MOLECULAR CHARACTERIZATION OF Mx 1 GENE IN QUAIL

THESIS

Submitted in partial fulfilment of the requirements for the degree
of
Master of Veterinary Science

in

ANIMAL GENETICS AND BREEDING

By
Dr. Diwesh Kumar Niraj
Roll No. 4644

To
DEEMED UNIVERSITY
INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR - 243 122 (U.P.)

2012

Dedicated to....

My beloved Parents

&

Guide

DIVISION OF ANIMAL GENETICS
INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR - 243 122, U.P. INDIA

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Dated : 23/06/2012

Certificate

Certified that the research work embodied in this thesis entitled "**Molecular characterization of Mx 1 gene in quail**" *submitted by* **Dr. Diwesh Kumar Niraj**, *Roll No.* **4644**, *for the award of* **Master of Veterinary Science** *degree in* **Animal Genetics and Breeding** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Diwesh Kumar Niraj**, *has worked for more than 21 months in this Institute and has put in more than 150 days attendance under me from the date of registration for the degree of* **Master of Veterinary Science** *of the Deemed University, as required under the relevant ordinance.*

Pushpendra

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We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **Master of Veterinary Science** *of this Institute.*

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Date : 25/01/12
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(Diwesh Kumar Niraj)

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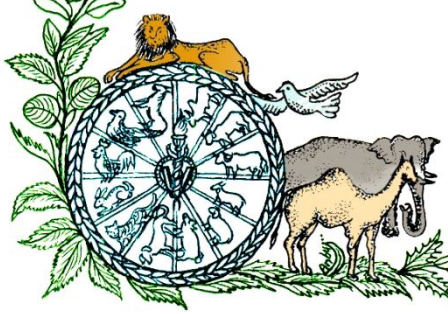
Abbreviations

%	:	Percent
°C	:	Degree Centigrade
/	:	Per
@	:	At the rate of
aa	:	Amino acid
bp	:	Base pair (s)
Da	:	Dalton
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxynucleotide Tri Phosphate
ds-DNA	:	Double stranded DNA
DW	:	Distilled Water
EDTA	:	Ethylene diamine tetra acetate
FAO	:	Food and Agricultural Organisation
Fig.	:	Figure
gm	:	Gram(s)
Hr/hr	:	Hour
Kb	:	Kilobase
KCl	:	Potassium Chloride
Mg^{++}	:	Magnesium ion
min	:	Minute(s)
mix	:	Mixture
ml	:	Milli liter
mg	:	Milli gram
OD	:	Optical density
l	:	Micro liter
M	:	Micro mole
g	:	Micro gram
mM	:	Milli mole
N	:	Normality
ng	:	Nano gram
nm	:	Nano meter

No. (no)	:	Number
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of hydrogen ion concentration
RBC	:	Red Blood Cells
WBC		White Blood Cells
RNA	:	Ribose Nucleic Acid
rpm	:	Revolution per minute
SDS	:	Sodium dodecyl sulphate
Ser	:	Serine
SSCP	:	Single Stranded Conformational Polymorphism
ss- DNA	:	Single stranded DNA
TBE	:	Tris Borate EDTA
TE	:	Tris EDTA
Tris	:	Tris hydroxymethyl amino methane
TEMED	:	NNN'N'- tetramethylene diamine
UV	:	Ultra Violet
V / cm	:	Volt / centimeter
W/V	:	Weight / Volume

Introduction



1 Introduction

India is the 2nd most populated country in the world with 11% of the total world population. On the other hand, it holds only 2.7% of total geographical area and stands at the 7th position in the world in this way. Therefore, the increasing demands of nutritious food, unemployment, and shrinkage of land, have drawn the attention of people towards the livestock production and poultry farming. Due to its least demanding nature in terms of infrastructure in the backyard set up, high nutritive value, less input cost and quick return of money, the poultry farming has grown up nicely into a dynamic agro-industry at a fast pace in just over last four decades.

The contribution of poultry sector to GDP is equivalent to nearly 10% of the total output from livestock and 2.8% of total agriculture (Draft National Poultry Policy of India, 2005). Egg production in India has risen to 45.2 billion in 2005 from a meager value of 5 billion in 1971 (Country Report India, www.fao.org). Poultry sector contributes 1.8 billion tonnes of poultry meat to national meat industry. Now, the poultry industry is a Rs. 45,000 crore industry, providing direct and indirect employment to over 4 million persons (Venky's India Ltd, 2009).

India has achieved 3rd rank in egg production and 6th in broiler meat production in the world with layer industry growing at about 5-7 % and broiler industry at 12-15% per annum (Basic animal husbandry statistics of India, 2010). Despite these facts, the per capita

availability of egg and chicken meat per annum is around 51 eggs and 2.26 kg chicken meat, respectively in 2009-10 which is very low as compared to the National Institute of Nutrition's recommendations of 180 eggs and 11 kg chicken meat, respectively (Basic animal husbandry statistics of India, 2010).

So, there is a huge gap between demand and supply which provides a good opportunity for further improvement in the poultry sector. The increase in poultry meat and egg production can be met by increased farming of alternative poultry species like Japanese quail, which are known for their ability to produce more eggs and better meat in comparison to chicken.

For economic production, good health and survival of birds along with optimum productive performance is fundamental and crucial factor which decides the success and feasibility of poultry production at any level. The losses and damages caused by poultry diseases are mortality, morbidity, negative effects of sub clinical infection, reduced genetic progress, drug residues hazardness for human beings and zoonoses.

Methods to combat diseases or pathogens are chemotherapy and vaccination, but these are not cost effective. Moreover, rapid changes in the pathogenicity of pathogens necessitate continuous development of vaccines and medicines. Furthermore, high costs of medicine, vaccination failure and problem of drug residues in poultry products are becoming the cause of concern among health conscious consumers. It is, therefore, needed that the future researches endeavors shall focus on utilization of diversified approaches such as development of disease resistant lines, well suited to Indian climatic conditions and has better adaptability and production capacity.

Disease resistance is an important trait of chicken that directly determines this economics. Besides the economic benefits acquiring from improving disease resistance, the possibility of reducing medication would be an attractive feature from the stand point of public health, ethics, product quality and animal welfare (Pinard *et al.*, 1992). The genetic variation for disease resistance has not been exploited due to easy access of vaccines and the random nature of infection.

The individual animal or bird in a population, even maintained in the same environment, feed and management reacts differently to a particular disease, showing variation in disease resistance traits. Disease resistance ultimately leads to lowering the cost of production and accelerates improvement in the economic viability in sustainable production systems.

The latest discoveries in the area of molecular and immunogenetics have tremendously added to the knowledge of genome organizations, number of genes, their structures, expression, regulation and nucleotide polymorphism. That is why scientific community is searching the genes responsible for disease resistance and trying to search a molecular marker which can be used for disease resistance. Indirect selection based on suitable genetic markers, represents an ideal approach for the improvement of disease resistance (Gavora and Spencer, 1983).

Avian immune system is a highly evolved and complex system involving different cell types and soluble factors that act in concert to elicit immune response against invading pathogens and thus play a central role in determining survivability. Immune response and disease resistance in chicken have been found to be affected by multiple genetic and environmental factors (Gavora, 1993).

The immunological defense may be grouped mainly into three different and interdependent functional types viz. (1) the cellular system, which provides cell mediated immunity or direct cellular intervention against pathogens, (2) the humoral system, which acts by producing humoral immunity or by antibody production and (3) the non-specific system (e.g. complement system, phagocytosis etc.). The primary cellular constituents of immune system are bursa (B), and Thymus (T) derived lymphocytes and inflammatory leukocytes (macrophages and granulocytes). It is the interaction between macrophages, T-cells and B-cells that is essential for generation and maintenance of an effective immune response that gives rise to immune protection from diseases.

Genetic control of the three major parts or systems of the immunological defense is independent and polygenic in nature (Biozzi *et al.,* 1982). These may be distinguished as three sets of host response genes such as: (a) the genes which control the susceptibility or resistance of an innate immunity (b) the genes which govern the specificity of acquired or adaptive immune response and (c) the genes which affect the quality of specific immune response.

Gelderman (1975) has proposed that expression of quantitative traits is controlled by many genes, each with a small effect. However, Lande (1981) suggested a major gene model which emphasizes larger contribution of a few genes in genetic variations. Such major genes are used as candidates for association studies for identification of markers. Genes responsible for immune responsiveness and disease resistance can be categorized into three categories *i.e.* genes coding for pathogen receptors, genes regulating phagocytic function and genes responsible for quality and quantity of antibody production (Lewin *et al.,* 1999).

Economically important disease like influenza cause huge morbidity and mortality in different poultry breeds. This ultimately affects the economics of production. There are many genes which are responsible for disease resistance like MHC, Interferon, Interleukins, Myxovirus resistance gene (Mx) *etc.* Mx 1 gene is an interferon induced gene which inhibits the proliferation of single stranded negative sense RNA virus. Ko *et al.* (2002) reported the antiviral activity of Mx1 gene in poultry. Subsequently, it was found that Mx 1 gene of chicken is around 21 Kb and the cDNA is 2118 bp long. Then Seyama *et al.* (2006) reported that polymorphism at 2032nd position of cDNA resulted in differential antiviral activity. If adenine is substituted in place of guanine it leads to increased antiviral activity. Antiviral activity of Mx 1 gene is also proved in different species like mouse, human and bovine. However, in duck Mx 1 gene has no antiviral activity (Bazzigher *et al.*, 1993). Qu *et al.* (2008) submitted Coturnix coturnix (common quail) Mx protein (Mx) mRNA, partial cds of 2142 bp length (NCBI Acc. No. EF575605). Mishra (2009) reported presence of polymorphism in Mx 1 gene in Aseel breed of chicken. Singh (2010) found polymorphism in Mx 1 gene in Kadaknath and RIR breeds of chicken. Ramesh (2011) reported presence of polymorphism in Mx 1 gene in Naked Neck and Tellicherry breeds of chicken. Till now polymorphism of Mx 1 gene has not been reported in commercial Japanese quail (*Coturnix coturnix japonica*).

In view of the importance of Mx 1 gene it would be most befitting to study these gene sequences and polymorphism with modern molecular genetic tools like PCR-RFLP and SSCP.

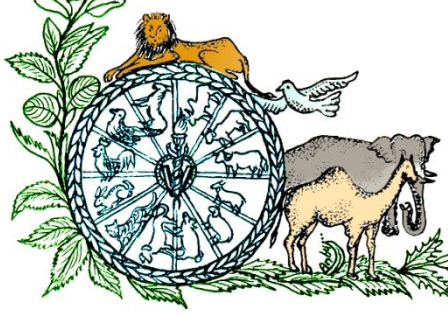
However, unlike chicken, Mx 1 gene has not been studied and characterized in the Japanese quail (*Coturnix coturnix japonica*). Therefore, it is hypothesized that identification of putative variations

in the Mx 1 gene may be useful to unravel the molecular mechanism for Myxovirus resistance in quail.

Keeping these facts in view the proposed study has the following objectives:-.

- **To identify and characterize various allelic variants of the Mx 1 gene in commercial Quail.**
- **To sequence the allelic variants and compare with the published data.**

Review of Literature



2 Review of Literature

Japanese quail *(Coturnix coturnix japonica)* is a migratory bird of Japan, now domesticated throughout the world as a good source of egg and meat due to fast growth rate, high rate of egg production and ease of management. The quails were introduced at Poultry Research Division (PRD) IVRI, Izatnagar under UNDP-ICAR collaboration educational project for the first time in India in 1974, to mitigate chronic protein deficiency among the Indian population (Ahuja, 1992). The introduction of the Japanese quail has opened a new path as an alternative for poultry farmers. The experimental quail farm of CARI, Izatnagar is maintaining five pure lines of quail namely CARl UTTAM, CARl UJJAWAL, CARl BROWN, CARl SWETA and CARl PEARL (quail layer).

Initially, the commercialization of domestic quail was banned in India under the Wild Life (Protection) Act, 1972. It hampered the development and popularization of quail farming. But now the Government of India is encouraging entrepreneurs to start quail farms, for which a government license is required to sell commercial Japanese quail considering the jungle variety of the bird, which is a protected species. The Ministry of environment and forests delegated the power to grant such license to the department of Animal husbandry. So, far more than 500 licenses have been issued in Maharashtra State alone (www.vethelplineindia.com). So, quail has tremendous scope to occupy a considerable portion of Indian poultry market in coming years as a profitable enterprise (Muthukumar and Roy, 2005-06).

Quail meat is having amazing tender taste, game flavour, low calorific value and high dry matter content. Lesser fat and low calorific value makes it an ideal food for health conscious consumers. Quail eggs and meat are renowned for being rich in vitamins, essential amino acids, unsaturated fatty acids and phospholipids, which are vital for human physical and mental development. Quail meat and eggs are fit to be included in the diets of children, pregnant mothers and geriatric and convalescent patients. Tikk and Tikk (1991) reported quail to be more efficient converters of feed to eggs than the domestic fowl by producing twice as much as egg mass per kg body weight. An adult Japanese quail weight is 150-200 gm and heavier weight of female is the uniqueness of this species. Layers of quail are capable of producing 280 eggs (hen housed average) annually in Indian condition and more than 300 eggs in European countries (Johri, 1997).

Quail has also been identified worldwide as a laboratory animal for research because of its salient characteristics that include rapid growth, short generation interval (upto 4 to 5 generation in a year), early sexual maturity (42 to 45 days) and high rate of lay. Quail is extensively used as a model organism for embryological and cancer research (Panda and Mohapatra, 1998). Recently, the Japanese quail has proven to be an excellent model organism for the production of transgenic avians using lentiviral vectors. The high degree of homology between chicken and quail genomes allows researchers to design highly specific DNA constructs for the production of transgenic birds (Poynter *et al.*, 2009). The Japanese quail was the only avian species which was chosen by NASA, in their space programme during late eighties.

The quail farming is steadily gaining popularity as it is considered a delicacy for high class society because the meat of Japanese quail has the aphrodisiac character and eggs are useful for

the treatment of TB, arthritis and Asthma patients (Harpal, 2005). The weekly production of broiler quails in India varied between 50,000 - 1, 00,000 as estimated by Agarwal, (1996). An exclusive quail market segment has lately been growing within the poultry meat sector to cater to the quality conscious meat consumers at affordable prices.

However, all these production performances are severely affected due to outbreak of various diseases, which not only reduce the productivity of birds, but also diminish the job opportunities of millions of unemployed efficient men power. Ultimately, it affects the country's whole economy to a large extent. Besides, there are several diseases, which are of zoonotic importance too.

Therefore effective measures to combat these diseases should be taken up. The advances in molecular biology have opened the new dimensions in the field of poultry health. As viral diseases can not be cured by therapy and incidences of vaccination failure are also present, the marker assisted selection (MAS) for disease resistance traits can serve the purpose.

2.1 EVIDENCES OF AVIAN FLU IN QUAIL

Quail, as a domesticated bird in different regions of the world, has time to time suffered from avian flu outbreak. An outbreak of bird flu on 11th Dec, 2006 resulted into loss of 2,70,000 quail population in Kimje, South Korea (Ministry of Agriculture and Forestry, Republic of Korea, 2006). Recently, an outbreak of bird flu on 1st Mar, 2009 in Central Japan caused loss of 3,20,000 quail birds (Ministry of Agriculture, Govt. of Japan, 2009). In India, information about the outbreak of avian flu in quail is not available in detail. However, commercialization of Japanese quail farming still being in the process, we have to be prepared to fight this deadly disease of zoonotic importance.

2.1.1 Susceptibility of Quail to influenza virus

Some studies related to influenza viral disease have been performed in quail, but study about genetic resistance of this species has not been done yet. Makarova *et al.* (2003) revealed that quail could function as amplifiers of influenza virus reassortants that have the potential to infect humans and/or other mammalian species.

Perez *et al.* (2003) conducted research to understand the molecular determinants for adaptation of H9 influenza viruses to land-based birds. The replication and transmission of several 1970s duck H9 viruses were tested in chickens and quail. Quails were more susceptible than chickens to these viruses. Generation of recombinant H9 viruses by reverse genetics showed that changes in the HA (haemagglutinin) gene of the viruses are sufficient to initiate efficient replication and transmission in quail.

2.2 HOST IMMUNE SYSTEM

In case of vertebrates, host defense against pathogens is mediated by two general systems: innate and acquired immunity. Innate immunity constitutes the first line of defense, providing a rapid response by the expression of germ-line encoded proteins that pre-exist or are induced within hours of infection. Adaptive immunity is a slower, but highly specific response mediated by B and T lymphocytes that confers effective and long-lasting protection against infection. Adaptive immunity is based on the generation of a large repertoire of antigen-recognition receptors by somatic gene rearrangement. Diversity has been considered the hallmark of adaptive immunity. In the last few years, however, evidence has accumulated supporting the importance of diversity (probably as a response to selective pressures) even within innate immunity (Hoffmann *et al.* 1999). Moreover, differences in innate immune mechanisms have been shown to be

critical in host susceptibility to infection (Cooke and Hill, 2001), making this an area of intense research.

The interferon (IFN) system is a major component in innate immunity against viruses. IFNs help to limit virus propagation by inducing an antiviral state in potential target cells and by enhancing adaptive immune responses (Stetson and Medzhitov, 2006). The type-1 interferon pathway plays a central role in the innate response to viral infection, inducing expression of multiple genes like protein kinase 2', 5' oligoadenylate synthatase and Mx gene (Stark *et al.*, 1998), known to participate in the antiviral response. Mx gene is one of the best characterized interferon-regulated antiviral genes. The Mx protein, in particular, has been shown to possess innate antiviral activity not only to influenza virus but several RNA viruses, such as Orthomyxovirus, Rhabdoviridae, Paramixoviridae, Bunyaviridae, Picornaviridae and Togaviridae (Haller *et al.,* 1998).

2.2.1 Historical Background of Mx1 gene

Study of Mx1 gene started with the study of Lindenmann (1962) in mouse, which showed that the inbred mouse strain A2G is resistant to doses of mouse adapted influenza virus that are lethal to other inbred strains. Study of Lindenmann was an interesting observation because innate resistance in A2G mice was dependent on a single dominant locus, named Mx1, that was expressed in a variety of cell types ranging from macrophages to hepatocytes and was exquisitely specific for Orthomyxovirus. Subsequent studies showed that the specific resistance of Mx1+ murine cells to influenza viruses is attributable to the IFN-induced protein Mx1. After virus infection, the Mx1 protein is rapidly expressed in the nuclei of cells in the area where virus replication occurs, thus blocking viral spread (Arnheiter *et al.*, 1990). The presence of a natural resistance gene to influenza was intriguing because mice are not natural hosts for Orthomyxovirus.

2.3 Mx 1 PROTEIN

The Mx protein, as an interferon-induced guanosine triphosphatase, not only confers resistant activity to *Orthomyxovirus* infection but also to *Rhabdoviridae, Paramixoviridae, Bunyaviridae, Picornaviridae and Togaviridae* families. The Mx 1 protein has been found in many organisms including yeast (Rothman *et al.*, 1990) and vertebrates ranging from fish to humans (Staeheli *et al.*, 1989; Staeheli, 1990; Pavlovic and Staeheli, 1991; Bazzigher *et al.*, 1993; Plant and Thune, 2004).

