recognition sequence of 5′ GT↓AC 3′, which cleaves the G allele when using primers NE–F2 and NE–R2/R. In addition, the PCR products obtained using the NE–F2 and NE–R2/S primers were digested with *Ssp*I (1U/μg) (New England Biolab). *Ssp*I has a recognition sequence of 5′ AAT↓ATT 3′ to cut the fragment at with the A allele. The digested fragments were visualized by 3% agarose gel in constant voltage 100 volt for 70 min followed by ethidium bromide staining. The PCR products digested by *Rsa*I but not *Ssp*I were identified as the G allele, which is considered as the sensitive allele, and those digested by *Ssp*I but not *Rsa*I as the A allele, which considered as the resistant allele. The frequency of resistant allele (A/Mx+ allele) and sensitive allele (G/Mx^{-} allele) were investigated. All samples of homozygous resistant and homozygous sensitive genotype and three samples of heterozygous were sequenced to confirm the single nucleotide substitution G2032A.

Statistical analysis

Estimated genotype frequencies, alleles frequencies, F_{IS} and Hardy–Weinberg equilibrium (HWE), based on the Chi–square (χ^2) test, for all populations were analyzed using Genepop (v 1.2) (Raymond and Rousset, 1995). The inbreeding coefficient (F_{IS}) was performed by FSTAT (v 2.9.3.2) (Goudet, 2002).

RESULTS AND DISCUSSIONS

Mx gene genotyping

Genomic DNA of three chicken populations; KU–Phuphan black–bone chickens (KP), crossbreed native broiler (BR), and commercial layer chicken (LA) was successfully amplified for the chicken *Mx* gene. The PCR products were 100 bp as previously reported in chickens (Luan *et al*., 2010; Sartika *et al*., 2011; Elfidasari *et al*., 2013). To identify the resistant (AA) and sensitive (GG) genotypes, the PCR products were cleaved by restriction endonucleases. The mismatched RFLPs using *Rsa*I yielded one visible fragment of either 100 bp for allele A without a recognition site or 73 bp for the G allele, and/or in the contrary for using *Ssp*I. Complete, partial and no digestion with *Rsa*I indicated sensitive, variable and resistant, to AIV, respectively. The homozygous resistant genotype contained 100/100 bp fragments (AA) for *Rsa*I while contained 73/73 bp fragments for *Ssp*I. Conversely, the homozygous sensitive genotype

contained 73/73 bp fragments (GG) for *Rsa*I while it contained 100/100 bp fragments for *Ssp*I. The heterozygous genotype contained 100/73 bp fragments (AG) for both *Rsa*I and *Ssp*I. Allele distribution and genotyping of *Mx* gene for some samples are shown in Figure 1.

Genotypes of 137 chickens were determined. Of these 8.76% (n = 12) had the homozygous resistant genotype (AA) where as 27.00% (n = 37) had the homozygous sensitive genotype (GG), and the remaining 64.23% (n = 88) had heterozygous genotype (AG). For each population, the homozygous resistant genotype was 6.94% and 20.00% for KP (n = 72) and BR (n = 35), respectively while this genotype was not found in LA (n = 30). In addition, the homozygous sensitive genotype was 43.10%, 8.6% and 10%, in KP, BR and LA, respectively. The heterozygous genotype was 50.00%, 71.43% and 90%, in KP, BR and LA, respectively. This is the first report of *Mx* genotyping in the KP. Although, the association between genotype and antiviral activity was not observed in this study, there are many reports to support the correlation between productivity trait and antiviral activity. Pagala *et al*. (2013) confirmed that *Mx* genotype could be associated with antiviral traits in Tolaki chickens. The AA and AG genotypes are more resistant against virus than the GG genotype. Homozygous sensitive genotype was highly found in KP population, the result suggested that our selective breeding program should be pondered about the selection intensity of production performance possibly to prevent the reduction of the favorable AA genotype. However, the *Mx* sensitive genotype could improve the productivity in chicken. Li *et al*. (2006) confirmed that the productive performance of chickens with the GG genotype was slightly better than that AA and AG genotypes. Contrary, BR population showed the number of chickens containing homozygous resistant genotype more than KP populations whereas this genotype was not found in LA. There are factors that could create important co-evolutionary of host and AIV in poultry (Fourment and Holms, 2015). Chicken host required to accumulate mutations essential to alter host range and adaptive resistant phenotype to viral subtype (Lee *et al*., 2015). Generally, the indigenous chicken breeds have gradually adapted to the local environment and survived long-term selection which they could contain the higher resistant genotype compared to commercial breeds (Zekarias *et al*., 2002; Seyama *et al*., 2006; Berlin *et al*., 2008). For LA population, the selection in commercial breeds is often carried out in a confined, pathogen-free environment to permit maximum expression of the production traits which effected on the change of *Mx* genotypes. Li *et al*. (2006) found that the commercial populations with very low frequencies of the positive antiviral allele were selected for increased growth or higher egg production, which might lead to a correlated reduction in frequencies of some disease-resistance alleles.

