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Princelina Bora
Research Scholars, Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

Arindom Bora
Research Scholars, Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

N Shyamsana Singh
Associate Professor and Head, Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

Prava Mayengbam
Associate Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

TC Tolenkhomba
Assistant Professor (SG), Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

Corresponding Author:
TC Tolenkhomba
Assistant Professor (SG), Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

Polymorphism in chicken antiviral Mx protein gene in native 'Zo-ar' chicken of Mizoram, India

Princelina Bora, Arindom Bora, N Shyamsana Singh, Prava Mayengbam and TC Tolenkhomba

Abstract
The Mx protein gene has been found to be associated with antiviral activity in chicken, in many previous researches. The aim of this study has been to assess and uncover the presence of polymorphism within the Mx protein gene in native chicken 'Zo-ar' of Mizoram, a state of India and to observe and analyse the allelic and genotypic frequency in order to screen the variation present in the studied population with respect to the Mx protein gene which is associated with disease resistant traits in poultry and indirectly associated with production and reproduction performance. DNA isolation has been done from blood samples collected 50 randomly chosen 'Zo-ar' chicken irrespective of their age or sex. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique has been used for the detection of Single Nucleotide Polymorphisms within the Mx gene. Three genotypes AA (0.14), AG (0.46) and GG (0.40) were found. The G allele (0.63) was observed to be predominantly present and the observed heterozygosity was found to be 0.46 in the population. Further, the population was found to be conforming to the Hardy-Weinberg Equilibrium with respect to the Mx protein locus. From the present study the prevalence of variation within the Mx locus could seen in the 'Zo-ar' chicken population of Mizoram which further shows possibility of genetic improvement in disease resistance and egg production traits provided proper selection methods and formulating of appropriate mating systems are done.

Keywords: Mx protein gene, 'Zo-ar' chicken, PCR-RFLP, heterozygosity variation, genetic improvement

Introduction
Mx protein is an interferon-induced antiviral mediator which was first identified due to its association with influenza virus resistance. It belongs to the GTPase superfamily [1]. The Mx gene is found in many organism starting from yeast, rat, fishes and also human which is reported by various sources to have association with resistance against viral activity by inhibiting the replication of different viruses inside the host's body. In chicken, the this gene have been demonstrated to cause influence in the resistance mechanism of the body against Vesicular stomatitis virus (VSV) and Avian influenza virus (AIV) by some studies [2, 3] after which many research have performed on this particular gene specially on the allele MxCDS1892 as it is also called as the "resistance allele" due to the polymorphic association of this allele with resistance against diseases [4].

There is a huge demand of meat and eggs from native chicken population among consumers of India in the recent times due to the flavourful meat of local chicken which contains less fat percentage as compared to that of the commercial broilers. Another advantage of native population is its hardiness and capacity of resistance against many diseases. However, it has been known that breeding only for increasing production leads to reduction in the disease resistance capacity of the birds [5] as both the traits are negatively genetically correlated. Thus, if attention is given on finding out information on the genetic constitution with respect to genes related disease resistance, an overall improvement can be achieved which will improve production without compromising on the innate disease resistance capacity.

The local chicken 'Zo-ar' of Mizoram are scavenging in nature, reared in the backyard of most of the village households for both games and table purpose [6] which have superior body weight than that of breeds like Naked neck and Frizzle fowls [7]. However, very little work has been carried out to exploit the genetic potential of these fowls [7, 8]. Genetic selection in order to improve production without compromising fitness which is an inborn advantage of the

native population is difficult to achieve with traditional methods like that of phenotypic selection. Therefore, if attention is given on finding out information on the genetic constitution with respect to genes related to disease resistance without decreasing performance for traits like egg production and body weight gain, an overall improvement can be gained which can cause increase in economic importance of the fowls.

Therefore, the principle objective of this study has been concentrated on detecting of polymorphism within the Mx protein gene in the local chicken 'Zo-ar' of Mizoram along with the observation of genetic variation present within the population and distribution pattern of the different genotypes along with the available amount of heterozygosity has been done which will help in determining the possibility of genetic improvement in the population regarding egg production and egg numbers.

Materials and Methods

Chickens and Blood samples

Blood samples (1 mL) were collected from 50 unrrelated, randomly chosen 'Zo-ar' chicken of Mizoram, India. The chickens were taken from backyard flocks reared in different districts of the state (Kolasib, Mamit and Aizawl) by the villagers. The samples were collected aseptically from the wing vein in EDTA vials and kept in ice pack immediately. Thereafter, the blood samples were brought directly to the laboratory and stored at -20˚C until further use.