2.3.1 Biochemical structure

As an interferon-associated protein, it contains conserved tripartite guanosine triphosphate binding sites and a leucine zipper (Horisberger *et al.*, 1990; Melen *et al.*, 1992; Pitossi *et al.,* 1993). Mx proteins belong to superfamily large GTPase, which includes the dynamins, the products encoded by the *Drosophila shibire* (Chen *et al.,* 1991), the yeast vacuolar sorting protein Vps1p (Rothman *et al.,*1990), and the GTP-binding protein from *Arabidopsis thaliana* (Van der Bliek and Alexander, 1999). The molecular weight of Mx protein is around 70–80 kDa.

Various Mx proteins differ widely with respect to intracellular distribution and biological activities (Bernasconi *et al.*, 1995) including endocytosis, intracellular vesicle transport and mitochondrial distribution. These proteins present a modular organization characterised by varied degrees of sequence conservation underlying their ability to self-assemble into higher order structures that resemble rings and helical stacks of rings, as in dynamins (Van der Bliek and Alexander, 1999). Mx proteins are characterised by putative tripartite GTP-binding domains (Richter *et al.*, 1995), a self-assembly sequence in the N-terminus (Nakayama *et al.*, 1993) and a GTPase effector domain (GED)

in the C-terminus. The highly conserved N-terminal GTPase domain is of 300 amino acids long, "middle" domain is of 150 amino acids, and the GTPase effecter domain (GED) is of 100 amino acids including two leucine zippers that have the capacity to form amphipathic helices. Studies with human Mx A showed that the GED is able to specifically contact the middle domain, and that this interaction is critical to constitute a functional GTPase domain, as well as for oligomerization (Schumacher and Staeheli, 1998; Di Paolo *et al.*, 1999; Haller and Kochs, 2002). When human Mx A and mouse Mx 1 were mutated by replacing lysine by methionine or alanine or by deleting it and tested for antiviral activity, GTP binding capacity and GTPase activity, it was found that functional GTP binding motif is necessary for virus inhibition (Pitossi *et al.*,1993).

Targeted mutagenesis has shown that GTPase activity is required for the antiviral activity of Mx proteins (Garber *et al.,* 1993; Melen *et al.,* 1994). However, the amino-terminal domain, which contains the GTP-binding domain, is not sufficient for antiviral activity, since molecules lacking the carboxy-terminal domain or displaying point mutations, deletions, or linker-insertion mutations in the carboxy-terminal domain are also devoid of antiviral activity. In some of these carboxy-terminal mutants, GTPase activity is also affected, but in others, it remains intact (Schwemmle *et al.*, 1995). These mutational analyses suggest a two domain-model with intra- or intermolecularly interacting domains. In fact, aggregates of amino- and carboxy-terminal proteolytic fragments of human Mx A protein have GTPase activity, in contrast to the corresponding isolated fragments, which lack such activity.

The human Mx A protein contains a tripartite GTP-binding domain consisting of GxxxxGKS, DxxS and TKxD, where X is any

amino acid (Horisberger *et al.*, 1992). Mx A forms oligomers and this oligomerization is important for GTPase activity. However, oligomerization may not be important for antiviral action (Jansen *et al.*, 2000). Therefore, GTP hydrolysis may not be required for antiviral activity. It may be possible that high molecular weight of Mx A oligomers represent a stable intracellular pool from which active Mx A monomers are recruited. Several domains have been proposed to be responsible for oligomerization. According to Melen *et al.* (1992), LZ1 and LZ2 of leucine zipper may be responsible. Schumacher and Staeheli (1998) proposed that carboxy-terminal region of Mx A may have role in oligomerization, which folds back into internal region within a central interaction domain.

Rat Mx 2 and rat Mx 3 are two alpha/beta interferon-inducible cytoplasmic GTPases that differ in three residues in the amino-terminal third, which also contains the tripartite GTP-binding domain and that differ in five residues in the carboxy-terminal quarter, which also contains a dimerization domain. While Mx 2 is active against vesicular stomatitis virus (VSV), Mx 3 lacks antiviral activity.

The chicken Mx protein is a predominantly cytoplasmic form and consists of 705 amino acids (Bernasconi *et al.*, 1995). Yang *et al.* (2006) reported expression of the Mx protein in high and low egg production chicken strains.

The protein sequence encoded by the Fugu Mx gene is 77%, 48%, and 51% identical to that of trout Mx 1, chicken Mx, and mouse Mx 1 genes, respectively. The Fugu Mx gene is expressed in a variety of tissues, with high expression detected in the heart, gill, kidney, intestine and brain. The Fugu Mx promoter was inducible by human IFN-â in the human hepatoma (Huh7) cells and by polyinosinic: polycytidilic acid in the top minnow hepatoma (PLHC-1) cells. Deletion

analysis of the promoter showed that both ISREs contributed to inducibility (Yap *et al.*, 2003).

Lee and Vidal (2002) carried out the detailed study about the amino acid composition and intracellular localization of Mx proteins in different species as shown in table 2.1.

Table 2.1 : Amino acid composition and intracellular localization of Mx proteins

Species	Gene	No. of amino acids	Intracellular localization
Human	Mx A	661	Cytoplasm
	Mx B	715	Nucleus and cytoplasm
Mouse	Mx 1	631	Nucleus
	Mx 2	655	Cytoplasm
Rat	Mx 1	652	Nucleus
	Mx 2	659	Cytoplasm
	Mx 3	659	Cytoplasm
Chicken	Mx	705	Cytoplasm
Duck	Mx	721	Nucleus and cytoplasm
Cattle	Mx 1	651	Cytoplasm
Pig	Mx 1	663	Cytoplasm

2.4 CHROMOSOMAL LOCATION AND STRUCTURE OF Mx 1 GENE

Several physical mapping techniques like Somatic Cell Hybridization, In Situ Hybridization (ISH), Fluorescent In Situ Hybridization (FISH) and Pulse Field Gel Electrophoresis (PFGE) have been employed in different species to locate the position of Mx 1 gene.

The Mx 1 gene in human is located on chromosome number 21 having 33016 bp. It consists of 17 exons and 16 intronic regions. The coding sequence is 1989 bp long (**NCBI Acc. No. AF135187**). The first four exons are non-coding (Tahzi-Ahnini *et al.*, 2000). The human Mx A promoter has three Interferon Stimulated Response Elements (ISREs). Out of these, two proximal ISREs contributed to IFN-

inducibility cooperatively, whereas the distal ISRE was non-functional (Ronni *et al.*, 1998). It is likely that the presence of multiple ISREs enhances the sensitivity of the promoter to induction by IFN.

In case of cattle, Mx 1 gene is present on chromosome number 1 and around 60000 bp long. It has 15 exonic regions and 14 intronic regions. The coding sequence is 2798 bp long followed by a polyadenylated tail (**NCBI Acc.No. NM_173940**).

The Mx 1 gene of porcine is located on chromosome number 13 having 15 exonic regions and 14 intronic regions. The whole gene is around 23200 bp and the total length of the coding sequence is 1991 bp (**NCBI Acc. No. AB164037**).

The ovine Mx 1 coding sequence is 1965 bp (**NCBI Acc no. NM_001009753**) long. The promoter possesses two ISRE sites, which are located upstream of the transcription start site and one site was located in intron A. In other species, Mx 1 promoters contained at least two functional ISRE, with exception of chicken Mx 1 promoter that has a single ISRE (Schumacher *et al.*, 1998).

Mouse Mx 1 gene is located on chromosome number 16 and it is 24817 bp long. There are 14 exons and 13 introns in it. Coding sequence of mouse is 1896 bp long (**NCBI Acc. No. BC011113**). The first exon of mouse Mx 1 gene is non-coding (Hug *et al.*, 1988).

The Fugu (a type of fish) Mx gene is 3.4 kb long and made up of 12 exons and 11 introns. In 5'-flanking region two ISRE are present at positions –51 to 38 and –97 to 85. The Fugu Mx promoter can be recognized by the trans-acting factors of a human cell. Hence, the short promoter regions of the Fugu will be very useful in understanding the molecular mechanism of gene regulation in the vertebrate immune system (Yap *et al.*, 2003).

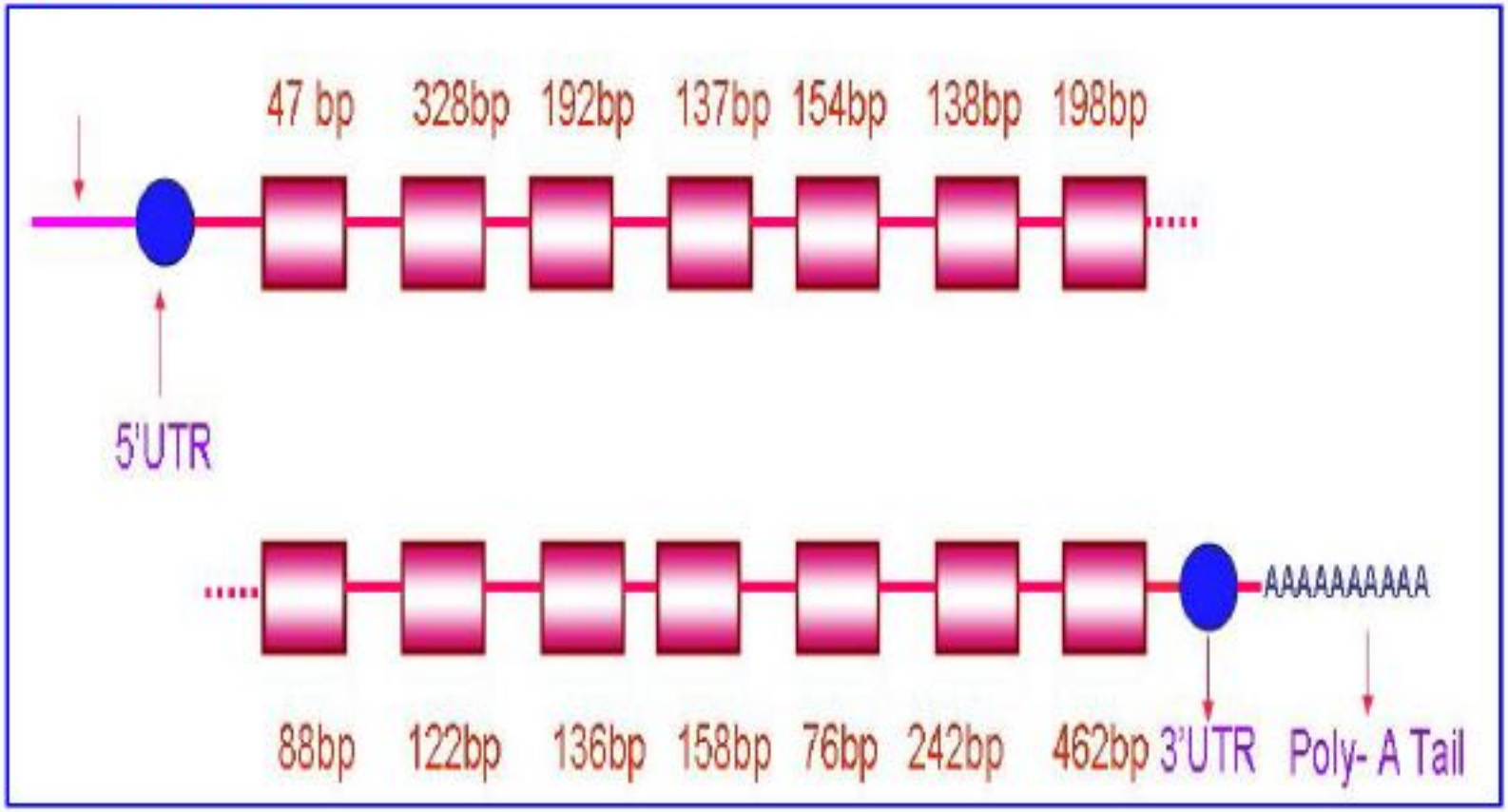
The chicken (*Gallus gallus*) Mx 1 gene has 21289 bp linear DNA present on chromosome number 1. It has 14 exonic regions and coding sequence is 2118 bp long (**NCBI Acc.No. DQ788613**) (Fig. 2.1). According to Li *et al.* (2007) the length of the full genomic DNA sequences of the Mx gene for Silkie (DQ788614), White Leghorn (DQ788615), 2 Rhode Island Red (DQ788613) and Red Jungle Fowl (DQ788616) individuals were 21,258, 21,289, 21,292 & 21,281 and 21,207 bp, respectively. The genomic organization of exonic region of WLH from the 5' to the 3' end was 237, 193, 138, 155, 139, 199, 79, 123, 142, 159, 77, 243, and 234 bp, respectively. The 5'-UTR was 140 bp but was separated by an inserted fragment of 664 bp into 2 parts, which were 48 and 92 bp, respectively. The 3'-UTR was 288 bp with a poly (A) signal at the end. The length of the promoter was 215 bp and included an IFN-stimulated response element. The genomic organization of the Mx gene for the other 3 individuals and Red Jungle Fowl were the same as that of the White Leghorn. In total, 237 mutation sites, including 24 singleton variable sites and 213 parsimony informative sites, were identified among 4 directly sequenced Mx genomic DNA sequences and the reference sequence.

Table 2.2. Size and chromosomal location of Mx 1 gene in different species

Species	Chromosomal Location	Size (bp)	NCBI Acc. No.
Human	21	33016	AF135187
Cattle	1	60000	NM_173940
Pig	13	23200	AB164037
Mouse	16	24817	BC011113
Chicken	1	21289	DQ788613

2.5 ANTIVIRAL ACTIVITY OF Mx 1 PROTEIN

Differential antiviral activity of Mx 1 protein has been proved in various species. The nuclear mouse Mx 1 protein primarily inhibits



Box shows exon and line shows intron (Acc no. DQ788615)

Fig. 2.1 : Structural organization of Mx 1 gene of chicken

the replication of influenza virus and *Orthomyxoviruses* (Garber *et al.*,1993; Haller *et al.*, 1998), the cytoplasmic human Mx A protein is active against a broad spectrum of viruses belonging to the families *Orthomyxoviridae* (Pavlovic *et al.*,1990), *Bunyaviridae* (Frese *et al.*, 1996), *Paramyxoviridae* (Zhao *et al.*,1996), *Rhabdoviridae* (Pavlovic *et al.*,1990) and *Togaviridae* (Landis *et al.*,1998).

Mx proteins of mouse, rat and human have reported to play important role in generating antiviral state in tissue culture cells (Pavlovic *et al.*, 1990). Mouse is the only species that has been shown to become resistant to influenza virus infection *in vivo* by Mx 1 protein (Arnheiter *et al.*, 1990). Transgenic mice that express high levels of the Mx 1 protein are resistant to influenza infection. The Mx 1 protein first reported in A2G strain of mouse by Lindenmann (1962) encodes a nuclear Mx 1 protein. Murine nuclear Mx 1 protein inhibits influenza virus replication (Staeheli *et al.*, 1986, Meir *et al.*, 1990), but cytoplasmic Mx 1 protein inhibits vesicular stomatitis virus replication and not the influenza virus (Zurcher *et al.*, 1992; Jin *et al.*, 1999, 2000). Arnheiter and Haller (1988) found when Mx^+ mouse cells were microinjected with the monoclonal anti-Mx antibody 2C12, interferon alpha/beta still induces Mx protein, but no longer inhibits efficiently the expression of influenza viral proteins as visualized by immunofluorescent labeling. However, interferon inhibition of an unrelated control virus, vesicular stomatitis virus, remains unchanged.

Nicholas *et al.* (2006) during investigation of the innate immune response within the brain to lyssavirus infection and Rabies virus found out that transcript levels for type 1 interferons (IFN-á and -â), the inflammatory mediator interleukin 6 (IL-6) and the antiviral protein Mx 1 levels increased in mice. Intracranial inoculation resulted in the early detection of virus replication and rapid expression within

the brain of the innate immune response genes. Transcripts for type 1 IFNs declined as the disease progressed. Peripheral, extraneural inoculation delayed the host response until virus entered the brain, but then resulted in a large increase in the level of IFN-â, IL-6 and Mx1 transcripts.

Human Mx A protein confers resistance to vesicular stomatitis virus and influenza virus in Mx A transfected mouse 3T3 cells. The study of Mx A protein on measles virus and vesicular stomatitis virus in human monocytic cell line U937 revealed that only vesicular virus transcription was inhibited whereas no difference was detected for measles virus. Similarly, no effect was observed on multiplication of two *Picornaviruses*, one *Thogatovirus* or *Herpes simplex virus type É.*

Cytoplasmic human Mx B and rat Mx 3 are without antiviral activity (Meier *et al.*, 1990; Pavlovic *et al.*, 1990). In addition, almost all laboratory mouse strains, carry nonfunctional Mx 1 and Mx 2 genes, except feral mouse strains which contain functional antiviral Mx 1 and Mx 2 proteins (Jin *et al.*, 1998; Jin *et al.*, 1999) and rainbow trout Mx proteins do not have antiviral activity except against some selected viruses (Trobridge *et al.*, 1997). Dittmann *et al.* (2008) found out that virus strains of avian origin were highly sensitive to Mx 1, whereas strains of human origin showed much weaker responses. Artificial reassortments of the viral components in a minireplicon system identified the viral nucleoprotein as the main target structure of Mx 1. The recently reconstructed 1918 H1N1 "Spanish flu" virus was much less sensitive than that of highly pathogenic avian H5N1 strain A/Vietnam/1203/04 when tested in a minireplicon system. Importantly, the human 1918 virus-based minireplicon system was almost insensitive to inhibition by human Mx A, whereas the avian influenza A virus H5N1-derived system was well controlled by Mx A.

These findings suggest that Mx proteins provide a formidable hurdle that hinders influenza A viruses of avian origin from crossing the species barrier to humans. They further imply that the observed insensitivity of the 1918 virus-based replicon to the antiviral activity of human Mx A is a hitherto unrecognized characteristic of the "Spanish flu" virus that may contribute to the high virulence of this unusual pandemic strain.

The antiviral activity of fish Mx proteins remains unclear. Trout Mx proteins failed to inhibit the replication of infectious hematopoietic necrosis virus (Trobridge *et al.*, 1997). While it has been reported that there is a correlation between Mx protein expression and protection against infectious pancreatic necrosis virus (IPNV) induced by IFN in Chinook salmon embryo (CHSE-214) cells, there is no clear evidence that the Mx protein is involved in the inhibition of IPNV (Nygaard *et al.*, 2000).

Nakatsu *et al.*, (2004) had shown that permanently transfected 3T3 cell lines expressing bovine Mx 1 mRNA have antiviral potential against VSV infection. Transfected cell clones expressing bovine Mx 1 mRNA showed a significantly smaller number of cells infected with VSV compared with the control cells.

The duck Mx, which was reported to be non-antiviral and found in the nucleus as well as in cytoplasm showed no enhanced influenza virus resistance (Bazzigher *et al.*, 1993).

Furthermore, the chicken Mx protein, a predominantly cytoplasmic form, was also reported to be devoid of antiviral activity (Bernasconi *et al.*, 1995). The active form of chicken Mx protein in natural conditions is expected to be effective against Newcastle disease virus (*Paramyxoviridae*) which is a close relative of influenza virus (Easterday, 1975).