Population examination of genetic polymorphism of *Mx* gene

The results from three populations of chickens, KP, BR, and LA identified by specific PCR-RFLP using multiple endonucleases indicated a polymorphism in the *Mx* gene. KU–Phuphan black–bone chicken populations were in the Hardy-Weinberg equilibrium (HWE) at this locus (P=0.27), whereas BR and LA populations were not in the equilibrium ($P<0.01$). Departure from the HWE may occur due to a variety of causes, including purifying selection, inbreeding and population substructure (Dorak, 2014). However, the Wright's inbreeding coefficient (F_{IS}) of all populations were not significant evidence for inbreeding with -0.143, -0.436 and -0.785 for KP, BR and LA, respectively. The F_{IS} is calculated as the difference between the observed and expected heterozygosity divided by the expected heterozygosity (Sulandari *et al*., 2009). 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 (Dorak, 2014). Generally, closed populations and high selection intensity, the genetic drift (allele A) could be the reasons of departure from the HWE (Li *et al*., 2006) which was found in LA population in this study. In addition, BR population might be under the influence of population substructure because they were collected from different areas leading to departure from HWE.

The frequency of the allele A of KP, BR and LA chicken populations were 0.319 (31.9%), 0.557 (55.7%) and 0.450 (45.0%), respectively, while the frequency of the allele G was 0.681 (68.1%), 0.443 (44.3%) and 0.550 (55.0%), respectively. Data was shown in Table 1. Our study found that the frequencies of the A allele in BR population were much higher than those found in LA and KP populations. Luan *et al.* (2010) suggested the frequency distribution of the allele A and the allele G were significantly correlated to geographical location, growth environment and selection pressure. The allele A frequency was lower in chicken raised in better environment contrasted to that in poor environment. The selected three populations were raised under different environments. LA

population was raised under intensive care in evaporative cooling system and KP population was raised under ambient temperature with intensive care, while BR population was raised free-range under ambient temperature. Moreover, the surveys of various native, commercial, and laboratory strains of chickens have reported rates of the allele A ranging from 59.2% to 72.4% and have suggested that the native breeds have a higher frequency of the allele A than commercial production chickens (Li *et al*., 2006; Seyama *et al*., 2006; Sulundari *et al*., 2009; Sartika *et al*., 2011). The frequency of the allele A in native chickens higher than that of all the other populations the reason for this may be the different distribution of the unique geographical location of the one. Under the long-term natural selection, the resistance increased (Luan *et al*., 2010). On the other hand, KP population showed that the frequency of the allele G was higher than those found in other populations. The result indicated that the selective breeding program should be conducted with regarded to prevent the reduction of the A allele. The high frequencies of the allele G could be readily reduced by modern breeding techniques. Nevertheless, there are many reports revealed the benefit of the allele G in chickens. Four highly selected commercial line (White Leghorn, White Plymouth Rock, Rhode Island Red and Dwarf White Plymouth Rock) with improving growth performance revealed the highly frequencies of the allele G ranging from 0.7258 to 0.9435 (Li *et al*., 2006).

Nucleotide substitution in the *Mx* gene

To confirm the resistant and sensitive genotypes, all samples of homozygous resistant and sensitive *Mx* gene and three samples of heterozygous were sequenced. The nucleotide substitution from G to A at position 2032 was detected. The resistant homozygous contained nucleotide A at this position, therefore, *Ssp*I recognized AATAAT could cut at this site, which generated 73 bp fragments, but *Rsa*I was not. Data was shown in Figure 2. Conversely, the sensitive homozygous exhibited nucleotide G at this position. *Rsa*I recognized GTAC could cut at this site, which generated 73 bp fragments, but *Ssp*I was not. Data was shown in Figure 3. The single nucleotide substitution at position 2032 (Ko *et al*., 2002) was responsible for the position 631 (Ser to Asn) variation of the MX protein (Seyama *et al*., 2006; Li *et al*., 2007 and Watanabe, 2007). Genetic resistance to avian influenza has previously been associated with an amino acid changed Ser631Asn of the chicken *Mx* gene and the Asn allele (A allele) confers viral resistance to avian influenza (Ko *et al*., 2002; Seyama *et al*., 2006; Davison *et al*., 2008; Sartika *et al*., 2011). Although, any antiviral activity expressed by the chicken *Mx* gene was more complicated (Pitossi *et al*., 1993) especially given the complex structural interactions and the numbers of polymorphism reported for the chicken *Mx* gene caused by exposure to or infection with virus (Wang *et al*., 2012; Fulton *et al*., 2014), the single amino acid substitution probably influences the antiviral activity of chicken *Mx* gene could be considered to confer positive antiviral response to the *Myxo* virus in chickens.

Figure 1 Allele distribution and genotyping of *Mx* gene by 3% agarose gel electrophoresis of mismatched PCR products digested by *Rsa*I or *Ssp*I. M is 100 bp standard marker, AA is homozygous resistant, AG is heterozygous resistant/sensitive, GG is homozygous sensitive.

Table 1 Genotyped frequencies, allele frequencies, and inbreeding coefficient (F_{IS}) of KU–Phuphan black–bone chickens (KP), crossbreed broiler (BR) and layer breed (LA).

