GENOTYPIC AND ALLELIC FREQUENCY ANALYSIS OF *Mx* GENE IN PHILIPPINE NATIVE AND COMMERCIAL CHICKENS (*Gallus gallus domesticus*)

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ABSTRACT

The study was conducted to analyze the genotypic and allelic frequencies of *Myxovirus* (*Mx*)-resistance gene in Philippine native chickens. DNA was extracted from blood and 101 bp of the *Mx* gene exon 13 was amplified and digested using PCR-RFLP. Digested products were visualized through polyacrylamide gel electrophoresis to determine the genotype of each chicken. Genotyping results showed that Philippine native chickens had higher frequencies of the favorable AA genotype and A allele than GG genotype and G allele, respectively. Similarly, commercial layer, but not broiler chickens, had higher frequencies of AA genotype and A allele. Moreover, the present study confirms the association of *Mx* genotype with genetic group, plumage color, and geographical location. Frequencies were not significantly different between sexes. Patani, Joloanon, and Labuyo genetic groups, black plumage color and native chickens from Mindanao were associated with AA genotype. Commercial broiler was more likely to be associated with GG genotype than AA and AG genotype.

Key Words: disease resistance, genotyping, *Mx* gene, Philippine native chicken, PCR-RFLP

INTRODUCTION

The prevalence of infectious diseases is one of the major issues poultry breeders are struggling to solve and those viral infectious diseases with characteristic rapid spread are the most destructive in the world (Ahmed *et al*., 2007, Gordon *et al*., 1971). Despite the availability of the advancing technological disease monitoring systems and vaccination, disease outbreaks are still a huge problem (Barcelo, 1994). Thus, inclusion of disease resistance in the breeding goal with successive selection of breeds with the resistance trait may reduce the number of outbreaks brought by viral infections (Pagala *et al*., 2013).

Molecular assisted selection (MAS) which uses genetic markers is one of the advanced methods for rapid selection of animals for a desired trait (Sartika *et al*., 2010). One of the candidate genes for disease resistance codes for the *Myxovirus* (*Mx*)-resistance protein. The *Mx* protein is found in many organisms (Aebi *et al*., 1989; Meier *et al*., 1990; Rothman *et al*., 1990; Bazzigher *et al*., 1993) and displays different intracellular localization and antiviral activities. *Mx* protein inhibits viruses of the families Orthomyxoviridae (agents of influenza) and Paramyxoviridae which can cause avian influenza, Newcastle disease and H1N1 disease (Dillon & Runstadler, 2010; Ko *et al*., 2002; Swayne & Halvorson, 2008). Selection of animals with the desired disease resistant genotype for *Mx* can be done to prevent the risk of disease outbreaks in poultry farms in the Philippines.

In the Philippines, there are different genetic groups of native chickens which include Banaba, Bolinao, Boholano, Camarines, Darag, Joloanon, Labuyo, Paraoakan and

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Patani. These chickens are identified in certain geographical locations and distributed in three island groups of the Philippines namely, Luzon, Visayas and Mindanao. They can be characterized by having brown or black plumage color, different from the white commercial layers and broilers. Apart from their distinct taste, they are known to be disease resistant and adapted to different environmental conditions. To our knowledge, there is no study that has genotyped local domesticated chicken breeds/strains for the presence of *Mx* gene, and has evaluated the association of genotypic frequencies with chicken's genetic group, plumage color, geographical location and sex. Association of these factors to disease resistance trait of poultry animals were previously reported (Sagesse *et al.* 2008, Wang *et al.* 2014, Psifidi *et al.* 2016, Molee *et al.* 2016, and Acar *et al.* 2017).

This study generally aims to analyze the genotypic frequencies and allelic frequencies of *Mx* gene in different genetic groups of Philippine native chicken. Specifically, to compare the genotypic and allelic frequencies of *Mx* gene between different genetic groups of native and commercial (broiler and layer) chickens and to determine the association of genotype with genetic group, plumage color, geographical location and sex.

MATERIALS AND METHODS

Purposive sampling was done for the six genetic groups of Philippine native chicken used in the study. A total of one hundred forty-three samples of Paraoakan, Darag, Boholano, Labuyo, Joloanon, Patani and ten samples each for commercial broiler and layers (Table 1) were obtained. Blood was carefully collected from the brachial vein of the wing using 1 cc syringe. Forty µL of fresh blood from a healthy individual were blotted onto FTA cards (Whatman™ FTA™) and were allowed to dry overnight.