2.6 MECHANISM OF ACTION OF Mx 1

The exact mechanism of action of Mx proteins is still a matter of debate and it is not clear whether the antiviral activity of Mx is a luxurious accident of some undefined cellular function or has evolved to inhibit in each species a set of species-specific pathogens. The broad activity of some Mx proteins suggests an intracellular target intersecting a range of viral pathways.

After infection of virus, interferon is released that induces the antiviral Mx protein and blocks the virus multiplication at different steps. Type I IFN activates expression of its target genes using the JAK/STAT signaling pathway (Fig. 2.2). Binding of Type I IFN to the Type I receptor causes phosphorylation of the receptor associated Jak-1 and Tyk-2 kinases, which leads to the subsequent phosphorylation of Stat-1 and Stat-2 proteins. The phosphorylated Stat-1 (p91) and Stat-2 (p113) form a heterotrimer with p48 (also known as IRF9) and the complex translocates to the nucleus to form interferon stimulated gene factor-3 (ISGF-3). The ISGF-3 complex binds to ISRE in the promoter regions of interferon stimulated genes (ISGs). In case of influenza virus Mx protein blocks the primary transcription of influenza and Thogoto virus in nucleus via an interaction of mouse Mx 1 and the PB2 subunit of the influenza polymerase (Stranden *et al.*, 1993).

Preliminary analysis indicates increased cytokine and interferon gene induction in Mx-Asn631 chicken lymphocytes compared to Mx-Ser631 chicken lymphocytes, which may provide insight into the increased AI protection observed in chickens with the MX-631Asn allele (Kapczynski, D.R.*et al.,* 2009).

The cytoplasmic Mx proteins from the mouse and rat inhibit vesicular stomatitis virus (VSV) but not influenza virus (Meier *et al.*,

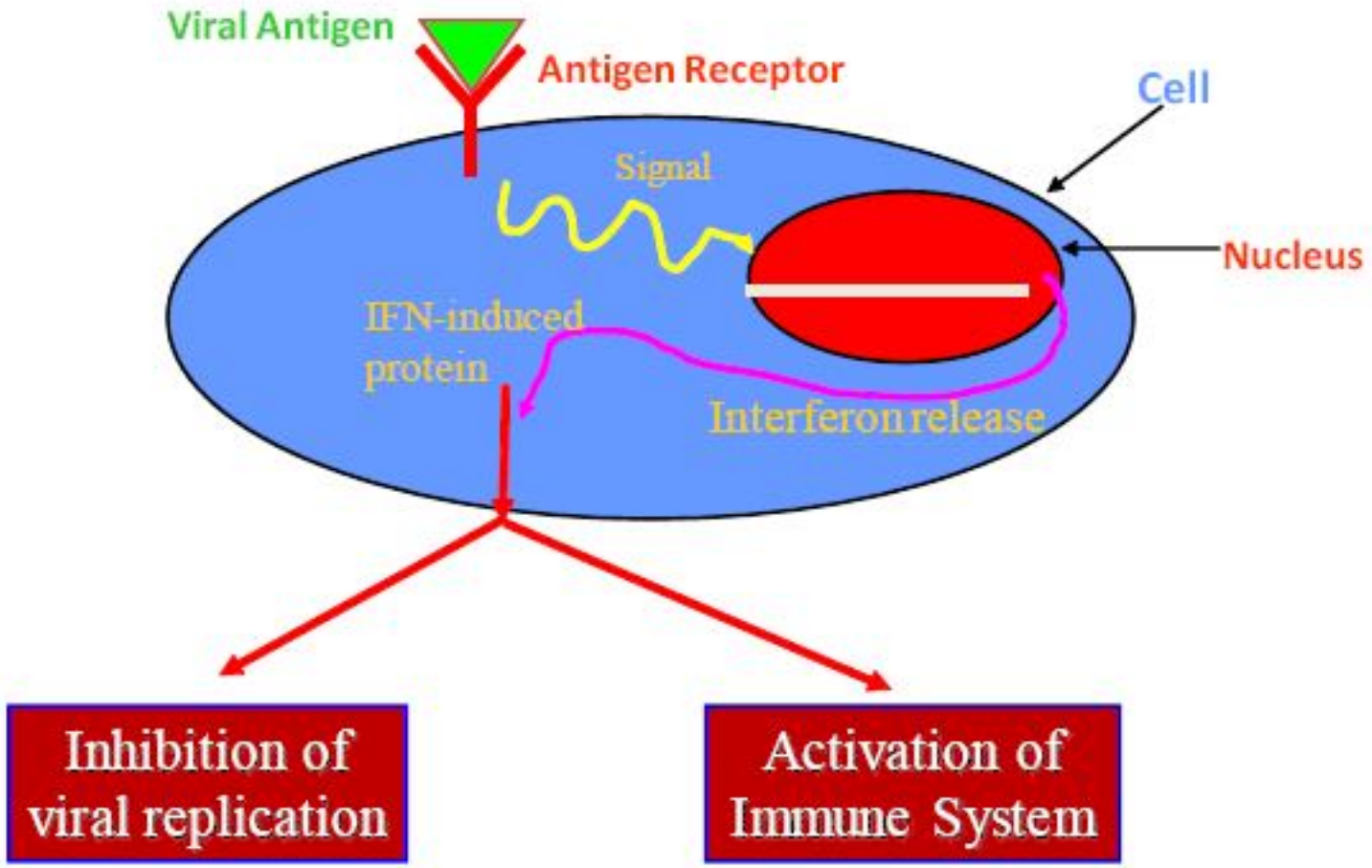


Fig. 2.2 : Mechanism of antiviral action of Mx 1 gene in chicken

1990; Zurcher *et al.*, 1992). Mx protein not only inhibits the vesicular stomatitis virus but several members of the *Bunyaviridae* family that typically replicate in the cytoplasm, at early stage of the viral cycle.

In contrast to mouse Mx 1, which resides in the cell nucleus, the cytoplasmic human Mx A protein does not inhibit primary transcription of influenza viruses but inhibits a subsequent step involved in genome amplification and secondary transcription (Pavlovic *et al.,* 1992). Upon full induction Mx A contribute 0.5%-1% of cytosolic proteins. Mx A can bind elements of cytoskeleton such as actin and tubulin and several larger cellular proteins. But these protein-protein interactions seem to be transitory.

Whether primary or secondary transcription is affected, it depends on the subcellular localization. When Mx A is moved into the nucleus by virtue of a foreign nuclear localization signal, like the murine Mx 1 protein, blocks primary transcription (Zurcher *et al.,* 1992), indicating that the Mx proteins of the two species act in a comparable way by recognizing the same or similar viral target structures.

Mx A blocks the post-transcription level through interaction with the nucleocapsid protein (NP) of *Thogotovirus* preventing the infecting particles from entering the cell nucleus (Kochs and Haller, 1999). Mx A inhibits mRNA synthesis of vesicular stomatitis virus (Staheli *et al.*, 1986). In case of influenza virus, it inhibits a poorly defined multiplication step which follows primary transcription but precedes genomic replication (Pavlovic *et al.,* 1992).

Mx A also inhibits the multiplication of measles virus. However in this case the protective effect of Mx A is cell type specific, blocking either viral RNA synthesis or synthesis of viral glycoprotein depending on the cell line used (Schneider-Schaulies *et al.*, 1994).

2.7 GENETIC POLYMORPHISM

Jin *et al.* (1998) found clear variation at position 498 in exon 4, after digestion by *Taq* I: one band (type 1;126 bp) was observed in SPR mice; two bands (type 2; 82 and 44 bp) were observed in the BALB/c, C57BL/6, CBA/J, CAST/Ei, BFM/2, and PGN2 strains; and three bands (type 3; 126, 82, and 44 bp) were seen in the SL/NiA, MOG,SWN, NJL, and ZBN strains. The PCR-RFLP that contained position 6422 in exon 14 and was digested with *Hha* I has an uncut single band (type 1; 334 bp) was observed only in CBA/J, while two bands (type 2; 227 and 107 bp) were seen in all of the other strains examined. The Mx 1 mRNA of Mx-mouse strains such as BALB/ c and C57BL/6 has a deletion of the coding region from exon 9 through 11 resulting in premature termination of the Mx 1 protein, and other Mx- strain CBA/J has a point mutation that converts to a termination codon at position 389 in exon 10.

In bovine, Ellinwood *et al.* (1998) found a variant Mx 1 cDNA with an 18 bp deletion that did not appear to be a genetic origin. Nakatsu *et al.* (2004) found 12 nucleotides variation, when they compared Holstein Mx 1 with Mx 1 cDNAs reported previously by Ellinwood *et al.* (1998). Ten nucleotide substitutions were synonymous mutations and a single nucleotide substitution at 458 resulted in amino acid exchange of isoleucine (ATT) and methionine (ATG) which is different from the previous finding of Ellinwood *et al.*(1998) that at position 1189 caused an amino acid substitution of leucine (CTG) to proline (CCG) at position 364. A 13 bp deletion-insertion mutation was also found in the 3' UTR. An 18 bp deletion-insertion is located at boundary between exon 3 and intron 3.

Nakajima *et al.* (2006) found the 3-bp deletion at position 1778–1810 in exon 13 of porcine Mx 1 gene. Comparison of the translated amino acid sequence of exon 13 with the published sequence of the

protein revealed that exon 13 of Mx 1 in the Meishan breed has lost the serine residue at amino acid 565. The 11-bp deletion found in exon 14 of Landrace breeds leads to a frameshift with 8 amino acid substitutions and a 23 amino acid extension in the carboxyl terminal region of the Mx 1 protein (Morozumi *et al.*, 2001).

Although fish are believed to have a highly evolved immune system, their interferon system has not been well characterised (Manning and Nakanishi, 1996). IFN-like activity has been shown in a number of fish species (Robertsen, 1998), but unlike mammals and birds, fish type I IFN has not been purified nor their genes been cloned. Recently, cDNAs for Mx genes have been cloned from several fish. Three full-length Mx cDNAs each have been cloned from rainbow trout (Trobridge and Leong, 1995; Trobridge *et al.*, 1997) and Atlantic salmon (Robertsen *et al.*, 1997). A single copy of Mx cDNA has been cloned from flounder and halibut (Jansen and Robertsen, 2000). These Mx genes have been shown to be inducible *in vivo* by injection of live fish with fish rhabdovirus (Trobridge and Leong, 1995) or polyinosinic: polycytidilic acid (poly I:C) (Trobridge and Leong, 1995; Robertsen *et al.*,1997; Jansen and Robertsen 2000). However, the gene organization and regulatory region of teleost Mx genes have not been well characterised.

A total of 237 single nucleotide polymorphisms were found in the chicken Mx gene by comparison among 4 directly sequenced Mx genomic DNA sequences (Li *et al.*, 2007), and the reference sequence was inferred from the chicken genome project. The genomic diversity of the chicken Mx gene showed large variation in different regions, with the highest diversity in the 5' untranslated region and the lowest in the 3' untranslated region.

Ko *et al.* (2002) reported that, among a number of naturally occurring mutations in the chicken Mx gene, only the serine to

asparagine mutation (631th position of amino acid sequence) caused by a single nucleotide polymorphism at position 2,032 (G to A) of Mx cDNA resulted in positive antiviral function *in vitro*. The substitution also showed skewed allele frequencies in different chicken populations, with a much higher frequency of the favourable allele 'A' in native breeds than in highly selected lines. Native breeds were in Hardy-Weinberg equilibrium whereas 3 out of 4 commercial populations were not in Hardy-Weinberg equilibrium (Li *et al.,* 2006). Selection, environment and negative correlation between production and disease resistance traits could contribute to highly skewed frequencies (Zekarias *et al.,* 2002). Disequilibrium may be due to high selection intensity, genetic drift and non-random mating.

Livant *et al.* (2007) identified three single nucleotide polymorphism (SNPs) in Mx 1 gene. The guanine to adenine substitution, which was found both at 1892 and 2019 positions of exon 14 (**NCBI Acc No. NM-204609**) as reported previously by Ko *et al.*, 2002 in other chicken breeds were at different positions i.e. 2032 and 2159, respectively. A third SNP was in the 3' UTR, immediately 3' to the stop codon (**NCBI Acc No. DQ788616**).

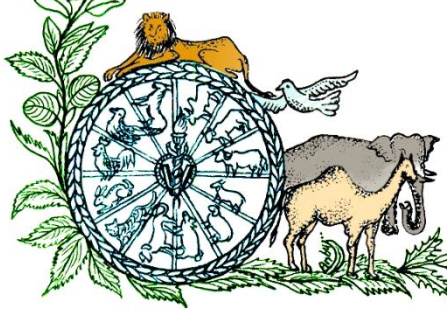
Mishra (2009) found two genotypes namely RS and SS in 100 bp amplified fragment exon 14 of Mx1 gene in Aseel breed of chicken. The genotype frequencies were as 0.655 and 0.345 for RS and SS genotypes, respectively. The allelic frequencies were as 0.33 and 0.67 for R and S alleles, respectively. Mishra (2009) also studied 284 bp of promoter region of Mx1 gene in Aseel breed of chicken and found three genotypes (AB, BC and AC) and three alleles (A, B and C). The frequencies of AB, BC and AC genotypes and A, B and C alleles were estimated. The AB genotype was most frequent (0.74) followed by AC (0.14) and BC (0.12) genotype. The allelic frequencies of A, B and C alleles were 0.44, 0.43 and 0.13, respectively.

Singh (2010) found three genotypes namely RR, RS and SS in 102 bp amplified fragment of exon 14 of Mx1 gene in Kadaknath and RIR breeds. In Kadaknath the genotype frequencies were 0.37, 0.43 and 0.20 for RR, RS and SS genotypes, and in RIR the genotype frequencies were 0.17, 0.36 and 0.47 for RR, RS and SS genotypes, respectively. The allelic frequencies were as 0.33 and 0.67 for R and S alleles, respectively. Singh (2010) also studied 284 bp of promoter region of Mx1 gene in Kadaknath and RIR breeds of chicken. In Kadaknath and RIR the genotype frequencies were found to be 0.26, 0.21 and 0.53 for CC, CE and CF genotypes, respectively.

Ramesh (2011) found two genotypes namely RS and SS in 102 bp amplified fragment of exon 14 of Mx1 gene in Naked Neck and Tellicherry breeds. The genotypes and gene frequencies were found to be 0.42, and 0.58 for RS and SS genotypes, respectively and 0.21and 0.79 for R and S alleles, respectively in Naked Neck. In Tellicherry the genotypes and gene frequencies were found to be 0.38 and 0.62 for RS and SS genotypes, respectively and 0.19 and 0.81 for R and S alleles, respectively. Ramesh (2011) also studied 284 bp of promoter region of Mx1 gene in Naked Neck and Tellicherry breeds of chicken. In Naked Neck the genotype frequencies were found to be 0.26, 0.21 and 0.53 for CC, CE and CF genotypes, respectively. In tellicherry breed frequencies of DI,JK, KK, KL and KM genotypes were estimated as 0.16, 0.14, 0.20, 0.28 and 0.22, respectively.

So far, no report is available in the literature regarding polymorphism study about the Mx 1 gene in quail. Only a sequence submission by Qu *et al.* (2008) was found for Coturnix coturnix (common quail) Mx mRNA, partial cds of 2142 bp length **(NCBI Acc. No. EF575605)**.

Materials and Methods



3 Materials and Methods

3.1 EXPERIMENTAL BIRDS

The blood samples of 250 quail birds maintained at experimental quail farm of CARI, Izatnagar were used under present investigation.

3.2 COLLECTION OF BLOOD SAMPLES

About 0.5 ml of blood was collected from the jugular vein of each bird in a sterile eppendorf tube containing 0.5 M EDTA (@100µl/ ml of blood) as an anti-coagulant under sterile conditions. Samples were collected randomly within different lines of quail. After collection of blood, the tubes were tightly capped and were shaken gently to facilitate thorough mixing of blood with the anti-coagulant. The tubes were then kept immediately in icebox containing ice and gel cool packs and were transported to the laboratory immediately. After reaching to the laboratory, samples were kept in deep freeze at –20°C till the isolation of genomic dna.

3.3 ISOLATION OF GENOMIC DNA

Genomic DNA was isolated from the blood samples by Phenol: chloroform extraction method as described by Sambrook and Russell (2001) with slight modification. The steps followed for genomic dna isolation samples were as follows:

I. The blood samples were taken out of deep freeze and were thawed at room temperature.

Fig. 3.1 : Japanese quail birds

II. From the thawed blood samples, 0.1ml blood was transferred to eppendorf tube and then 1ml of PBS was added to it.

III. It was centrifuged @3000 rpm for 5 min at room temperature.

IV. The reddish tinged supernatant containing plasma and WBC was discarded by careful pipetting.

V. To the pellet containing RBC, 750µl of RBC lysis buffer was added and inverted to & fro for 15 min.

VI. 10il of Proteinase K (stock con. 20mg/ml) and 40 µl SDS (10%) were added in the above tube and then kept in water bath at 37°C overnight.

VII. Next day in the morning, the content in each tube was divided into half in another eppendorf tube so that each sample is now having a duplicate.

VIII. Equal volume of Tris- saturated Phenol (750µl) was added and mixed gently for 10 minutes.

IX. Two phases were separated by centrifuging at 5000 rpm for 5 min and the upper aqueous phase was transferred to a new micro-centrifuge tube and the duplicated samples are combined into one eppendorf tube.

X. Extraction with phenol was (steps VIII and IX) repeated twice.

XI. The aqueous phase was then extracted twice with equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1).

XII. Lastly, the aqueous phase was extracted twice with equal volume of Chloroform: Isoamyl Alcohol (24:1).

XIII. DNA was precipitated from the aqueous phase by adding 2-2.5 volume of chilled absolute ethanol followed by gentle mixing.

XIV. The precipitated DNA was centrifuged at 10000 rpm for 10 min and the DNA pellet was washed twice with 70% ethanol.

XV. Finally, DNA pellet was air dried for 1-2 hrs to remove all the traces of ethanol and was subsequently dissolved in 100 µl distilled water.

XVI. The eppendorf tubes were kept in water bath at 60°C for 2 hr to inhibit DNase activity and to dissolve pellet properly.

XVII. After 2 hr of incubation, the DNA was cooled and stored at 4°C for one week for its proper dissolving in water and then at -20°C for further use.

3.4 CHECKING OF QUALITY, PURITY AND CONCENTRATION OF DNA

The genomic DNA isolated from the blood samples was checked for quality, purity and concentration. Only the DNA samples of good quality, purity and concentration were used for further analysis.

3.4.1 Quality of genomic DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8% w/v agarose. At first the gel casting tray was prepared by sealing its both end with adhesive tape and then the comb was arranged over it in such a way that there remains a gap of at least 0.5 mm between the tips of the comb teeth and floor of the casting tray, so that the wells were completely sealed by agarose.

Subsequently, 0.8% agarose (w/v) suspension in 0.5X TBE buffer was made and heated on an electric heater until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling it to 60°C, ethidium bromide (10 mg/ml) @ 5 µl per 100 ml of agarose solution was added to a final concentration of 0.5 mg/ ml and was mixed gently.