Genomic DNA isolation, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Isolation of DNA was done from the collected blood samples using commercially available GeneJET Genomic DNA Purification Mini Kit (K0782, Thermo Fisher Scientific) following the protocols attached with the kit. Quantity and quality checking of the isolated DNA was done using a Nanodrop Spectrophotometer (Thermo Scientific, USA). Further, gel electrophoresis was done on 0.75% agarose gel for checking any shearing of the extracted DNA. The optical density ratio (OD_{260}/OD_{280}) of the DNA samples was checked and only those samples having an OD_{260}/OD_{280} ratio of 1.7 to 1.9 were subjected to further analysis for molecular and quantitative data.

A 25 µL PCR mixture was prepared for amplification which contained 200 µM of each dNTPs, 10X PCR buffer, 2U Taq DNA polymerase, 5 pM of each forward and reverse primer [9] as shown in Table 1, 2mM of $Mgcl_2$ and 60 ng of extracted genomic DNA.

PCR amplification of the Mx gene was done using the following thermo-cycles: Pre Denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 45 sec, annealing at 56ºC for 30 sec, elongation at 72ºC for 45 sec and post extension at 72ºC for 5 min. The obtained amplicon size was 101 bp. The PCR products were then subjected to restriction endonuclease digestion using *Hpa*I restriction endonuclease enzyme by incubating at 37ºC for about 8 hours. The digested products were then separated in 2% agarose gel for visualising the bands in 0.5 X TAE containing 1.0 µM ethidium bromide. Observation of the separated bands was done under UV trans-illuminator and photographs of the gel were taken using Gel Doc system for further interpretation. The primers and restriction enzymes used for PCR-RFLP analysis have been mentioned in Table 1.

Table 1: Gene, PCR amplicon size, primer sequence, annealing temperature for PCR and restriction endonuclease (RE) enzyme used for RFLP analysis

Gene		Primer sequence (5´ - 3´)	T^A (°C)	Product size (bp)	RE	Incubation
Mx	F	GAGTACCTTCAGCCTGTTTT	56	101	*Hpa*I	37 ºC 8 hrs
	R	ATCTGATTGCTCAGGCGTTAA				

Statistical Analysis

Gene and genotypic frequencies were obtained using the POPGENE 32 software [10] for population genetics analysis following the given formulae:

$$Genotype\ frequency = \frac{D}{N}$$

$$Gene\ frequency = \frac{2D + H}{2N}$$

Where,

D = Number of individuals having the particular genotype in the population

H = Number of the heterozygous genotype in the population

N = Total number of the individuals in the population

The tests for Hardy Weinberg equilibrium were performed using exact probability (P-values) tests provided in POPGENE 32 version from the genotypic frequency which was earlier obtained.

The observed heterozygosity and expected heterozygosity were also calculated using the POPGENE 32 software following the mentioned formulae.

The observed heterozygosity (H_o) has been calculated as the actual percentage of heterozygosity occurring in the sample population.

$$Ho = \frac{number\ of\ heterozygotes}{total\ number\ of\ samples} X100$$

The expected heterozygosity or genetic diversity was measured [11] by the formula mentioned below,

$He=1-\sum P^2_i$ (Where, P_i is the frequency of i^{th} allele).

Polymorphism Information content (PIC) values were obtained in this study by using the formula mentioned below [12]:

$$PIC = 2\sum_{i=1}^{k-1}\sum_{j=i+1}^{k} x_i x_j (1 - x_i x_j)$$

Where,

k= number of alleles

x_i= allele frequency at the homozygous loci

$x_i x_j$= allele frequency at the heterozygous loci

Ethical approval

All the methods and processes applied in this study have been done following standard rule specified under animal ethics regulations in the country. The protocols have also been approved by the Institutional Animal Ethics Committee (IAEC) of India. The reference number for approval is CVSC/CAU/IAEC/19-20/P-21.

Results

The PCR amplification of Mx protein gene resulted in amplicon size of 101 bp. Further, RE digestion of this product with the restriction endonuclease enzyme *Hpa*I detected the presence of polymorphism, by giving rise to three different genotypes which are AA (101 bp), AG (101 and 82 bp) and GG (82 bp). All the genotypes AA, AG and GG are shown in Figure 1.