At least 20 discs of dried blood in the Whatman™ FTA cards were punched out using Harris™ 1.2 mm micropuncher and washed three times with 200 µl Whatman™ FTA purification reagent and then dried overnight. The two discs from the washed sample

Table 1. List of Philippine native chicken genetic group and commercial chicken strains used in the study.

Genetic Group	Plumage color	Location	Male	Female	Total
Boholano	Brown	Visayas	9	19	28
Darag	Brown	Visayas	12	34	35
Joloanon	Black	Mindanao	11	9	20
Labuyo	Brown	Luzon, Visayas, Mindanao	13	2	15
Paraoakan	Black	Luzon	8	23	31
Patani	Black	Visayas, Mindanao	3	11	14
Layer	White	Luzon	0	10	10
Broiler	White	Luzon	-	-	10

of each individual were placed in the PCR tube and were eluted for ten minutes at 90°C using Applied Biosystems Veriti™ thermocycler after addition of 50 µl sterile nanopure (SNP) water.

PCR-RFLP method was employed to genotype the G/A nucleotide position 1, 892 in the 13th exon of *Mx* using PCR-RFLP mismatched primers published by Ommeh *et al.* (2010) (Forward primer: 5′ GAG TAC CTT CAG CCT GTT TT 3′; Reverse primer: 5′ ATC TGA TTG CTC AGG CGT TAA 3′). The amplicon was 101 bp. The reverse primer produced a mismatch at the 3′ end by introducing an A nucleotide at its second last position. The polymorphic G/A site was located at position 80 bp (based on nine poly Ts) of the amplified fragment. *Hpa*I (5′ GTT| AAC 3′ or 3′ CAA|TTG) cuts at position 82 bp when an allele G is present. The mismatch RFLPs yield one visible fragment of either 101 bp for allele A without a recognition site or 82 bp for allele G (Figure 1).

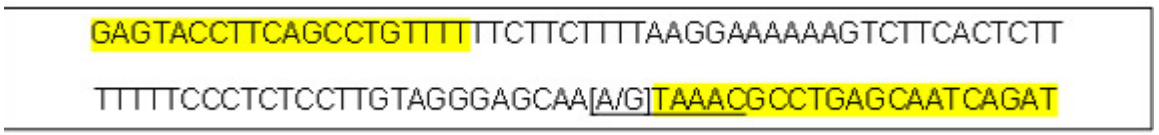

Fig. 1. Nucleotide sequence of the *Mx* gene fragment amplified through PCR. Primers are highlighted and the G/A 2032 polymorphism causing the S631N mutation is in square brackets. The Hpa I restriction site is underlined.

The *Mx* fragment was amplified through polymerase chain reaction (PCR) with the following reaction mixture : 1 µl of genomic DNA, forward and reverse primer at 0.5 µM each, 0.8U *Taq* polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1X buffer A in a final volume of 20 µl. Amplification was done using 94°C of initial denaturation for 5 minutes followed by 30 cycles of 94 °C denaturation for 30 seconds, 52 °C annealing for 30 seconds and 72 °C for 30 seconds, then a single incubation at 72 °C for 1 minute and 30 seconds followed by a hold at 4 °C. PCR was done in Applied Biosystems Veriti™ thermocyclers and amplicons were visualized by electrophoresis.

Aliquots of PCR products were digested by *Hpa*I. Digestion reaction mixture includes 10 µL PCR product, 1 U *Hpa*I restriction enzyme, sterile nanopure water and 10X buffer in a final volume of 25 µl. These were incubated at 37°C for 5 hours and 20 minutes at 65°C afterwards. Digested products were visualized by CBS™ Polyacrylamide Gel Electrophoresis (PAGE) through an Ethidium Bromide stained 12% acrylamide gel in 1X TAE buffer.

Genotyping was done using the photograph results of the de-stained polyacrylamide gel viewed through the Biorad™ Gel Doc XR machine. Observed genotypic and allelic frequencies were then computed. Exact test was carried out using "HardyWeinberg" package (Engels, 2009; Graffelman, 2017) to test for deviation of genotypic frequencies from Hardy-Weinberg proportions. Further, Chi-square test of independence was done to determine the possible association of genetic group, sex, geographical location and plumage color of chicken with *Mx* genotypes. Significant association results from chi-square test of independence were then subjected to correspondence analysis as previously reported (Park *et al.*, 2007) to provide graphical representation (biplot) of cross tabulations

between the said factors and the *Mx* genotypes, respectively. Correspondence analysis was carried out using "FactoMineR" and "devtools" packages. Tests were run at 5% level of confidence in R studio (R Core Team, 2016).