The agarose solution was poured in to the sealed casting tray. The gel was prepared to about 4 mm thicknesses. The agarose gel was allowed to set completely at room temperature and then the comb was

gently removed. The adhesive tape was also removed and gel casting platform was submerged in the electrophoresis tank containing 0.5 X TBE buffer.

For loading the samples, 5 µl autoclaved triple distilled water along with 2 µl diluted DNA was taken and after mixing it with 2 µl of 6 X gel loading dye (xylene cyanol and bromophenol blue), it was loaded into the well with the help of microtips. A marker DNA was also run in one of the well. Electrophoresis was performed at 5V/cm (40-50 V) for 3 hrs. Once the electrophoresis was over, the gel was visualized under UV transilluminator and documented by photography. Only DNA samples showing intact band and devoid of smearing were used for further analysis.

3.4.2 Purity of genomic DNA

The purity of genomic DNA was checked using UV-Spectrophotometry. About 6 µl of genomic DNA of each sample was dissolved in 294 µl of triple distilled water and spectrophotometric reading at OD_{260} and OD_{280} were taken against 300 µl triple distilled water as blank.

Only the genomic DNA samples lying in the ranges of OD ratio (260:280) between 1.7 to 1.9 were considered good and were used for further study (PCR amplification) and those showing value beyond this range were reprocessed by phenol: chloroform extraction method.

3.4.3 Concentration of genomic DNA

For estimating the concentration of genomic DNA following formula was employed

$$\text{DNA concentration (mg/ml)} = \frac{OD_{260} \text{ x dilution factor x } 50}{1000}$$

(1 OD value at 260nm is equivalent to 50 ng dsDNA/µl)

3.5 AMPLIFICATION OF THE Mx 1 GENE

Five separate fragments of Mx 1 gene, one for RFLP study and other four for SSCP study, were selected for amplification in commercial Japanese quail based on assumptions of similarity with Mx 1 gene of chicken due to homology. The RFLP fragment of 100 bp spanning over a part of intron 13 to part of exon 14 was amplified using the forward (M_1) and reverse (M_2 & M_3) primers as reported by Seyama *et al.* 2006 (Table 3.1). Another four fragments for SSCP study were selected from different exonic regions of Mx 1 gene viz. i). fragment-I of 185 bp consisting of Exon 3 region, ii). fragment-II of 148 bp consisting of Exon 5 region, iii). fragment-III of 161 bp consisting of Exon 7 region and iv). fragment-IV of 176 bp consisting of Exon 13 region (Fig. 3.2). The primers for these four fragments were designed with the help of Lasergene Software (DNASTAR) taking consensus sequences of common quail (Coturnix coturnix) mRNA partial cds (NCBI Acc. No. EF575605) and chicken Mx 1 gene sequences available publicly at NCBI (Acc no. DQ788613, Acc no. DQ788614, and Acc no. DQ788615).

3.5.1 PCR primers

Two pair of primers for amplification of 100 bp RFLP fragment of Mx 1 gene of Japanese quail was selected from the literature (Seyama *et al.*, 2006). The sequences of primers are given in Table 3.2. After received the lypholized primers from the commercial suppliers the stock primers were diluted with required DNase free water (Biogene, USA) so as to make a stock primer concentration of 100 p mole/µl. The working primer solution was further prepared by 10 fold dilution from the stock solution to have a final concentration of 10 p mole/µl. Then both the stock and working primers were stored at -20°C till use.

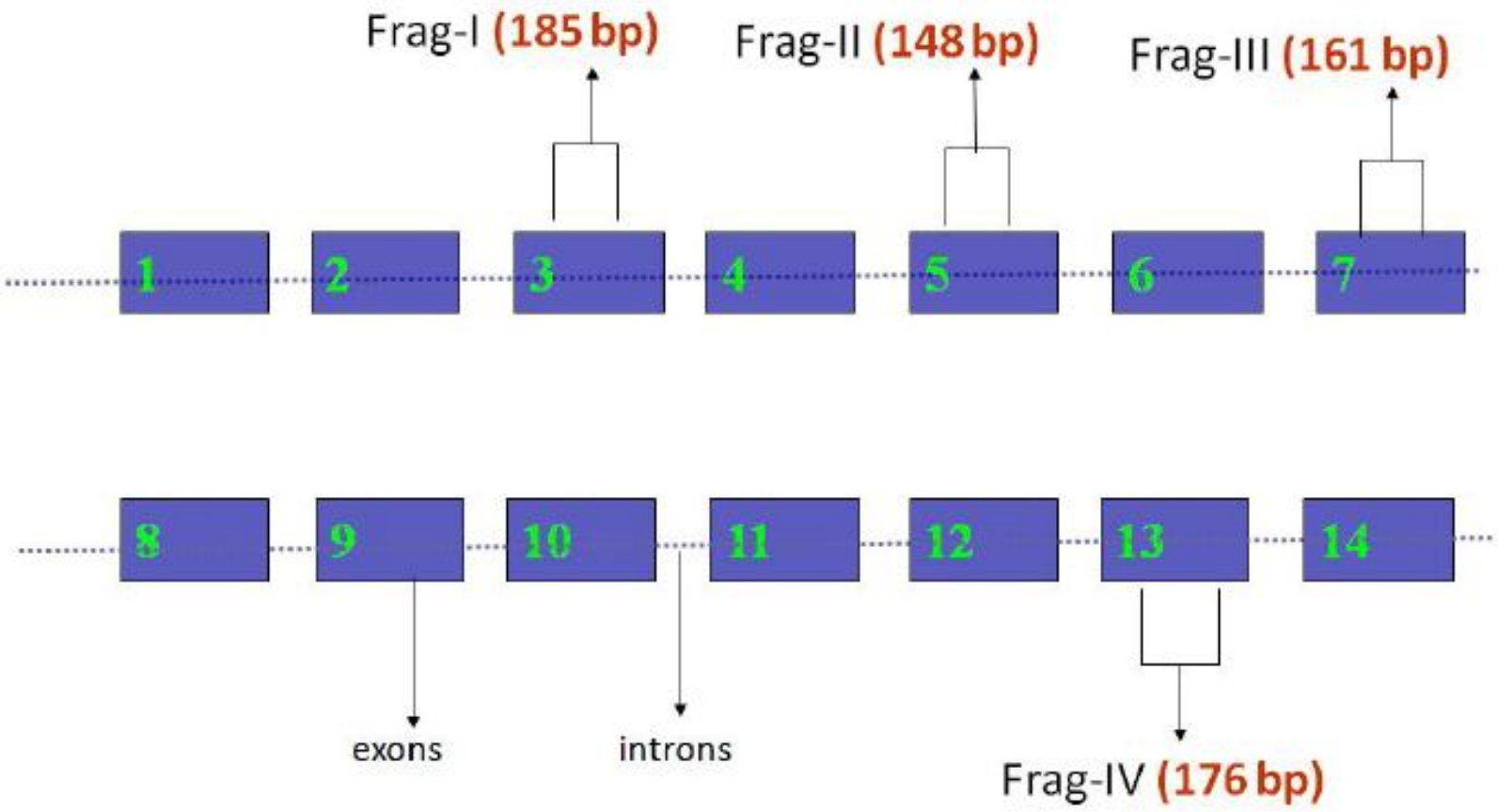


Fig. 3.2 : Regions of Mx 1 gene selected for amplification in Japanese Quail

Table 3.1 : Primer sequences used to amplify Mx 1 gene in Japanese quail

Primers	Sequences	Length (bp)	Amplified region
M_1 (F)	5'-CCTTCAGCCTGTTTTTCTCCTTTTAGGAA-3'	29	RFLP
M_2 (R)	5'-CAGAGGAATCTGATTGCTCAGGCGTGTA-3'	28	Frag.
M_3 (R)	5'-CAGAGGAATCTGATTGCTCAGGCGAATA-3'	28	(100bp)
E3 QF	5'- GCAGCAGAACACAGCTTTCA -3'	20	SSCP Frag.I
E3 QR	5'- CTAGGAAGAGCAACACCAGAC -3'	21	(185bp)
E5 QF	5'- CAGGATATAGTGGCTAGCAC -3'	20	SSCP Frag.II
E5 QR	5'- GGTCATTATCTTGTGGCTGGTTCC -3'	24	(148bp)
E7 QF	5'- TCCTCACTAAACCAGATCTGGTG-3'	23	SSCP Frag.III
E7 QR	5'- TTGCTGGATTACAGAGGCCAAGGA-3'	24	(161bp)
E13 QF	5'- GCAAGCAACAGCTGCGAAAA-3'	20	SSCP Frag.IV
E13 QR	5'- AAACCATTTCCAGGGCAAAGCTGG-3'	24	(176bp)

Apart from the above mentioned primers, some other set of primers were also tried previously for different regions of Mx 1 gene in Japanese quail. But unfortunately we could not standardize the PCR programme with those primers. Even after vigorous experiments, the amplification was unclear and/or multiple bands with poor quality. So further study with those primers could not be completed for the concerned fragments. List of those primers have been given separately in Table 3.2.

Table 3.2: Initially tried primer sequences to amplify Mx 1 gene in Japanese quail.

Primers	Sequences	Length (bp)	Amplified region
M4	5'-ACCTGTGCCATCTGCCCTCTGA-3'	22	Promoter region
M5	5'-CACAGCAAGGAGAAACAATTAACTACAT-3'	28	Frag. (284bp)
M18 F	5'-TGCAGGAGAAGGAGAAACCACAG-3'	23	Promoter
M19 R	5'-ACACAGCAAGGAGAAACAAT-3'	20	region
M20 F	5'-CTGCAGGAGAAGGAGAAACC-3'	20	Frag. (258bp)

Promx QF	5'-GAGAAGGAGAAACCACAGGACAAGGA-3'	26	Promoter region
Promx QR	5'-ATACACAGACTCCACCTACATGAC-3'	24	Frag.(200bp)
IN 13 QFI	5'-CTTGAATAGCAACTCCATACCGTG-3'	24	Partial Exon 14
E14 Q R1	5'-AGGACAGTAGAGAGGATGATCAGAGG-3'	26	region (181bp)
IN 13 QF II	5'-CACCTCTTAATAGAGTACCTTCAGCCTG-3'	28	Partial Exon 14
E14 Q R2	5'-GCAAACTATCTCCATAGTCGTG-3'	22	region (163bp)
IN 13 QF III	5'-TGAATAGCAACTCCATACCGTG-3'	22	Partial Exon 14
E14 Q R1	5'-AGGACAGTAGAGAGGATGATCAGAGG-3'	26	region (178bp)

3.5.2 Reaction Mixture

Various combinations of reaction chemicals were tried to optimize the concentration of each component. The standardized concentrations of the components used in the reaction mixture are given for five fragments, in Table 3.3 to 3.7.

Table 3.3 : PCR reaction mixture for RFLP fragment of 100 bp

Sl.No	Reaction components	Amount 1X (25 µl)	Final concentration
1	dNTPs mix (10 mM)	1 µl	100 mM
2	Primers		
	Forward Primer (M_1)	5 µl	50 ng
	Reverse primer (M_2 or M_3)	5 µl	50 ng
3	$MgCl_2$ (25 mM)	3 µl	3 mM
4	10 x PCR assay buffer	2.5 µl	1 x
5	Taq DNA polymerase (1U/ml)	1 µl	1 unit
6	Genomic DNA	1 µl	80-100 ng
7	Nuclease free water	6.5 µl	
	Total	**25 µl**	

Table 3.4. PCR reaction mixture for SSCP fragment-I (185 bp)

Sl.No	Reaction components	Amount 1X (25 µl)	Final concentration
1	dNTPs mix (10 mM)	1 µl	100 mM
2	Primers		
	Forward Primer (E3 QF)	1 µl	10 p mole
	Reverse primer (E3 QR)	1 µl	10 p mole
3	$MgCl_2$ (25 mM)	2 µl	2mM
4	10 x PCR assay buffer	2.5 µl	1 X
5	Taq DNA polymerase (1U/ml)	1 µl	1 unit
6	Genomic DNA	1 µl	80-100 ng
7	Nuclease free water	15.5 µl	
	Total	**25 µl**	

Table 3.5. PCR reaction mixture for SSCP fragment-II (148 bp)

Sl.No	Reaction components	Amount 1X (25 µl)	Final concentration
1	dNTPs mix (10 mM)	1 µl	100 mM
2	Primers		
	Forward Primer (E5 QF)	1 µl	10 p mole
	Reverse primer (E5 QR)	1 µl	10 p mole
3	$MgCl_2$ (25 mM)	2 µl	2mM
4	10 x PCR assay buffer	2.5 µl	1 X
5	Taq DNA polymerase (1U/ml)	1 µl	1 unit
6	Genomic DNA	1 µl	80-100 ng
7	Nuclease free water	15.5 µl	
	Total	**25 µl**	

Table 3.6. PCR reaction mixture for SSCP fragment-III (161 bp)

Sl.No	Reaction components	Amount 1X (25 µl)	Final concentration
1	dNTPs mix (10 mM)	1 µl	100 mM
2	Primers		
	Forward Primer (E7 QF)	1 µl	10 p mole
	Reverse primer (E7 QR)	1 µl	10 p mole
3	$MgCl_2$ (25 mM)	2 µl	2mM
4	10 x PCR assay buffer	2.5 µl	1 X
5	Taq DNA polymerase (1U/ml)	1 µl	1 unit
6	Genomic DNA	1 µl	80-100 ng
7	Nuclease free water	15.5 µl	
	Total	**25 µl**	

Table 3.7. PCR reaction mixture for SSCP fragment-IV (176 bp)

Sl.No	Reaction components	Amount 1X (25 µl)	Final concentration
1	dNTPs mix (10 mM)	1 µl	100 mM
2	Primers		
	Forward Primer (E13 QF)	1 µl	10 p mole
	Reverse primer (E13 QR)	1 µl	10 p mole
3	$MgCl_2$ (25 mM)	2 µl	2mM
4	10 x PCR assay buffer	2.5 µl	1 X
5	Taq DNA polymerase (1U/ml)	1 µl	1 unit
6	Genomic DNA	1 µl	80-100 ng
7	Nuclease free water	15.5 µl	
	Total	**25 µl**	

3.5.3 Setting up of PCR reactions

In the first step, a PCR master mix, containing all the reaction components except genomic DNA, was prepared. Care was taken to add *Taq* DNA polymerase at the end and the whole process was carried in ice under sterile conditions. After all the components were added, the master mix was mixed gently, followed by spinning by table top micro centrifuge. Master-mix of 24 µl was added to each labeled PCR tubes of 0.2 ml capacity kept in ice. Finally, 1 l (80-100 ng) of good quality diluted genomic DNA was added to each tube. The contents of the tube were gently mixed and spinned at 2000 rpm for 10 sec. Finally, the PCR tubes were kept in a preprogrammed thermocycler (Eppendorf gradient, Germany). PCR products obtained, after the completion of the programme, were kept at 4°C in refrigerator till further analysis.

3.5.4 PCR programme

Several combinations of PCR programmes were tried before setting on one programme giving the best amplification of the desired fragments. The standardized programmes for five fragments of Mx 1 gene in quail are given in Table 3.8 to 3.12.

Table 3.8. PCR programmes for RFLP fragment of 100 bp

	Steps	Temperature (°C)	Time
1	Initial denaturation	94	4 min
2	Denaturation	94	1 min
3	Annealing	**57.6**	30 sec
4	Extension	72	30 sec
5	Repeat	Step 2 to 4 for 35 cycles	
6	Final extension	72	10 min
7	Hold	4	5 min

Table 3.9. PCR programmes for SSCP fragment-I (185 bp)

	Steps	Temperature (°C)	Time
1	Initial denaturation	94	4 min
2	Denaturation	94	1 min
3	Annealing	**61**	1 min
4	Extension	72	1 min
5	Repeat	Step 2 to 4 for 35 cycles	
6	Final extension	72	10 min
7	Hold	4	5 min

Table 3.10. PCR programmes for SSCP fragment-II (148 bp)

	Steps	Temperature (°C)	Time
1	Initial denaturation	94	4 min
2	Denaturation	94	1 min
3	Annealing	**58.5**	1 min
4	Extension	72	1 min
5	Repeat	Step 2 to 4 for 35 cycles	
6	Final extension	72	10 min
7	Hold	4	5 min

Table 3.11. PCR programmes for SSCP fragment-III (161 bp)

	Steps	Temperature (°C)	Time
1	Initial denaturation	94	4 min
2	Denaturation	94	1 min
3	Annealing	**59.2**	1 min
4	Extension	72	1 min
5	Repeat	Step 2 to 4 for 35 cycles	
6	Final extension	72	10 min
7	Hold	4	5 min

Table 3.12. PCR programmes for fragment-IV (176 bp)

	Steps	Temperature (°C)	Time
1	Initial denaturation	94	4 min
2	Denaturation	94	1 min
3	Annealing	**61.2**	1 min
4	Extension	72	1 min
5	Repeat	Step 2 to 4 for 35 cycles	
6	Final extension	72	10 min
7	Hold	4	5 min

3.5.5 Checking of the amplified product

Horizontal submarine agarose gel electrophoresis was carried out to check the amplified product. A 1.5% w/v agarose (Low EEO) gel was prepared in 0.5 X TBE and subsequently 5 ml PCR product mixed with 1 ml of 6 X gel loading dye (Bromophenol blue and xylene cyanol) was loaded along with 100 bp ladder (Gene Ruler ™ Plus) as a marker in a separate lane. The electrophoresis was done at 60 volt for 2 hr. The amplified product in the gel was observed under UV transilluminator and documented by photography.

3.6.1 Restriction enzyme digestion of 100 bp RFLP fragment

To identify the restriction fragment length polymorphism, two restriction enzymes (*Rsa* I for M_1/M_2 amplified product and *Ssp* I for M_1/M_3 amplified product) were used for 100 bp fragment of Mx 1 gene (Seyama *et al.*, 2006). The RE digestion was carried out in 20µl as per the manufacturer's instruction. The compositions of various reaction components with their respective amount are given in Table 3.13 and 3.14.

Table 3.13. *Rsa* I RE digestion of 100 bp RFLP fragment of Mx 1 gene

Sl No	Reaction components	Amount (1x)
1	*Rsa* I (10U/µl)	1µl
2	10x Tango buffer	2µl
3	Nuclease free water	7µl
4	PCR product	10µl

Table 3.14. *Ssp* I RE digestion of 100 bp RFLP fragment of Mx 1 gene

Sl No	Reaction components	Amount (1x)
1	*Ssp* I (10U/µl)	1µl
2	10x buffer G	2µl
3	Nuclease free water	7µl
4	PCR product	10µl

All the digestion components were placed on ice and added one by one as per the order given in the Tables in a 0.5 ml eppendorf tube, except PCR product. The master mix was dispensed into 0.2 ml PCR tubes and PCR products were added to corresponding tubes. The tubes were spun in the microfuge for a while and incubated as per the conditions recommended for each REs. The restriction enzyme digestion was carried out for 16 hours at 37°C in a water bath for both the enzymes. After digestion the digested products were kept in refrigerator at 4°C till further study.