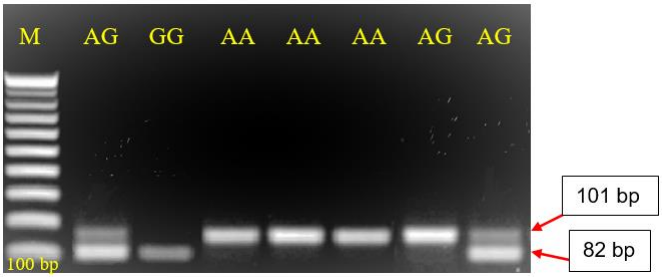

Fig 1: Genotypes of Mx gene digested with RE *Hpa*l in 2% agarose gel

Lane M: 100 bp gene ruler

The genotypic frequency distribution of Mx gene among the local chicken of Mizoram has been shown in Table 2. The genotypes AG and GG were almost equally distributed among the population with a genotypic frequency of 0.46 and 0.40, respectively while the genotype AA was rarely found (0.14). Moreover, out of the two alleles, the allele G was the predominant allele among the presently studied population with a frequency of 0.63 (Table 3). The native 'Zo-ar' chicken population was found to be in Hardy-Weinberg equilibrium with respect to the Mx locus**.**

The observed heterozygosity (0.46) and the expected heterozygosity (0.47) were found to be almost equal in the studied population. The PIC value of the Mx gene locus in the 'Zo-ar' chicken was found to be 0.47 (Table 2)

Table 2: Genotypic frequency distribution and heterozygosity of Mx gene

Parameters		'Zo-ar' chicken (n=50)
Genotypic Frequencies	AA	0.14 (7)
	AG	0.46 (23)
	GG	0.40 (20)
χ^2 value		0.03^{NS}
Observed Heterozygosity		0.46
Expected Heterozygosity		0.47
PIC		0.47

n = Number of animals; NS = Not significant; the figures in parenthesis are the number of animals

Table 3: Allele frequency of Mx locus

Locus	Allele	Allelic frequency
Mx	A	0.37
	G	0.63

Discussion

The digestion of Mx gene PCR amplified fragment of 101 bp with the restriction endonuclease enzyme *Hpa*I showed the presence of polymorphism in the locus, by yielding three different genotypes which are AA (101), AG (101 and 82 bp) and GG (82 bp). The genotypes AG (0.46) and GG (0.40) were almost equally distributed while the genotype AA (0.14) was rarely found. The allele G was present with a higher frequency (0.63) as compared to A allele (0.37.

Similar results of higher AG genotype frequency (0.50) were seen in Red jungle fowls, followed by GG (0.37) and AA (0.12) as in present study, which might be due to the close resemblance of the native chicken of Mizoram with the Red jungle fowl where the G allele (0.60) was also seen to be predominantly present. Contrary to the present results, the AG genotype was found to have a lower frequency in two Chinese native chicken breed namely, Xinghua chicken (0.10) and Silkie chicken (0.07) where both the population showed a higher AA (0.90) genotype frequency [13].

The lower frequency of A allele in the present study was found to be in contrast to the findings in native Chinese chicken populations [13] where the A allele was the major allele in most of the chicken breeds such as, Xinghua chicken (0.95), Silkie chicken (0.90), Langshan (0.95) and Wengchang (0.91). Moreover, in Indonesian indigenous chicken [9] also the A allele was predominantly reported having a frequency of 0.63. Likewise, in Gereze chicken the A allele appeared to be predominant and had a gene frequency of 0.98 among the population [14]. Investigation of the chicken Mx protein gene was also done in two native breeds of India viz. Kadaknath and Aseel and compared with White Leghorn. A non-synonymous mutation at the position 2032 was reported in this study too where G2032A SNP was found out responsible for variation of amino acid (Serine to asparagine). The frequency of the resistant allele A was found to be higher in all the three populations [16].

In previous studies [9, 15] the A allele was found to be associated with disease resistance and anti-viral activity in birds. In Indonesian indigenous chicken [9] the A allele of Mx locus was found to have association with disease resistance and anti-viral activity in birds specially against diseases like New Castle disease virus and Avian Influenza virus whereas, the G allele was the susceptible one. This polymorphism was due to the reason that the allele A of the Mx protein gene was found to be encoding for asparagine at the 631st amino acid of the chicken Mx gene which was regarded to be resistant against Vesicular stomatitis virus (VSV) and Avian influenza Virus (AIV). The G allele on the other hand was seen to be encoding for serine at the same position which was found to be susceptible for both Vesicular stomatitis virus and Avian influenza virus [9]. Moreover, the A allele was favourably associated with body weight at 40 days of age but was unfavourably associated with leg defects in Z line on commercial broilers and higher mortality in X line [15].

Conclusions

The present finding of moderate presence of A allele (0.37) is indicative of the fact that the Mizo native chicken has the genetic potential for disease resistance and antiviral activity, which may be exploited for further improvement by genetic selection. The population conforming to the Hardy-Weinberg equilibrium suggests the absence of selection with respect to disease resistance traits and also absence of migration in the breeding tract. Thus, the knowledge generated on the allelic

and genotypic distribution pattern in the native 'Zo-ar' chicken population of Mizoram, India can be useful for future genetic improvement of the population.

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