RESULTS AND DISCUSSION

Genotyping of *Mx* gene in one hundred forty-three (143) Philippine native and twenty (20) commercial chickens was done. A 101 bp PCR product was amplified from exon 13 of *Mx* gene and cut using a restriction enzyme. Three banding patterns can be recognized for the three genotypes AA, AG and GG (Figure 2). Previous studies showed that the presence of allele A and genotype AA in *Mx* gene confers viral disease resistance while having allele G and genotype GG increase the chance of disease susceptibility to viral infection (Ko *et al*., 2002; Watanabe, 2003; Benfield *et al*., 2008; Dillon and Runstadler, 2010; Sartika *et al*., 2010; Pagala *et al*., 2013).

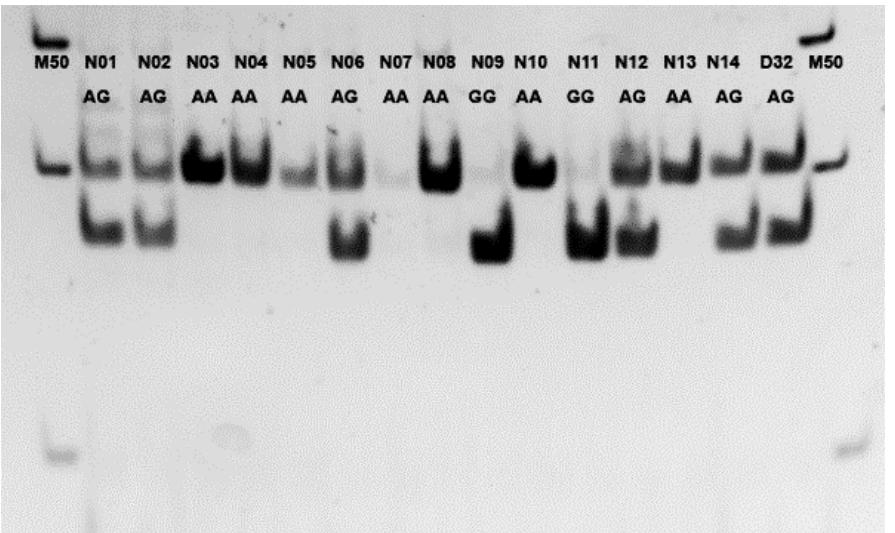

Fig. 2. Genotyping result of *Mx* gene in polyacrylamide gel. AA=*Mx* + resistant; GG= *Mx*- susceptible; AG= *Mx*+/- resistant/susceptible. M50= 50bp DNA ladder.

The results showed that genotypic and allelic frequencies of *Mx* gene in different genetic groups of Philippine native chicken appear to follow a similar trend. Paraoakan, Boholano and Darag had AG as the highest frequency followed by the genotype AA while the genotype GG had the lowest frequency (Table 2). Joloanon and Patani, which are said to be resistant to diseases as testified by small hold farmers in the southern part of the Philippines, and Labuyo had genotype AA as the highest frequency, followed by AG and GG genotypes. All genetic groups of Philippine native chicken had higher frequency of A than G allele.

Exact test reveals that all genetic groups of Philippine Native chickens except Darag follow Hardy-Weinberg proportions (Table 2). Departure of populations from Hardy-Weinberg expected proportions suggests evolutionary forces (i.e., assortative mating,

Table 2. Hardy-Weinberg test for *Mx* gene in six genetic groups of Philippine native chickens and in broiler and layer chickens.

Genetic Group	Observed Genotype			Allele frequency	Expected Genotype			p-value
	AA	AG	GG		AA	AG	GG	
Boholano	0.21	0.64	0.14	A= 0.54 G= 0.46	0.29	0.50	0.22	0.25^{ns}
Darag	0.17	0.71	0.11	A= 0.53 G= 0.47	0.28	0.50	0.22	0.02^{*}
Joloanon	0.55	0.45	0	A= 0.78 G= 0.22	0.60	0.35	0.05	0.53^{ns}
Labuyo	0.53	0.33	0.13	A= 0.70 G= 0.30	0.49	0.42	.09	0.54^{ns}
Paraoakan	0.35	0.58	0.06	A= 0.65 G=0.35	0.42	0.46	0.13	0.24^{ns}
Patani	0.50	0.36	0.14	A= 0.68 G= 0.32	0.46	0.44	0.10	0.55^{ns}
Layer	0.70	0.30	0	A= 0.85 G= 0.15	0.72	0.26	0.02	1.0^{ns}
Broiler	0	0.10	0.90	A= 0.05 G= 0.95	0.0	0.10	0.90	1.0^{ns}

^{ns} Not significant; Follow Hardy-Weinberg proportions; P > 0.05; * Significant; Does not follow Hardy-Weinberg proportions; P<0.05.

selection, migration, etc.) may be operating (Hartl *et al*., 1997). In the case of Darag, this genetic group might be under the influence of current selection or genetic improvement programs (Cabarles, 2013; Contreras *et al*., 2014; Tomambo *et al*., 2016).