3.6.2 Agarose gel electrophoresis of RE digested PCR product

The digested PCR product was electrophoresed in 4% w/v agarose gel for 2 hours at 60V in 0.5X TBE buffer. About 15 µl of digested product was mixed with 3 µl of 6X gel loading dye and then loaded into the corresponding wells. After completion of gel electrophoresis, the digested products were visualized by keeping the gel over UV transilluminator and documented by gel documentation system (Syngene, USA) at different zoom levels. For better comparison, an undigested sample was also loaded to the gel. Around 5µl (500ng) of 100 bp DNA marker was also run parallel to the digested product in one of the wells to determine the size of the fragments obtained. Photographs of all the gels were stored in computer system and used for further analysis of genotype pattern of different animals.

3.7 SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) OF FOUR FRAGMENTS OF MX 1 GENE IN JAPANESE QUAIL

Single-Strand Conformation Polymorphism (SSCP), or Single-Strand Chain Polymorphism, is used to identify the conformational difference of single-stranded nucleotide sequences of identical length as induced by differences in the sequences under certain experimental conditions. This property allows distinguishing the sequences by means of gel electrophoresis, which separates the different conformations.

The PCR-SSCP technique was used to study the polymorphism by screening the single nucleotide polymorphisms in fragment I (185 bp), fragment II (148 bp), fragment III (161 bp) and fragment IV (176 bp) of Mx 1 gene in quail using single strand conformation polymorphism technique. Polyacrylamide gel of 15% concentration was made for SSCP study. The composition used in preparation of polyacrylamide gel mix is given in Table 3.15.

Table 3.15. Composition of polyacrylamide gel

Sl No	Reagents	Quantity in 30 ml (15%) approx
1	Acrylamide: Bis (49:1)	9.0 ml
2	5X TBE	6 ml
3	Glycerol	1 ml
4	10% APS	250 µl
5	TEMED	27 µl
6	Autoclaved double distilled water	Upto 30 ml

Procedure:

1 Glass plates and spacer combs were washed with detergent, rinsed initially under running tap water till no remains of detergent were left and finally in double distilled water before drying. Before use, glass plates, spacers and comb were cleaned with ethanol and dried.

2 Glass plates were fitted by putting the 1 mm spacer between the two plates at its position and then clamp is applied. Flat end of glass plates was sealed by adhesive tape initially. Finally this end was sealed by pouring 0.5% agarose into the space between the glass plates and left for sometime for solidification of agarose. Then the freshly prepared gel mix was poured into the space between plates and finally comb was inserted immediately.

3 The above gel was allowed to polymerize at room temperature for 1h.

4 After polymerization, adhesive tape was removed from sealed end and gel was put in electrophoresis tank with notched plate facing towards the buffer reservoir.

5 The reservoir of the electrophoresis tank was filled with 1X TBE the comb is removed carefully and the gel was given a pre-run at 200 V for 30 min, in a vertical gel electrophoresis system.

6 About 6 µl of PCR product was taken in a 0.2 ml PCR tube and 6 µl formamide dye and 6 µl denaturing solution was added in it and mixed properly. Then, PCR tubes were sealed with the paraffin films.

7 PCR product and formamide dye mix were denatured at 95°C for 10 min (By keeping on 94°C hot water on heater) and snapped cool on ice for 15 min.

8 The product was loaded in gel carefully. The electrophoresis was performed at 4°C temperature for 13-16 h at 130 constant volt. The current, voltage and running time was standardized as per the size and composition of PCR product.

9 After running was over, gel was kept for silver staining to visualize the banding pattern.

3.7.1 Silver staining

Silver staining was carried out according to Basam *et. al.* (1991) with some modifications.

Removal of plate

1. The gel along with plate was placed on a clean surface by keeping the notched plate facing upwards.
2. The upper plate was separated gently by taking care to avoid breakage of the gel.
3. The first row loaded was marked by cutting the upper portion of the gel from that side.
4. The gel along with the plate was placed in a suitable sized tray. The gel was removed by flooding with distilled water so as to take the gel into the tray. Then DW was removed and the gel was fixed.

Fixing the Gel

5. About 200 ml of freshly prepared 10% ethanol was added in the tray, shaken gently for 5 min and the plate was removed carefully after the gel got detached. Then fixing of the gel with the ethanol solution was done by agitating it slowly for 5 min in the shaker.
6. The gel was rinsed 2 times for 1 min each with double distilled water.
7. Then the gel was oxidized by adding about 200 ml of freshly prepared 3% nitric acid in the tray, shaken gently for 3 min or until the tracking dye was no longer visible.
8. Again the gel was rinsed 2 times for 1 min each with double distilled water.

Staining by Silver nitrate

9. 300 ml of 0.2% silver nitrate solution was added to the tray and agitated slowly for 30 min.
10. The gel was rinsed 2 times for 30 sec each with double distilled water so as to remove all the traces of ethanol.

Developing the Gel

11. For developing the gel 400 ml of 3% sodium carbonate (12 gm in 400 ml) with 500 µl of 37% formaldehyde (added 15

minutes before) freshly prepared solution was used. At first about 100 ml of this solution was poured in the tray and gel is washed with it by shaking for about 30 sec to 1 min. Then immediately rest 300 ml of the solution was poured in the tray and the gel is gently shaken till development of the distinct bands.

12. The reaction was stopped by adding 100 to 200 ml of 10% glacial acetic acid.

The gel was visualized and documented by the gel documentation system and scanned by the scanner.

3.8 GENE AND GENOTYPE FREQUENCY

The genotypes were detected by seeing the PCR- RFLP and SSCP pattern of each sample in the gel. The gene and genotypes frequencies were estimated by standard procedure (Falconer and Mackey, 1996):

$$\text{Genotype frequency} = \frac{\text{Total No. of individual of a particular genotype}}{\text{Total No. of individuals of all genotypes}}$$

$$\text{Gene frequency} = \frac{D + \frac{1}{2}H}{N}$$

Where,

D= No. of homozygotes

H = No. of heterozygotes

N= Total no. of individual

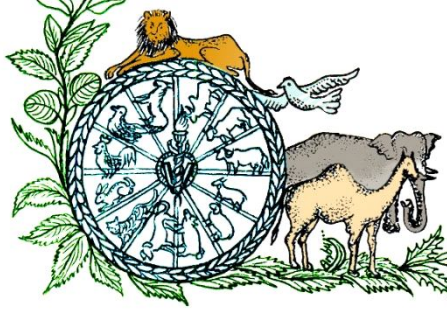
3.9 DNA SEQUENCING

Various genotypes of the four SSCP fragments i.e. 185 bp (Exon 3 region), 148 bp (Exon 5 region), 161 bp (Exon 7 region) and 176 bp (Exon 13 region) of Mx 1 gene in Japanese quail were selected for sequencing. Sequencing was performed for all the genotypes by automated dye terminator cycle sequencing method (ABI PRISM).

3.10 ANALYSIS OF Mx 1 GENE SEQUENCES OF CHICKEN

The sequences obtained from different birds were first blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of Mx 1 gene. Nucleotides as well as derived amino acid sequences were then aligned with that of the reported Mx 1 gene seuences of common quail (NCBI Acc. No. EF575605) and different breeds of chicken viz. RIR (NCBI Acc. No. DQ788613), SILKIE (NCBI Acc. No. DQ788614) and WLH (NCBI Acc. No. DQ788615), using the clustal W method of MegAlign Programme of Lasergene Software (DNASTAR). Percentage similarity and phylogenetic analysis were also performed to determine the evolutionary relationship.

Results and Discussion



4 Results and Discussion

In the present investigation, an attempt has been made to identify different allelic patterns of Mx1 gene in commercial Japanese quail (*Coturnix coturnix japonica*), by PCR-RFLP and PCR-SSCP techniques. A total number of five fragments were selected for study. For RFLP study, one fragment of 100 bp consisting of partial intron 13 and partial exon 14 was amplified. For SSCP study, four fragments as i). fragment-I of 185 bp consisting of Exon 3 region, ii). fragment-II of 148 bp consisting of Exon 5 region, iii). fragment-III of 161 bp consisting of Exon 7 region and iv). fragment-IV of 176 bp consisting of Exon 13 region of Mx 1 gene was amplified in each bird.

Various genotypes were identified and the gene and genotype frequencies were calculated. The different genotypes of Mx 1 gene were sequenced and the sequence data were compared with the available NCBI GenBank sequences.

4.1 POLYMORPHISM STUDY BY RFLP AND SSCP

Birds of commercial Japanese quail were selected (developed at CARI, Izatnagar) randomly to minimize any type of biasness in the results. For polymorphism study, genomic DNA was isolated and then checked for quality and purity. All the fragments of Mx 1 gene were amplified using suitable primers and then subjected to RFLP and SSCP analysis after optimizing various components.

4.1.1 Genomic DNA

Genomic DNA was isolated mostly from fresh blood and a few from frozen (-20°C) blood samples. Although, all the samples yielded sufficient amount of DNA, it was found that yield from fresh samples were appreciably higher than the frozen samples. This difference was probably due to damage of some RBC during the process of freezing and thawing. So, if condition permits, it is better to isolate genomic DNA from fresh blood or from refrigerated (4°C) blood within 1-2 days.

4.1.1.1 Quality and quantity of genomic DNA

Quality of genomic DNA was checked by following two methods.

i) Electrophoretic mobility

Under electrophoretic method, quality of genomic DNA samples isolated was assessed by submarine horizontal agarose (0.8%) gel electrophoresis. Most of the samples isolated showed clear and distinct band of genomic DNA under UV transilluminator indicating its good quality (Fig 4.1).

ii) Spectrophotometry

Quality was also judged by using UV spectrophotometry. The absorbance of the sample was seen in UV spectrophotometer at the wavelength of 260 nm and 280 nm. The ratio of optical density at the two wavelengths (OD260/OD280) indicates the purity of sample. OD ratio between 1.7 to 1.9 indicates good quality of genomic dna.

4.1.2 PCR optimization

PCR of genomic DNA is a complex series of chemical reactions where relative contributions to the overall progress vary between early, middle and late cycles. The crucial chemical variables (molecular balance between product, template, thermostable DNA polymerase,

primers and dNTPs changed with each cycle) decide the net synthesis of product during thermal cycling. Hence, an effort to get the best possible amplification, optimization of the reaction conditions were done by using different combinations of reaction mix. and different annealing temperature. The reaction combination giving the best results was chosen.

4.1.2.1 PCR reaction mixture

By trying several concentrations and combinations of each component, an optimum concentration of each component was decided to produce best result. The final concentrations of different components for 25 µl of reaction mixture are presented in Table 4.1.

The important step in optimizing the PCR programme is the optimization of annealing temperature. Several annealing temperatures varying from 55-65°C were tried for all the four exonic fragments. It was concluded that the best results could be observed at 57.6°C for 100 bp RFLP fragment. For SSCP fragments, the standardized annealing temperatures were 61°C for 185 bp fragment-I, 58.5°C for 148 bp fragment-II, 59.2°C for 161 bp fragment-III and 61.2°C for 176 bp fragment-IV of Mx 1 gene.

4.2 PCR-RFLP OF 100 bp FRAGMENT OF Mx 1 GENE

The PCR programme of Seyama *et al.* (2006) was used for the present study with slight modifications. To optimize the PCR conditions different annealing temperatures, initial denaturation time, and number of cycles were used. There occurred much difficulty in standardization of the PCR amplification for this fragment. After several trials, it was found that annealing temperature of 57.6°C for 30 sec for Mx 1 with initial denaturation of 94°C for 4 min followed by denaturation at 94°C for 1 min, extension (72°C, 30 sec) and a final extension at 72°C for 10 min and 35 cycles could produce amplified

Table 4.1. Optimized PCR reaction components for five fragments of Mx 1 gene in Japanese quail.

Sl No	Components	Concentrations				
		RFLP	SSCP			
		100 bp Frag	Frag.I (185 bp)	Frag.II (148 bp)	Frag.III (185 bp)	Frag.IV (176 bp)
1	dNTPs	100 mM (each)	100 mM (each)	100 mM (each)	100 mM (each)	100 mM (each)
2	Primers	50 pm (each)	10 pm (each)	10 pm (each)	10 pm (each)	10 pm (each)
3	$MgCl_2$	3 mM	2 mM	2 mM	2 mM	2 mM
4	Genomic DNA	80-100 ng	80-100 ng	80-100 ng	80-100 ng	80-100 ng

product for 100 bp fragment. But along with the 100 bp product there was presence of another undesired band just above 50 bp length, which was seen when the PCR product was run in 2% horizontal agarose gel electrophoresis. Initially we thought it as primer dimer, but even after taking different primer concentrations and/or varying the other ingredients of PCR reaction mixture, we could not succeed in removing this undesired band.

4.2.1 Amplification of the gene

The 100 bp (partial intron 13 and exon 14) fragment of Mx 1 gene was amplified in commercial Japanese quail by using the PCR primers M1, M2 and M3 (Fig.4.2).

The 100 bp amplified fragment was also reported by the same set of primers in WLH breed of chicken (Seyama *et al.*, 2006), Aseel (Mishra, 2009), Kadaknath and RIR (Singh, 2010) and Naked Neck and Tellicherry (Ramesh-2011).

4.2.2 PCR-RFLP

Digestion of 100 bp PCR product with *Rsa* I and *Ssp* I restriction enzymes in quail (Fig. 4.3) gave ambiguous results. Due to the presence of undesired bands of length just above 50bp in the PCR product, RE digestion always gave two bands, as like undigested PCR products which were used as control, by running in 2-4% range of horizontal agarose gel electrophoresis. So it was difficult to conclude whether the genotype is heterozygous or homozygous for one allele. Seyama *et al.* (2006) and Singh (2010) reported three genotypes (RR, RS and SS) in Jungle fowl and Kadaknath breeds, respectively, while, Mishra (2009) and Ramesh (2011) found two genotypes viz. RS and SS in Aseel breed and Naked Neck and Tellicherry breeds of chicken, repectively.

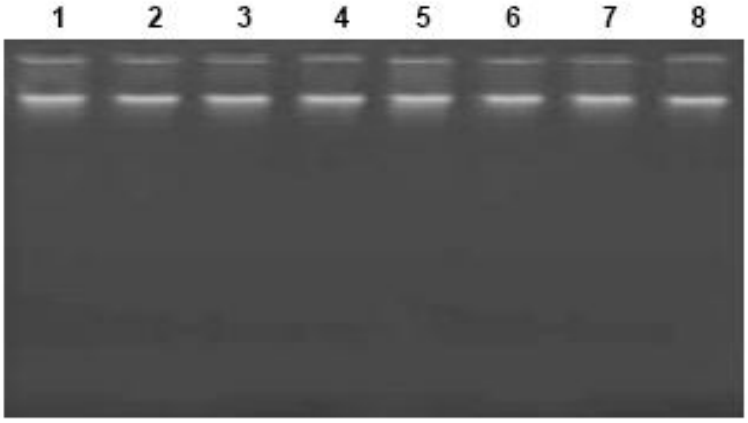


Fig. 4.1 : Genomic DNA of Japanese quail

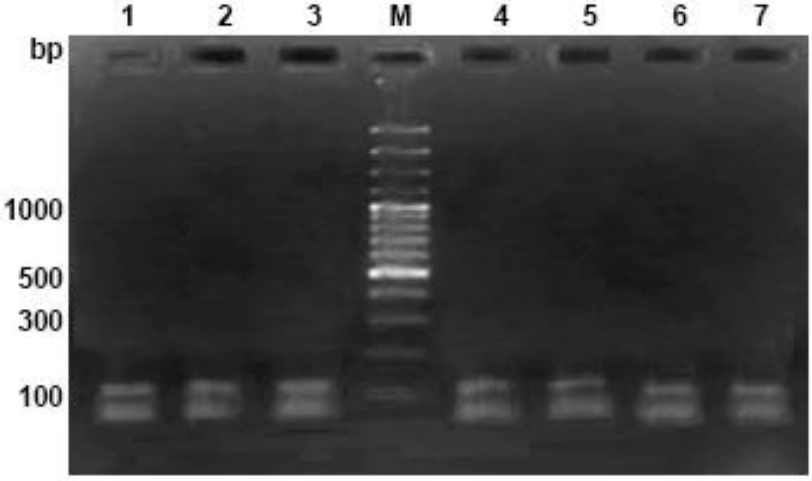


Fig. 4.2 : **Specific PCR amplification of 100 bp RFLP fragment of *Mx 1* gene of Japanese quail**
Lane M : 100 bp DNA ladder; **Lane 1-3 :** Amplification of 100 bp fragment by M1 and M2 primers; **Lane 4-6 :** Amplification of 100 bp fragment by M1 and M3 primers

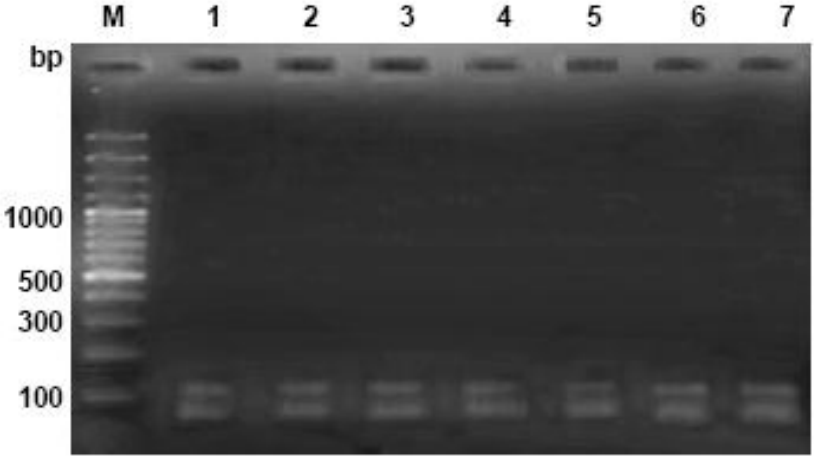


Fig. 4.3 : **PCR-RFLP digestion of 100 bp fragment of *Mx 1* gene in Japanese quail**
Lane M : 100 bp DNA ladder; **Lane 1-3 :** Digested PCR products of M1 and M2 primers; **Lane 4-6 :**Digested PCR products of M1 and M3 primers; **Lane 7 :** Undigested PCR product as control

4.2.3 Gene and Genotype Frequency

The 100 bp fragment of exon 14 of Mx 1 gene gave ambiguous result. So gene and genotype frequencies were not clear. Consequently further studies, like sequencing of this fragment could not be done.

4.3 PCR-SSCP of 185 bp (Frag.I), 148 bp (Frag.II), 161 bp (Frag.III) AND 176 bp (Frag.IV) of Mx 1 GENE IN JAPANESE QUAIL

SSCP is considered as a simple and reliable technique to find out polymorphism at single nucleotide level. The PCR-SSCP technique was used to study the polymorphism in the four different regions of Mx 1 gene of quail. These findings were compared with published sequences of common quail and different breeds of chicken viz. SILKIE, WLH and RIR.

4.3.1 SSCP optimization

Optimization of SSCP is necessary when this technique is carried out under a different set of laboratory conditions. Hence, an effort to get the best possible results, several combinations were tried to optimize the SSCP conditions as well as silver staining of DNA out of which the combination giving best result was chosen for the study.

i) Polyacrylamide gel mix.