Populations	No.	Genotype frequency			Gene frequency		Chi-square test	*P*-value	F_{IS}
		AA	AG	GG	A	G			
KP	72	0.069	0.500	0.431	0.319	0.681	5.074	0.279	-0.143
BR	35	0.200	0.714	0.086	0.443	0.557	16.509	0.002	-0.436
LA	30	0.000	0.900	0.100	0.550	0.450	46.051	0.000	-0.785

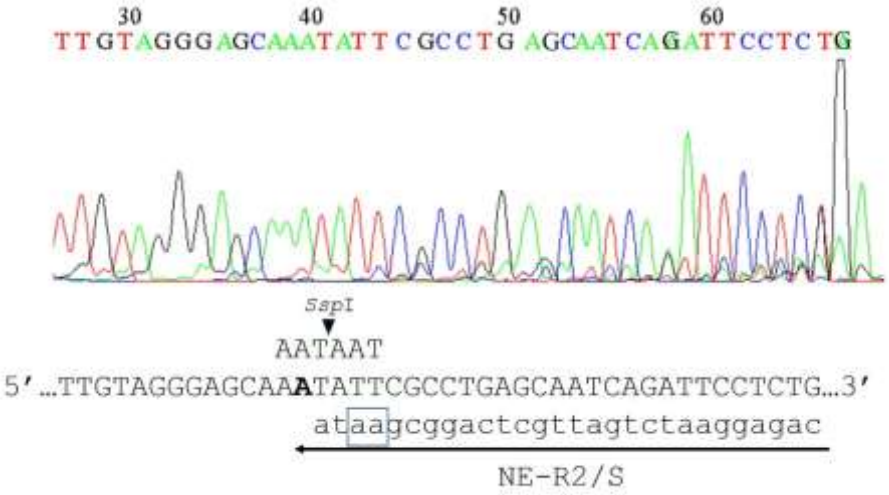

Figure 2 NE-R2/S reverse primer was used for mismatched PCR-RFLP, distinguishing the difference in nucleotide variation at position 2032 marked by bold capital letter. Lowercase letter underline with arrow indicated nucleotides of the mismatched primer. The cleavage site of *Ssp*I endonuclease, which recognize the sequences of AATATT, are indicated by a closed triangle above the sequence. Boxed letter indicated mismatched nucleotide.

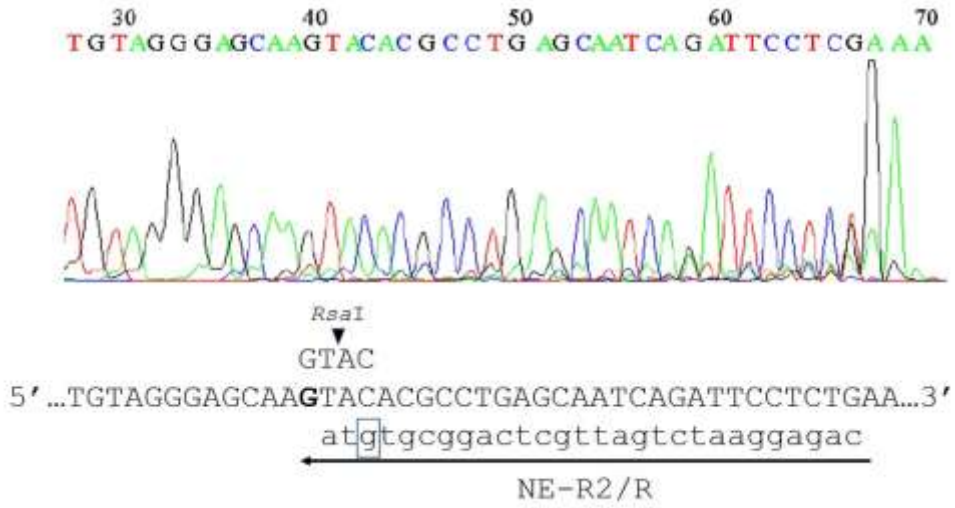

Figure 3 NE-R2/R reverse primer was used for mismatched PCR-RFLP, distinguishing the difference in nucleotide variation at position 2032 marked by bold capital letter. Lowercase letter underline with arrow indicated nucleotides of the mismatched primer. The cleavage site of *Rsa*I endonuclease, which recognize the sequences of GTAC, are indicated by a closed triangle above the sequence. Boxed letter indicated mismatched nucleotide.

Implication for genetic selection

The single nucleotide substitution G2032A showed genetic polymorphism among the three chicken lines. This study provided important data for future genetic assessments of KU–Phuphan black–bone (KP) chickens. In the KP population, the *Mx* gene exhibited polymorphic alleles and thus considered as a valuable population. However, The KP showed highly gene frequency of the *Mx* G allele meanwhile it has been reported that virus resistance trait could negatively affect commercial productivity. Careful evaluation of the allelic effects should be conducted prior to commercial application of the *Mx* genotyping for genetic selection. Moreover, a relative small sample size used in this study might lower the accuracy of the result. It is necessary to detect this polymorphism in larger sample size and more populations with various domestication backgrounds and selection history to reveal the distribution of the *Mx* gene.

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