Commercial layer and broiler chickens, on the other hand, also follow Hardy-Weinberg proportions (Table 2). In comparison, the frequency of genotype AA is higher in commercial layers than broilers. Moreover, the frequency of GG genotype of broilers is higher than layers. The frequency of the allele A was observed to be higher in layers than the broilers and that of allele G was higher in broilers than the layers.

To assess the association of chicken's *Mx* genotype with the genetic group, chi-square test of independence was done. Results showed significant relationship between chicken's genotype and its genetic group (P<0.001). Correspondence analysis biplot (Figure 3A) graphically shows that Patani, Labuyo, Joloanon genetic groups and commercial layers are more likely to be associated with genotype AA while Boholano, Darag, and Paraoakan are associated with genotype AG. Viral disease resistance of breeds with AA and AG genotypes were previously reported (Ko *et al*. 2002, Watanabe 2000, Benfield *et al*., 2008, Dillon and Runstadler 2010, Sartika *et al*. 2010 and Pagala *et al*., 2013). Although the present study had no parallel experiment to test viral resistance of chickens, the association of genetic groups with the favorable genotype (Figure 3A) is indicative of their genetic potential. Our findings support the testimonies of farmers that native chickens have higher disease resistance than commercial chickens. On the other hand,

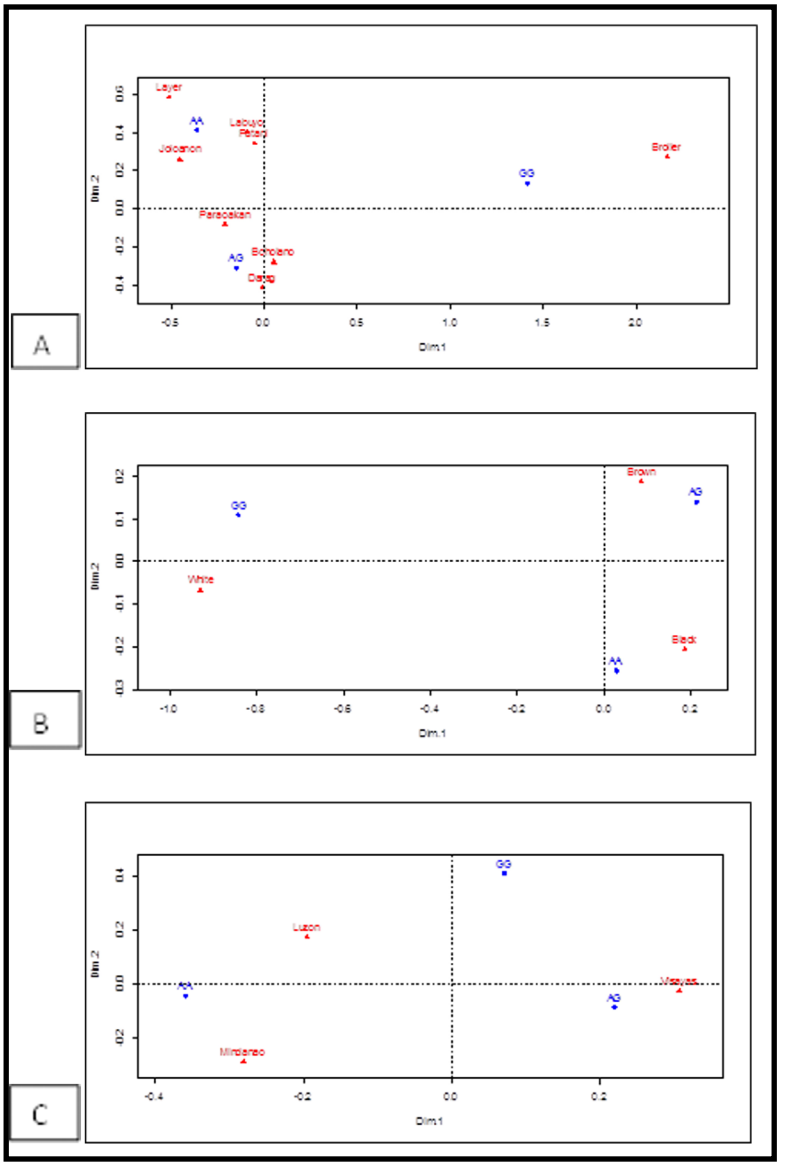

Fig. 3. Correspondence Analysis Biplot between Mx genotype and A) genetic group B) plumage color, and C) geographical location.