In SSCP analysis, single stranded DNA is separated by polyacrylamide gel electrophoresis under denaturing condition on the basis of their secondary confirmation. Out of several chemicals used in polyacrylamide gel mix, concentration of acrylamide: bisacrylamide is most important because they form solid matrix by cross-linking with each other. So, optimization of acrylamide: bisacrylamide concentration were tried to get optimum concentration. Several concentrations viz 29:1, 49:1, 50:1 and 100:1 were tried out of which 49:1 concentration

was found to yield most satisfying result for all the fragments and used for the study. Glycerol provides the hydroxyl group, which causes the sharpening of bands in the gel. Polyacrylamide gel both with glycerol and without glycerol was tried. It was found that polyacrylamide gel with glycerol gave better result than without glycerol.

ii) Amplified Product and dye concentration

Different concentration ratio of amplified product and denaturing formamide dye was loaded in polyacrylamide gel for optimization. It was found that 6 µl amplified product with 6 µl formamide dye and 6 µl of denaturing solution gave best result and used in the study.

iii) Optimization of voltage, current and time

It is necessary to optimize voltage and time for running the polyacrylamide gel for SSCP study. The constant voltage at different levels like 100volt, 110volt, 120volt, 130volt, 140volt and 150volt were tried. After analyzing the result it was concluded that the gel run at 130volt constant voltage for 16hr, 13hr, 14hr and 15hr gave best result for fragment-I of 185 bp (Exon 3), fragment-II of 148 bp (Exon 5), fragment-III of 161 bp (Exon 7) and fragment-IV of 176 bp (Exon 13), respectively and were used in the present study.

Using optimized condition, SSCP of the four fragments from different exonic regions of Mx 1 gene was carried out for all the samples under study. All the samples showed bright, well, demarcated DNA band after silver staining.

4.3.2 SSCP analysis of 185 bp Frag.I of Mx 1 gene in Japanese quail

To detect polymorphism, the fragment-I of 185 bp length in Exon 3 region of Mx 1 gene was amplified and subjected to PCR-SSCP analysis for all the samples (Fig-3.2 of materials and methods).

4.3.2.1 Amplification of 185 bp Frag.I

The 185 bp fragment of Mx 1 gene was amplified in all the Japanese quail birds included in this study using the designed PCR primers E3 QF and E3 QR with optimized PCR programme and reaction conditions, which was confirmed after sequencing (Fig. 4.4). All birds gave satisfying level of amplification.

4.3.2.2 PCR-SSCP

The amplified product (185 bp) was subjected to SSCP analysis and different SSCP patterns were observed. The PCR product of different birds gave three different patterns depending on their single strand confirmation. Thus the SSCP analysis revealed 3 patterns i.e. AA, BC and AC. The SSCP patterns of 185 bp fragment are shown in Fig. 4.5. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.3.2.3 Gene and Genotype frequency

The gene and genotype frequency observed are presented in Table 4.2.

Table 4.2 Gene and genotype frequency of 185 bp Frag.I

Sl No	Genotype	Frequency
1	AA	0.22
2	BC	0.38
3	AC	0.40
	Alleles	**Frequency**
1	A	0.42
2	B	0.19
3	C	0.39

The genotype frequency of heterozygote AC and BC were highest with 0.40 and 0.38, respectively whereas allele A with frequency 0.42

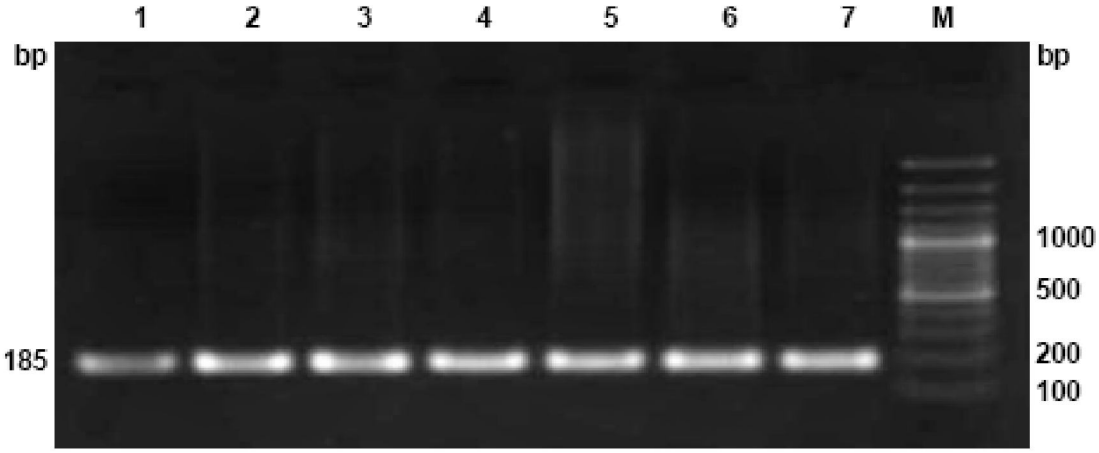


Fig. 4.4 : **Specific PCR amplification of 185 bp fragment of Mx 1 gene of Japanese quail**
Lane M : 100 bp DNA ladder
Lane 1-7 : Amplification of 185 bp fragment

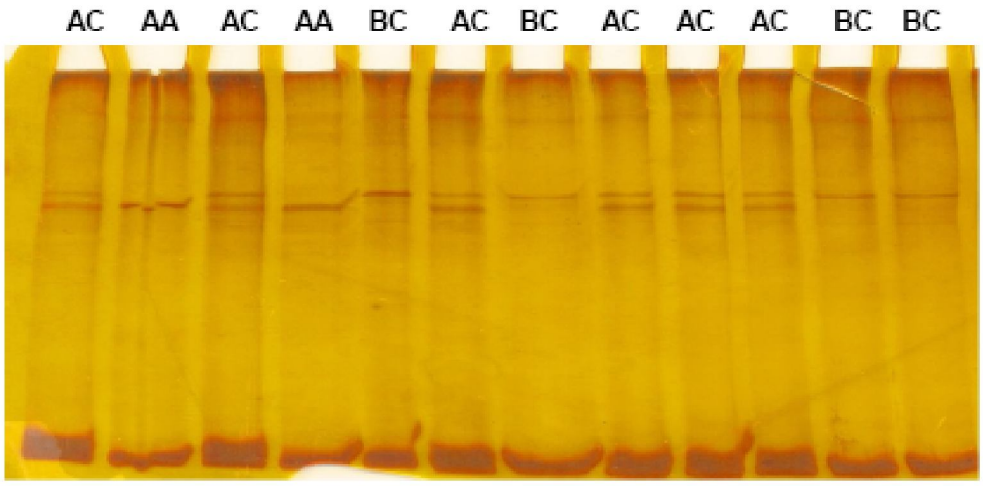


Fig. 4.5 : PCR-SSCP Genotypes of 185 bp (Frag-I) of *Mx 1* gene in Japanese quail

was at highest followed by allele C with 0.39 level frequency in the experimental population of Japanese quail used for study .This indicates that the natural selection may be favouring the AC and BC heterozygotes for this fragment in general. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.3.3 SSCP analysis of 148 bp Frag.II of Mx 1 gene in Japanese quail

To detect polymorphism, the fragment-II of 148 bp length in Exon 5 region of Mx 1 gene was amplified and subjected to PCR-SSCP analysis for all the samples (Fig-3.2 of materials and methods).

4.3.3.1 Amplification of 148 bp Frag.II

The 185 bp fragment of Mx 1 gene was amplified in all the Japanese quail birds included in this study with the designed PCR primers E5 QF and E5 QR with optimized PCR programme and reaction conditions, which was confirmed after sequencing (Fig. 4.6). All birds gave satisfying level of amplification.

4.3.3.2 PCR-SSCP

The amplified product (148 bp) was subjected to SSCP analysis and polymorphism was detected by observing SSCP patterns. The PCR product of different birds gave various patterns depending on their single strand conformation. Each bird showing different conformation pattern was assigned a specific genotype. The SSCP analysis revealed four patterns i.e. AA, BB, CC and DD. The SSCP patterns of 148 bp fragment are shown in Fig. 4.7.

4.3.3.3 Gene and Genotype frequency

The gene and genotype frequency observed are presented in Table 4.3. The genotype frequency of BB genotype was highest (0.44),

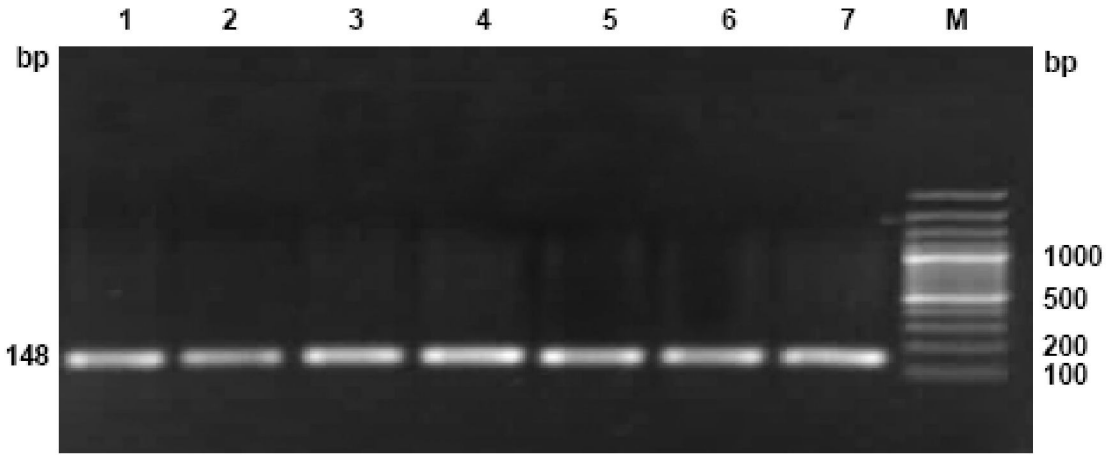


Fig. 4.6 : **Specific PCR amplification of 148 bp fragment of *Mx1* gene of Japanese quail**
Lane M : 100 bp DNA ladder
Lane 1-7 : Amplification of 148 bp fragment

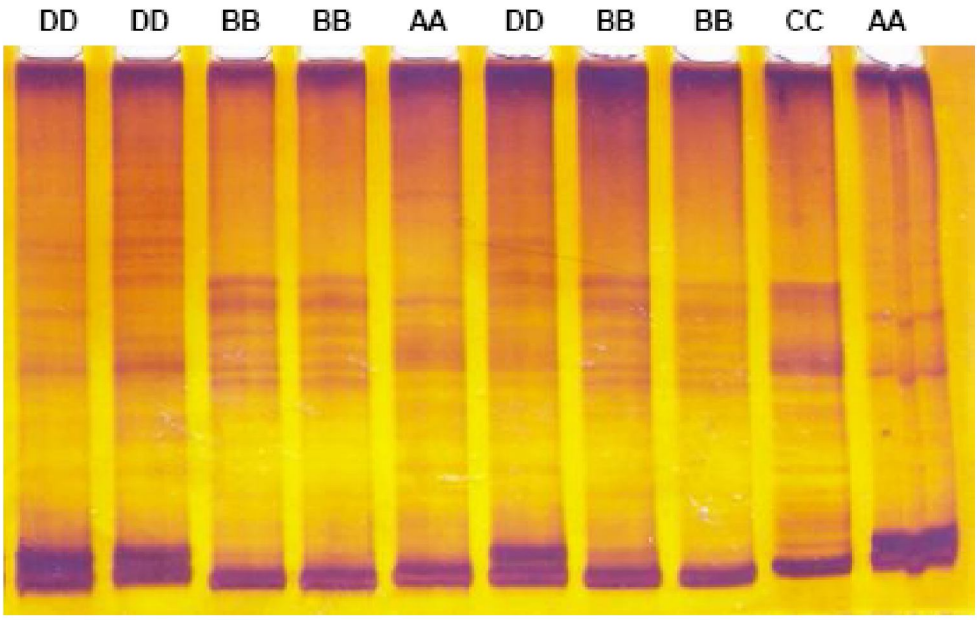


Fig 4.7 : **PCR-SSCP Genotypes of 148 bp (Frag-II) of *Mx 1* gene in Japanese quail**

followed by AA (0.23), CC (0.18) and DD (0.15) (Table 4.3). The heterozygous genotypes of A, B, C and D alleles could not be found in this population.

Table 4.3 Gene and genotype frequency of 148 bp Frag.II

Sl No	Genotype	Frequency
1	AA	0.23
2	BB	0.44
3	CC	0.18
4	DD	0.15
	Alleles	**Frequency**
1	A	0.23
2	B	0.44
3	C	0.18
4	D	0.15

There were four genotypes found in this population, out of which BB genotype was predominant with 0.44 frequency. The allelic frequency of B allele was highest (0.44), followed by A (0.23), C (0.18) and D (0.15). This indicates the natural selection may be favouring the BB genotype for this fragment in general. The heterozygote genotypes of A, B, C or D allele could not be found in the population. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.3.4 SSCP analysis of 161 bp Frag.III of Mx 1 gene in Japanese quail

To detect polymorphism, the fragment of 161 bp length in Exon 7 region of Mx 1 gene was amplified and subjected to PCR-SSCP analysis for all the samples (Fig-3.2 of materials and methods).

4.3.4.1 Amplification of 161 bp Frag.III

The 161 bp fragment of Mx 1 gene was amplified in all the Japanese quail birds included in this study with the designed PCR

primers E7 QF and E7 QR with optimized PCR programme and reaction conditions, which was confirmed after sequencing (Fig. 4.8). All birds gave satisfying level of amplification.

4.3.4.2 PCR-SSCP

The amplified product (161 bp) was subjected to SSCP analysis and SSCP patterns were observed. All the PCR products of different birds gave similar pattern depending on their single strand conformation. Thus the SSCP analysis revealed single pattern i.e. AA, which indicated that the 161 bp fragment is monomorphic. The SSCP pattern of 161 bp fragment is shown in Fig. 4.9.

4.3.3.3 Gene and Genotype frequency

The gene and genotype frequency observed are presented in Table 4.4.

Table 4.4 Gene and genotype frequency of 161 bp Frag.III

Sl. No.	Genotype	Frequency
1	AA	1.0
	Alleles	**Frequency**
1	A	1.0

There was a single allele 'A' found in this population. Thus both the gene and genotype frequency came to be 1.0 for this fragment. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.3.5 SSCP analysis of 176 bp Frag.IV of Mx 1 gene in Japanese quail

To detect polymorphism, the region i.e. 176 bp length in Exon 13 region of Mx 1 gene was amplified and subjected to PCR-SSCP analysis for all the samples (Fig-3.2 of materials and methods).

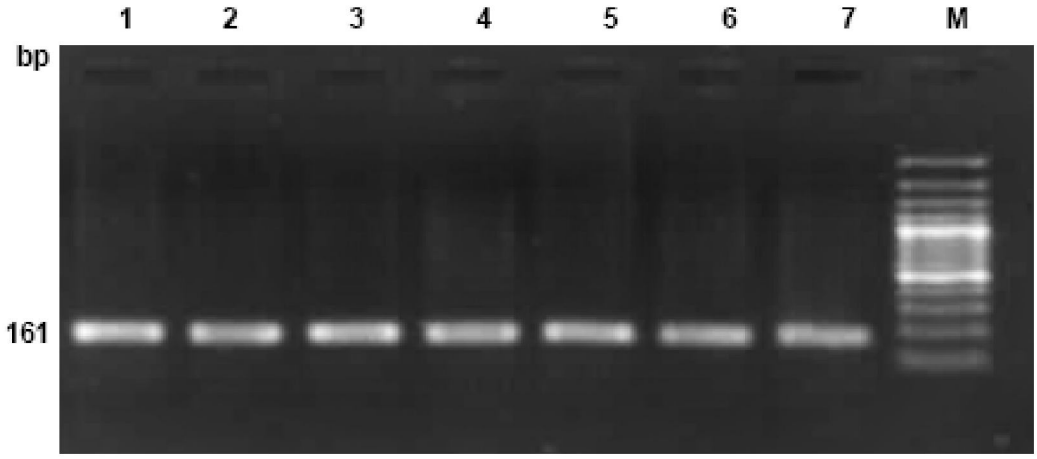


Fig. 4.8 : Specific PCR amplification of 161 bp of Mx1 gene of Japanese quail
Lane M : 100 bp DNA ladder
Lane 1-7 : Amplification of 161 bp fragment

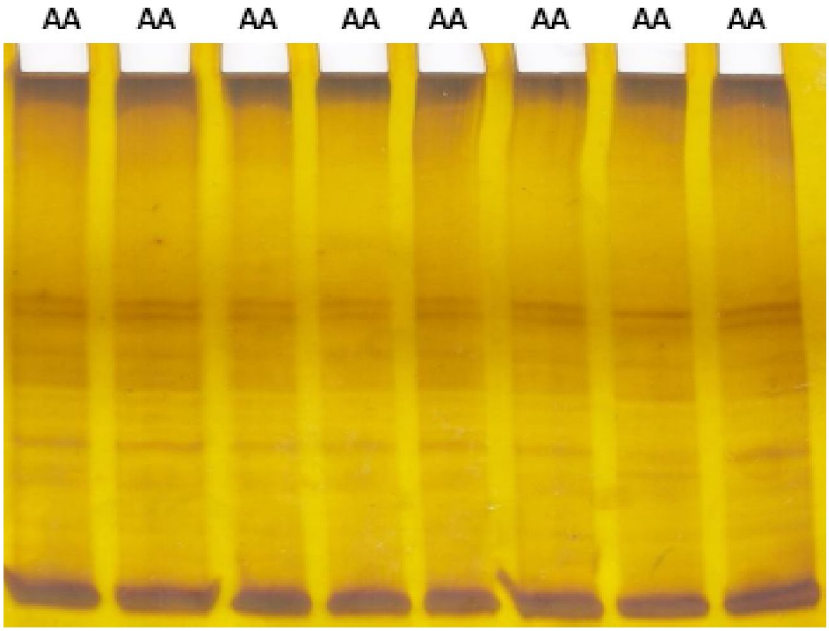


Fig. 4.9 : PCR-SSCP Genotypes of 161 bp (Frag-III) of Mx 1 gene in Japanese quail

4.3.5.1 Amplification of 176 bp Frag.IV

The 176 bp fragment of Mx 1 gene was amplified in all the Japanese quail birds included in this study with the designed PCR primers E13 QF and E13 QR with optimized pcr programme and reaction conditions, which was confirmed after sequencing (Fig. 4.10). All birds gave satisfying level of amplification.

4.3.5.2 PCR-SSCP

The amplified product (176 bp) was subjected to SSCP analysis and different SSCP patterns were observed. The PCR product of different birds gave two different patterns depending on their single strand confirmation. Thus the SSCP analysis revealed 2 patterns i.e. AA and BB. The SSCP patterns of 176 bp fragment are shown in Fig. 4.11. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.3.5.3 Gene and Genotype frequency

The gene and genotype frequency observed are presented in Table 4.5.