commercial broiler chickens are related with genotype GG. In commercial broilers, more emphasis had been placed on its genetic potential for higher production, rather than their acclimatization to odd environments or ability to resist diseases. Studies reveal selection for body weight reduces humoral immune response of broiler chickens (Klasing & Ohnstone, 1991; Shlosberg *et al.*, 1991; Miller, *et al.*, 1992; Rauw, 1998; Mauck, *et al.*, 2005; McKay, 2008). In contrast, layer breeding programs focused on robustness and disease resistance, as reflected in significant improvements in livability and welfare. Furthermore, considerable

attention is also given to egg production, egg's uniform size and color since they have been known to be associated with disease resistance trait (Kuhnlein, 1997; Fulton, 2004; Van der Klein, 2015).

Independence of plumage color with genotypic and allelic frequencies was also evaluated. The study shows that chickens with black plumage had the highest frequency of the genotype AA (0.40) and allele A (0.66) followed by the brown plumage (AA = 0.32, A = 0.61). The frequency of genotype AA (0.35) is relatively high in white commercial chickens; however, frequency of genotype GG (0.45) is higher. In addition, the frequency of allele A of white chickens (0.45) is lower than allele G (0.55). Chi-square test of independence reveals that there is significant relationship between chicken's genotype and its plumage color ($P< 0.001$). Correspondence analysis biplot (Figure 3B) graphically shows that black chickens are more likely to be associated with AA genotype while brown chickens are more associated with AG genotype, and white chickens with GG genotype. It appears that the plumage color of the Philippine native chickens is indicative of the favorable genotype for viral resistance.

Genotypic and allelic frequency analysis also evaluated the possible relationship of chicken's *Mx* genotype with geographical location. Chi-square test of independence reveals that there is significant relationship between chicken's genotype and geographical location ($P<0.05$). It is observed that the frequency of genotype AA and allele A are higher in chicken samples from Mindanao with 0.50 and 0.73 respectively, followed by chicken samples from Luzon with 0.43 and 0.68 respectively. Samples from Visayas had the least frequency of genotype AA (0.20) but had high frequency of genotype AG (0.65). In addition, the results showed that the Mindanao samples had the least frequency of genotype GG (0.03) and allele G (0.27) followed by Luzon samples (GG = 0.07, G = 0.32) while Visayas samples had the highest frequency for the said genotype (GG= 0.14) and allele (G= 0.47). Correspondence analysis biplot graphically shows that chickens coming from Luzon and Mindanao are more likely associated with genotype AA while those coming from Visayas are more likely associated with genotype AG (Figure 3C). This data suggests that native chickens distributed at different geographical locations of the country are more related with A allele than G allele.

Genotypic and allelic frequencies categorized by sex were also evaluated. The genotypic and allelic frequencies of cocks (AA=0.38, AG=0.54, GG=0.09, A=0.64, G= 0.36) and hens (AA=0.32 AG=0.57 GG=0.10, A=0.61, G=0.39) follow a similar trend, where genotype AG has the highest frequency. Also, allele A is more frequent than allele G for both sexes. Chi-square test of independence shows that there was no significant relationship between *Mx* genotype and sex ($P>0.05$).

In summary, genotyping at nucleotide position 1,892 at exon 13 of coding sequence of *Mx* gene showed general trends of genotypic and allelic frequencies in different populations of Philippine native and commercial chickens (broiler and layer). For native chickens, the frequency of the favorable AA genotype was higher than GG. Moreover, allele A of Philippine native chicken is more frequent than allele G. Broiler chickens were observed to have higher frequency of genotype GG than genotype AA while layers were observed to be more associated with genotype AA and allele A. Using the chi-square test of independence, *Mx* genotype was found to be associated with genetic group, plumage

color, and geographical location but not with sex.

While an exact test was done for small sample size, this study may be improved by increasing the number of samples. Performance and viral disease resistance traits in chickens may be analyzed together with molecular data to determine association of *Mx* genotypes with viral disease resistance traits in chickens.

ACKNOWLEDGMENTS

The work described in this paper was done with the support from Department of Agriculture-Bureau of Agricultural Research Scholarship Grant and Bureau of Animal Industry. Consultation with Prof. Consuelo Amor Estrella, Asst. Prof. Medino Gedeun Yebron and Asst. Prof. Jorge Michael Dominguez.

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