Table 4.5 : Gene and genotype frequency of 176 bp Frag.IV

	Genotype	**Frequency**
1	AA	0.36
2	BB	0.64
	Alleles	**Frequency**
1	A	0.36
2	B	0.64

The genotype frequency of BB genotype was highest with 0.64 and its allele frequency was highest with 0.64 in the population. This indicates the natural selection may be favouring the BB genotype for this fragment in general. The heterozygote genotypes of A or B allele

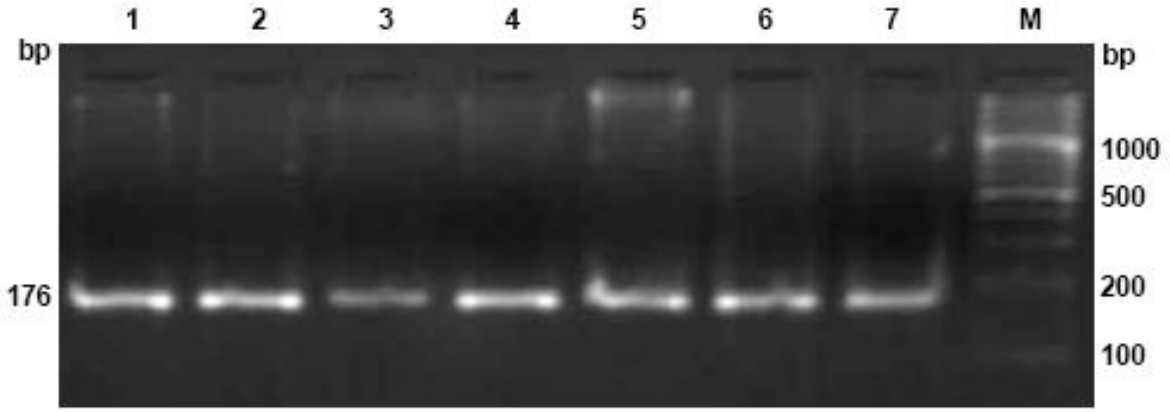


Fig 4.10 : **Specific PCR amplification of 176 bp of Mx1 gene of Japanese quail**
Lane M : 100 bp DNA ladder
Lane 1-7 : Amplification of 176 bp fragment

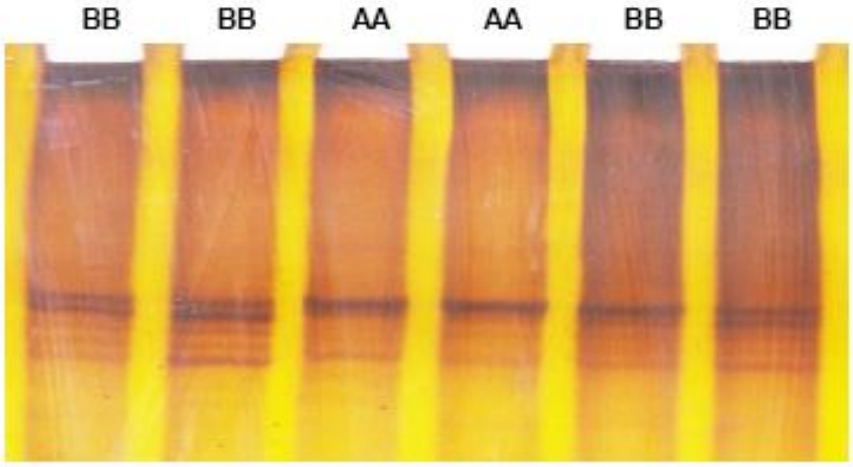


Fig. 4.11 : PCR-**SS**CP Genotypes of 176 bp (Frag-IV) of Mx 1 gene in Japanese quail

could not be found in the population. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

4.4 NUCLEOTIDE SEQUENCE ANALYSIS

For sequencing, various SSCP genotypes/patterns of 185 bp, 148 bp, 161 bp and 176 bp fragments of Mx 1 gene were selected on the basis of their SSCP patterns. These alleles were sequenced by Sanger's dideoxy chain termination sequencing method in Automatic ABI Prism DNA sequencer. Sequencing confirmed the amplification of all the fragments of Mx 1 gene in Japanese quail. The sequences obtained were subjected to NCBI BLAST and the similar sequences were downloaded from the Internet. After comparing with other available sequences of common quail and also different breeds of chicken (viz. RIR, SILKIE and WLH) the amplified fragments were confirmed. All the sequences for different fragments were aligned using MEGALIGN program of DNASTAR software.

4.4.1 Nucleotide sequencing of 185 bp Frag.I of Mx 1 gene in Japanese quail

Three different SSCP patterns of 185 bp fragment in Exon 3 region of Mx 1 gene of Japanese quail were sequenced. However the sequencing results confirmed all three having similar sequences, indicating presence of a single allele in the population (Fig.4.12). The sequencing confirmed the amplification of 185 bp fragment of this gene. The sequences obtained were subjected to NCBI BLAST and the similar sequences were downloaded from the database.

4.4.1.1 Comparative study of 185 bp Frag.I

The sequence of 185 bp fragment of Mx 1 gene of Japanese quail was aligned along with the published sequence of common quail and different breeds of chicken viz. RIR, SILKIE and WLH, using

MEGALIGN programme of DNASTAR software (Fig.4.13, Table 4.6). After analyzing, it was found that the variations at several places exist between quail group and chicken group sequences and the Japanese quail sequence matches exactly with the Common quail for this fragment. At 114th position, all the breeds have cytosine, whereas RIR breed has thymine at this position.

4.4.1.2 Percentage similarity and phylogenetic tree study for nucleotide sequence of 185 bp Frag.I

Percentage similarity study and phylogenetic tree creation was carried out between nucleotide sequences of Mx 1 gene of Common quail (NCBI Acc No. EF575605) and different breeds of chicken (NCBI Acc No. DQ788613, DQ788614 and DQ788615) along with Japanese quail by using dnaSTAR software, are presented in Fig. 4.14 & Fig. 4.15. The Japanese quail shows 100% homology with the Common quail sequence and are placed in separate cluster under phylogenetic tree.

4.4.1.3 Comparison of amino acid sequences of 185 bp Frag.I

The amino acid sequence of the 185 bp fragment of Mx 1 gene of Japanese quail was deduced from its nucleotide sequence and was compared with those of the published sequences available in NCBI database. The alignment report, percentage similarity and phylogenetic tree of the deduced amino acid sequence of this region is shown in fig.4.16, fig.4.17 & fig.4.18. There are differences of amino acids at 12 different sites within this region between quail and chicken group. There is 100% homology of Japanese quail with Common quail. Thus quail group form one cluster while chicken group form another cluster under phylogenetic tree.

4.4.2 Nucleotide sequencing of 148 bp Frag.II of Mx 1 gene

The four alleles (A, B, C and D) of 148 bp fragment of Exon 5 region of Mx 1 gene of Japanese quail were sequenced

```
          10        20        30        40        50        60
          |         |         |         |         |         |
GCAGCAGAACACAGCTTTCACAATGAATATGACATAAAGATTCGACCTTGCATTGACCTT 60
GTTGAGAGCTTGAGAAGGCTTGATATAGGAAACAAACTGATGTTGCCTATGATCACAGTG 120
ATTGGAGACCAGAACTCTGGGAAAAGCTCTGTCCTTGAAGCTTTGTCTGGTGTTGCTCTT 180
CCTAG 185
```

Fig. 4.12 : Nucleotide sequence of 185 bp fragment of *Mx 1* gene of Japanese quail

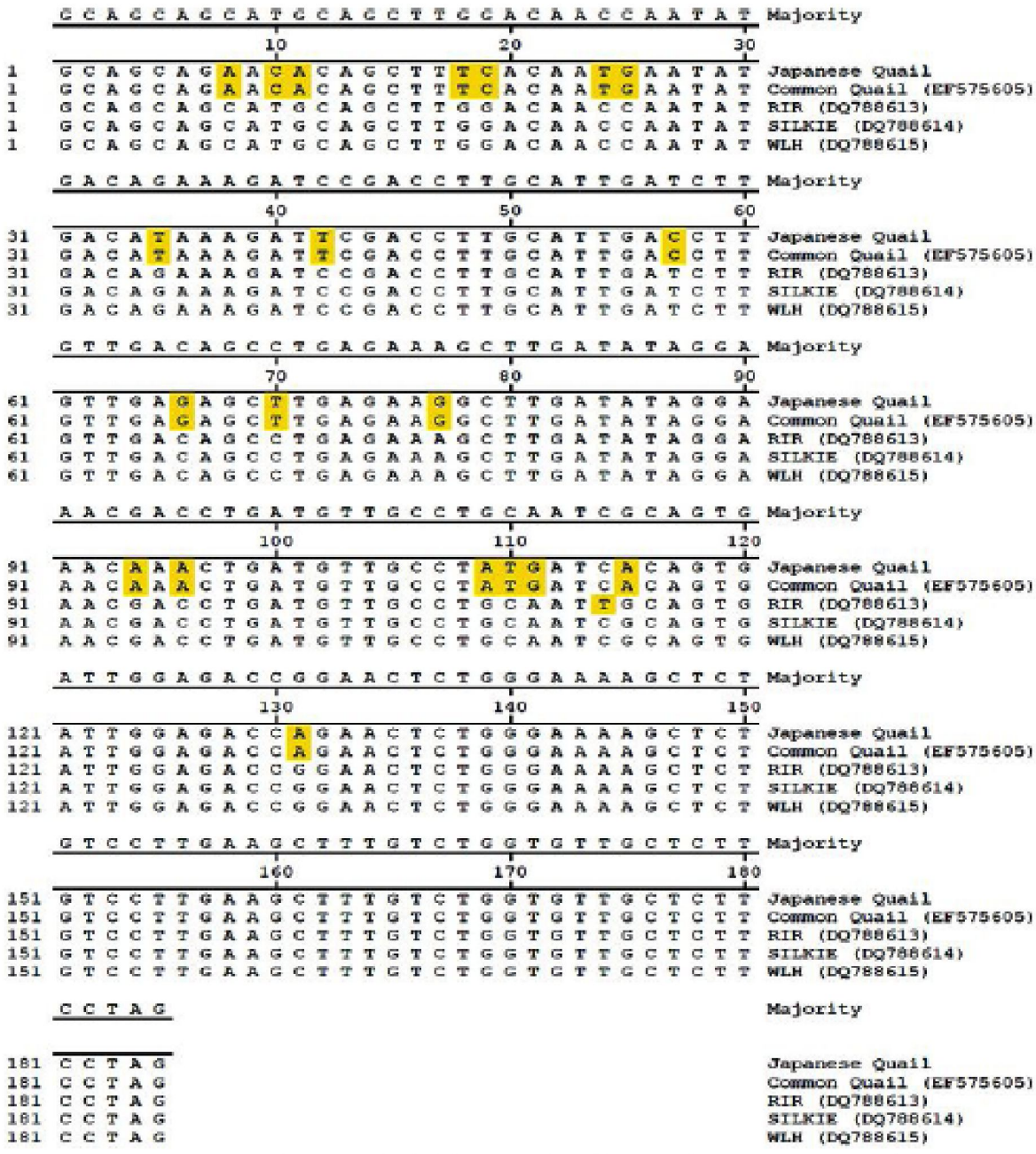


Fig. 4.13 : Nucleotide sequence alignment of 185 bp fragment of *Mx 1* gene of Japanese quail with available sequences of Common quail and different breeds of chicken

Percent Identity

	1	2	3	4	5	
1		100.0	88.6	89.2	89.2	1
2	0.0		88.6	89.2	89.2	2
3	12.5	12.5		99.5	99.5	3
4	11.8	11.8	0.5		100.0	4
5	11.8	11.8	0.5	0.0		5
	1	2	3	4	5	

Divergence

1 Japanese Quail
2 Common Quail (EF575605)
3 RIR (DQ788613)
4 SILKIE (DQ788614)
5 WLH (DQ788615)

Fig. 4.14 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of 185 bp *Mx 1* gene fragment

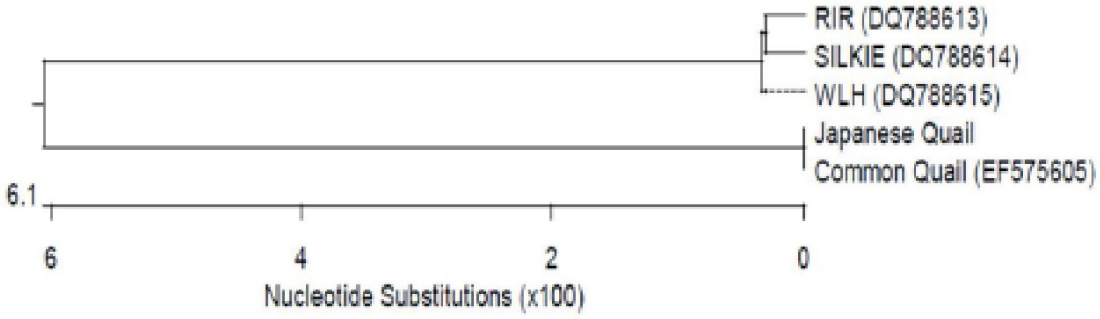


Fig. 4.15 : Phylogenetic tree based on 185 bp fragment of *Mx 1* gene in Japanese quail

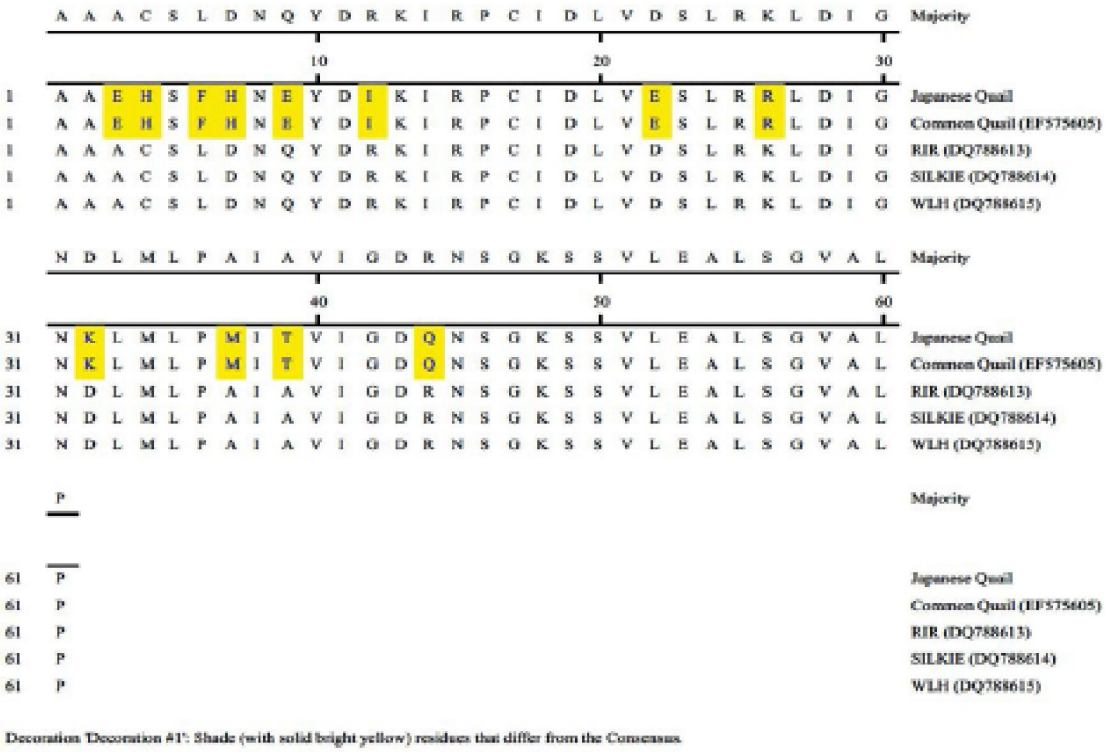


Fig. 4.16 : Amino acid sequence alignment of 185 bp fragment of *Mx 1* gene in Japanese quail

Percent Identity

	1	2	3	4	5	
1		100.0	80.3	80.3	80.3	1
2	0.0		80.3	80.3	80.3	2
3	22.9	22.9		100.0	100.0	3
4	22.9	22.9	0.0		100.0	4
5	22.9	22.9	0.0	0.0		5
	1	2	3	4	5	

Divergence

1 Japanese Quail
2 Common Quail (EF575605)
3 RIR (DQ788613)
4 SILKIE (DQ788614)
5 WLH (DQ788615)

Fig. 4.17 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of Amino acid sequence alignment of 148 bp *Mx 1* gene fragment

Table 4.6 : Comparative analysis of 185 bp Frag.I of Japanese quail with Common quail and different breeds of chicken

Sl No	Breeds	Nucleotide Position																			
		8th	10th	11th	18th	19th	24th	25th	35th	42nd	57th	66th	70th	77th	94th	96th	109th	110 th	111th	114th	115nd	131st
1	Japanese quail	**A**	**C**	**A**	**T**	**C**	**T**	**G**	**T**	**T**	**C**	**G**	**T**	**G**	**A**	**A**	**A**	**T**	**G**	**C**	**A**	**A**
2	Common quail	**A**	**C**	**A**	**T**	**C**	**T**	**G**	**T**	**T**	**C**	**G**	**T**	**G**	**A**	**A**	**A**	**T**	**G**	**C**	**A**	**A**
3	RIR	C	T	G	G	G	C	C	G	C	T	C	C	A	G	C	G	C	A	T	G	G
4	SILKI	C	T	G	G	G	C	C	G	C	T	C	C	A	G	C	G	C	A	C	G	G
5	WLH	C	T	G	G	G	C	C	G	C	T	C	C	A	G	C	G	C	A	C	G	G

(Fig.4.19). The sequencing confirmed the amplification of 148 bp fragment of this gene. The sequences obtained were subjected to NCBI BLAST and the similar sequences were downloaded from the database.

The allele wise nucleotide differences are presented in Table 4.7. From the alignment of the allelic variants, it was found that there are differences at 7 positions among the alleles. Allele B has thymine at 72nd position whereas other alleles have cytosine at that position. At 113th and 117th position A and C alleles have guanine and thymine respectively whereas B allele and D allele have adenine and guanine respectively at those positions. Similarly, allele A has cytosine, cytosine, adenine and guanine at positions 119^{th,} 122^{th,} 123rd and 124th where as other alleles have adenine, thymine, guanine and thymine at the respective positions.

Table 4.7 Allele-wise differences for 148 bp Frag.II of Mx 1 gene in Japanese quail

Sl. No.	Alleles	Nucleotide Position							
		72nd	113th	117th	119th	121st	122nd	123rd	124th
1	**A**	C	**G**	**T**	**C**	**G**	**C**	**A**	**G**
2	**B**	**T**	A	G	A	A	T	G	T
3	**C**	C	**G**	**T**	A	A	T	G	T
4	**D**	C	A	G	A	A	T	G	T

4.4.2.1 Comparative study of 148 bp Frag.II

All the four alleles of 148 bp of Japanese quail were aligned with the published sequences of common quail and different breeds of chicken (RIR, SILKIE and WLH) using MEGALIGN programme of DNASTAR software (Fig.4.20, Table 4.8). After analyzing, it was found that the variations at several places exist between quail group and chicken group sequences. At 35th position, RIR breed has cytosine

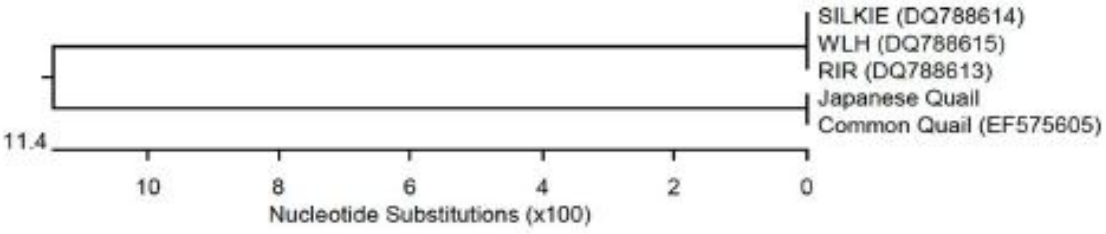


Fig. 4.18 : Phylogenetic tree on the basis of Amino acid sequence alignment of 148 bp *Mx 1* gene fragment in Japanese quail

Sequence of Mx1 gene of Japanese quail, 148 bp (A Allele)

```
          10        20        30        40        50        60
CAGGATATAGTGGCTAGCACTAGTGGTAACATTAGTGGAGAACTAATTTCCCTTGAAATC 60
TGGTCCCCGAACGTCCCAGACCTGACACTAATTGATCTTCCTGGAATTGCCAGAGTTGCC 120
GCAGGGAACCAGCCACAAGATAATGACC 148
```

Sequence of Mx1 gene of Japanese quail, 148 bp (B Allele)

```
          10        20        30        40        50        60
CAGGATATAGTGGCTAGCACTAGTGGTAACATTAGTGGAGAACTAATTTCCCTTGAAATC 60
TGGTCCCCGAATGTCCCAGACCTGACACTAATTGATCTTCCTGGAATTGCCAAAGTGGAC 120
ATGTGGAACCAGCCACAAGATAATGACC 148
```

Sequence of Mx1 gene of Japanese quail, 148 bp (C Allele)

```
          10        20        30        40        50        60
CAGGATATAGTGGCTAGCACTAGTGGTAACATTAGTGGAGAACTAATTTCCCTTGAAATC 60
TGGTCCCCGAACGTCCCAGACCTGACACTAATTGATCTTCCTGGAATTGCCAGAGTTGAC 120
ATGTGGAACCAGCCACAAGATAATGACC 148
```

Sequence of Mx1 gene of Japanese quail, 148 bp (D Allele)

```
          10        20        30        40        50        60
CAGGATATAGTGGCTAGCACTAGTGGTAACATTAGTGGAGAACTAATTTCCCTTGAAATC 60
TGGTCCCCGAACGTCCCAGACCTGACACTAATTGATCTTCCTGGAATTGCCAAAGTGGAC 120
ATGTGGAACCAGCCACAAGATAATGACC 148
```

Fig. 4.19 : Nucleotide sequence of four allelic variants of 148 bp fragment of *Mx 1* gene of Japanese quail

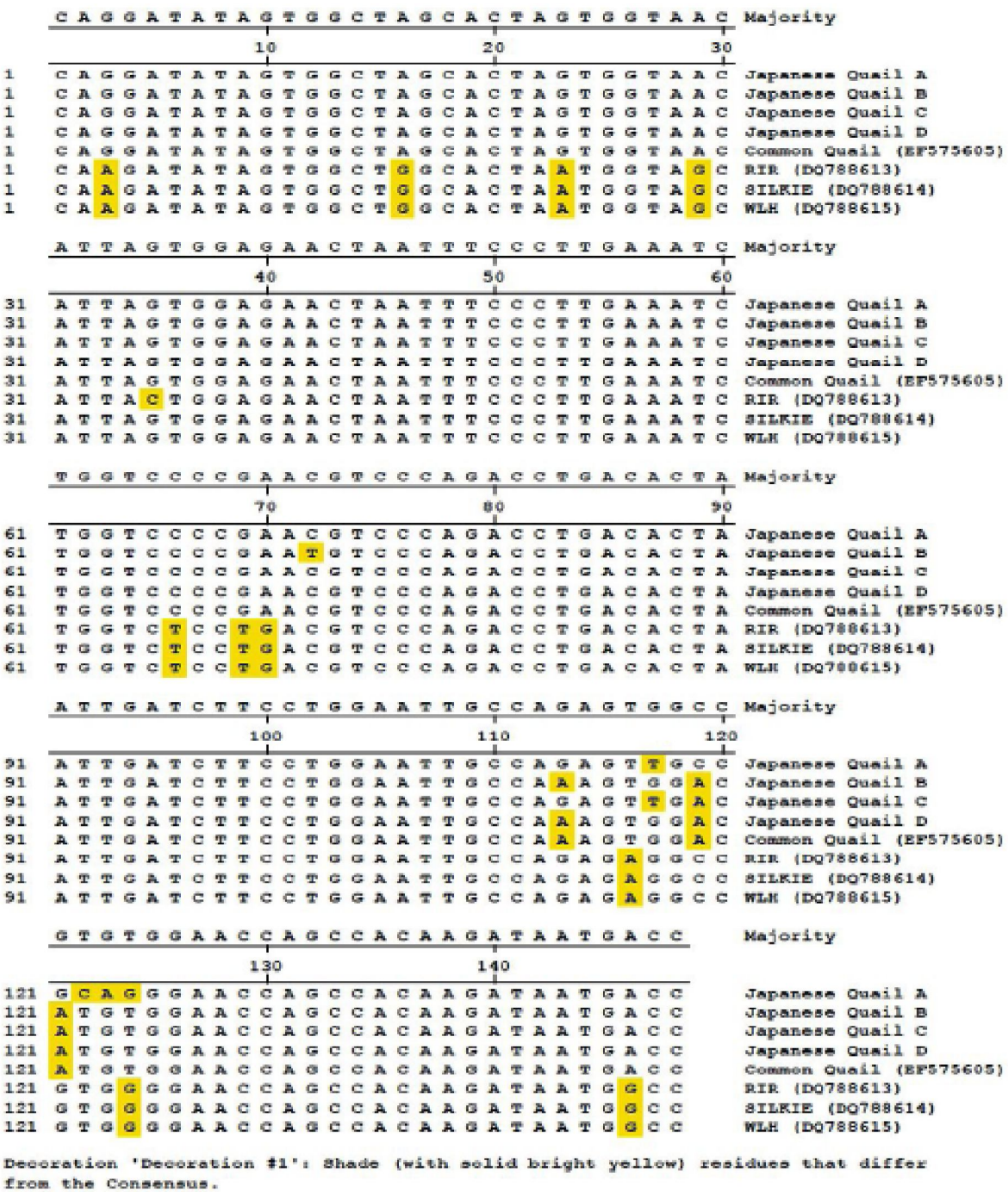


Fig. 4.20 : Nucleotide sequence alignment of different allelic variants of 148 bp fragment of *Mx 1* gene of Japanese quail with available sequences of Common quail and different breeds of chicken

Table 4.8 : Comparative analysis of different alleles of 148 bp Frag.II of Japanese quail with Common quail and different breeds of chicken

Sl No	Breeds	Nucleotide Position																	
		3rd	16th	23rd	29th	35th	66th	69th	70th	72nd	113th	116th	117th	119th	121st	122 nd	123 rd	124 th	146 th
1.	Japanese Quail																		
	A Allele	G	A	G	A	G	C	G	A	C	**G**	T	**T**	**C**	**G**	**C**	**A**	**G**	A
	B Allele	G	A	G	A	G	C	G	A	**T**	A	T	G	A	A	T	G	T	A
	C Allele	G	A	G	A	G	C	G	A	C	**G**	T	**T**	A	A	T	G	T	A
	D Allele	G	A	G	A	G	C	G	A	C	A	T	G	A	A	T	G	T	A
2	Common Quail	G	A	G	A	G	C	G	A	C	A	T	G	A	A	T	G	T	A
3	RIR	A	G	A	G	C	T	T	G	C	G	A	G	C	G	T	G	G	G
4	SILKI	A	G	A	G	G	T	T	G	C	G	A	G	C	G	T	G	G	G
5	WLH	A	G	A	G	G	T	T	G	C	G	A	G	C	G	T	G	G	G

whereas all others have guanine in this position. At 72nd position B allele of Japanese quail has thymine whereas all others have cytosine in that position.

4.4.2.2 Percentage similarity and phylogenetic tree study between alleles

Percentage similarity study was carried out between nucleotide sequences of Mx 1 gene of Common quail (NCBI Acc No. EF575605) and different breeds of chicken (NCBI Acc No. DQ788613, DQ788614 and DQ788615) along with Japanese quail by using DNASTAR software, are presented in Fig. 4.21. The allele D of Japanese quail has 100% homology with the Common quail sequence. The allele B and allele D of this fragment for Japanese quail formed a separate cluster with Common quail in phylogenetic tree (Fig. 4.22).

4.4.1.3 Comparison of amino acid sequences of 148 bp Frag.II

The amino acid sequences of the different alleles of 148 bp fragment of Mx 1 gene of Japanese quail was deduced from its nucleotide sequence and was compared with those of the published sequences available in NCBI database. The alignment report, percentage similarity and phylogenetic tree of the deduced amino acid sequence of this region is shown in Fig.4.23, Fig.4.24 & Fig.4.25. Apart from different variations as shown in the figure, the B allele and D allele of Japanese quail is showing 100% homology with Common quail amino acid sequence. The phylogenetic tree has placed, in a single cluster, the D allele and B allele of Japanese quail together with common quail, followed by C allele of Japanese quail.

4.4.3 Nucleotide sequencing of 161 bp Frag.III of Mx 1 gene

The single pattern shown by SSCP analysis for 161 bp fragment of Exon 7 region of Mx 1 gene of Japanese quail was sequenced (Fig.4.26). The sequencing confirmed the amplification of 161 bp

Percent Identity

	1	2	3	4	5	6	7	8		
1		94.6	96.6	95.3	95.3	91.2	91.9	91.9	1	Japanese Quail A
2	5.6		98.0	99.3	99.3	89.9	90.5	90.5	2	Japanese Quail B
3	3.5	2.1		98.6	98.6	90.5	91.2	91.2	3	Japanese Quail C
4	4.9	0.7	1.4		100.0	90.5	91.2	91.2	4	Japanese Quail D
5	4.9	0.7	1.4	0.0		90.5	91.2	91.2	5	Common Quail (EF575605)
6	9.4	11.0	10.2	10.2	10.2		99.3	99.3	6	RIR (DQ788613)
7	8.7	10.2	9.4	9.4	9.4	0.7		100.0	7	SILKIE (DQ788614)
8	8.7	10.2	9.4	9.4	9.4	0.7	0.0		8	WLH (DQ788615)
	1	2	3	4	5	6	7	8		

Divergence

Fig. 4.21 : Similarity and divergence between different allelic variants of Japanese quail, Common quail and different breeds of chicken on the basis of 148 bp *Mx 1* gene fragment

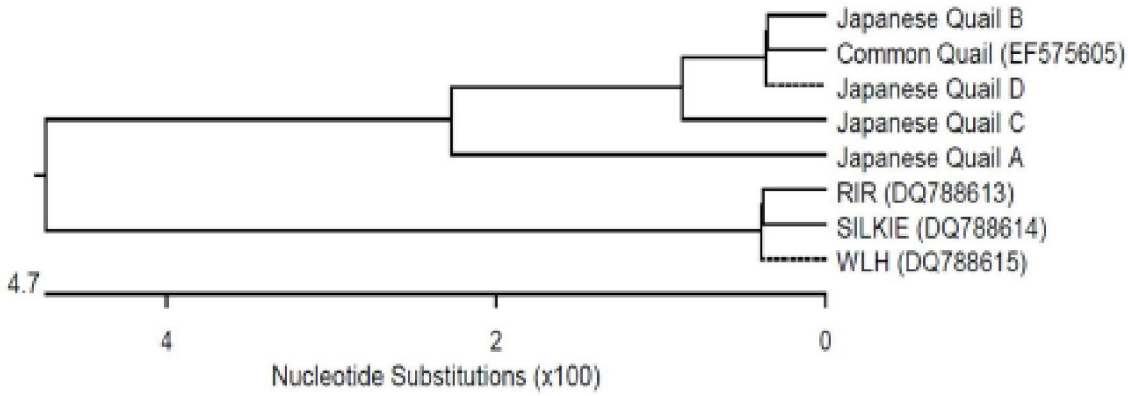


Fig. 4.22 : Phylogenetic tree based on 148 bp fragment of *Mx 1* gene in Japanese quail

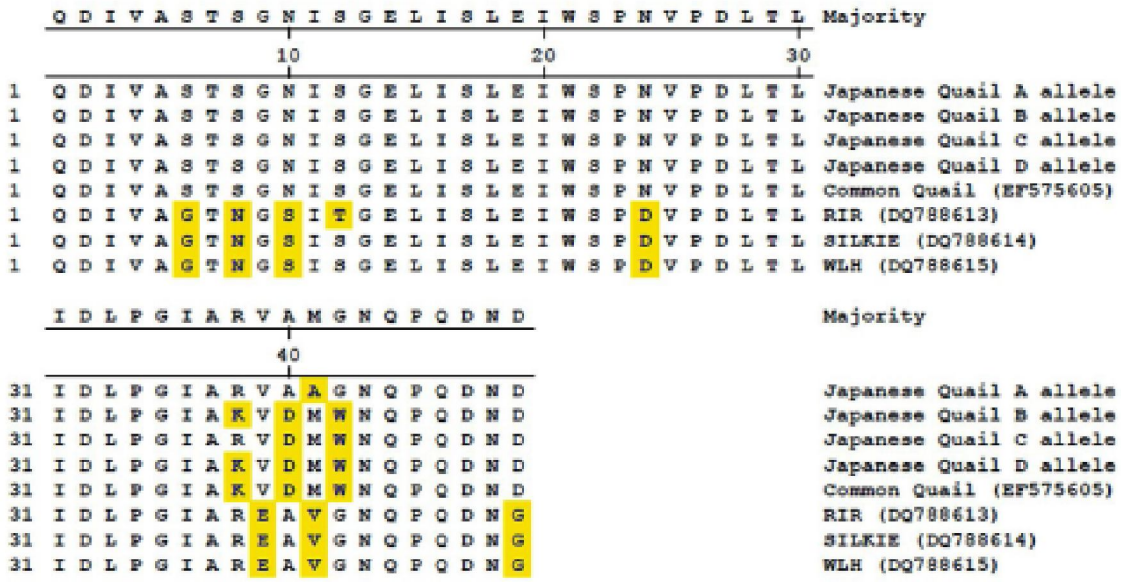


Fig. 4.23 : Amino acid sequence alignment of 148 bp fragment of *Mx 1* gene in Japanese quail, common quail and different breeds of chicken

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	
1		91.8	93.9	91.8	91.8	83.7	85.7	85.7	**1**
2	8.7		98.0	100.0	100.0	77.6	79.6	79.6	**2**
3	6.4	2.1		98.0	98.0	79.6	81.6	81.6	**3**
4	8.7	0.0	2.1		100.0	77.6	79.6	79.6	**4**
5	8.7	0.0	2.1	0.0		77.6	79.6	79.6	**5**
6	18.5	26.7	23.9	26.7	26.7		98.0	98.0	**6**
7	15.9	23.9	21.1	23.9	23.9	2.1		100.0	**7**
8	15.9	23.9	21.1	23.9	23.9	2.1	0.0		**8**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	

1 Japanese Quail A allele
2 Japanese Quail B allele
3 Japanese Quail C allele
4 Japanese Quail D allele
5 Common Quail (EF575605)
6 RIR (DQ788613)
7 SILKIE (DQ788614)
8 WLH (DQ788615)

Fig. 4.24 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of Amino acid sequence alignment of 148 bp *Mx 1* gene fragment

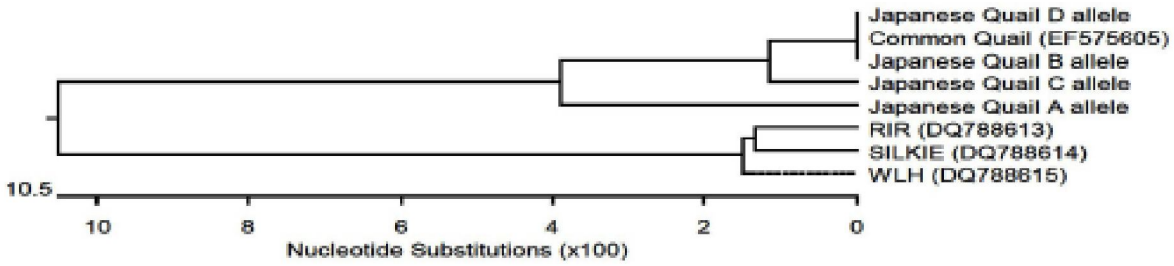


Fig. 4.25 : Phylogenetic tree on the basis of Amino acid sequence alignment of 148 bp *Mx 1* gene fragment in Japanese quail

```
          10        20        30        40        50        60
TCCTCACTAAACCAGATCTGGTGGACCACAGAACTGAAGGGACTGTCCTTAGGATAATGC 60
AAAATGAGGTCGTTCCACTCAGAAAAGGTTATATGATTGTGAAGTGTCACGGGCAACAGG 120
ATGTCAACAACGAATTGTCCTTGGCCTCTGTAATCCAGCAA 161
```

Fig. 4.26 : Nucleotide sequence of 161 bp fragment of *Mx 1* gene of Japanese quail

fragment of this gene. The sequences obtained were subjected to NCBI BLAST and the similar sequences were downloaded from the database.

4.4.3.1 Comparative study of 161 bp Frag.III

The sequence of 161 bp fragment of Mx 1 gene of Japanese quail was aligned along with the published sequence of Common quail and different breeds of chicken viz. RIR, SILKIE and WLH, using MEGALIGN programme of DNASTAR software (Fig.4.27, Table 4.9). After analyzing, it was found that the variations at several places exist between quail group and chicken group sequences and the Japanese quail sequence matches exactly with the Common quail for this fragment. At 57^{th} and 150^{th} position, Japanese quail, Common quail and RIR have adenine and guanine respectively, whereas SILKIE and WLH breed have guanine and adenine at the respective positions.

4.4.3.2 Percentage similarity and phylogenetic tree study between alleles

Percentage similarity study was carried out between nucleotide sequences of different breeds of Mx 1 gene (NCBI Acc No. DQ788613, DQ788614 and DQ788615) along with Japanese quail by using dnaSTAR software are presented in Fig. 4.28. Japanese quail shows 100% homology with the Common quail sequence and WLH breed has 100% homology with SILKIE breed of chicken for this fragment. Japanese quail forms separate cluster with common quail under phylogenetic tree Fig. 4.29.

4.4.1.3 Comparison of amino acid sequences of 161 bp Frag.III

The amino acid sequence of the 161 bp fragment of Mx 1 gene of Japanese quail was deduced from its nucleotide sequence and was compared with those of the published sequences available in NCBI database. The alignment report, percentage similarity and phylogenetic tree of the deduced amino acid sequence of this region is shown in Fig.4.30, Fig.4.31 & Fig.4.32.

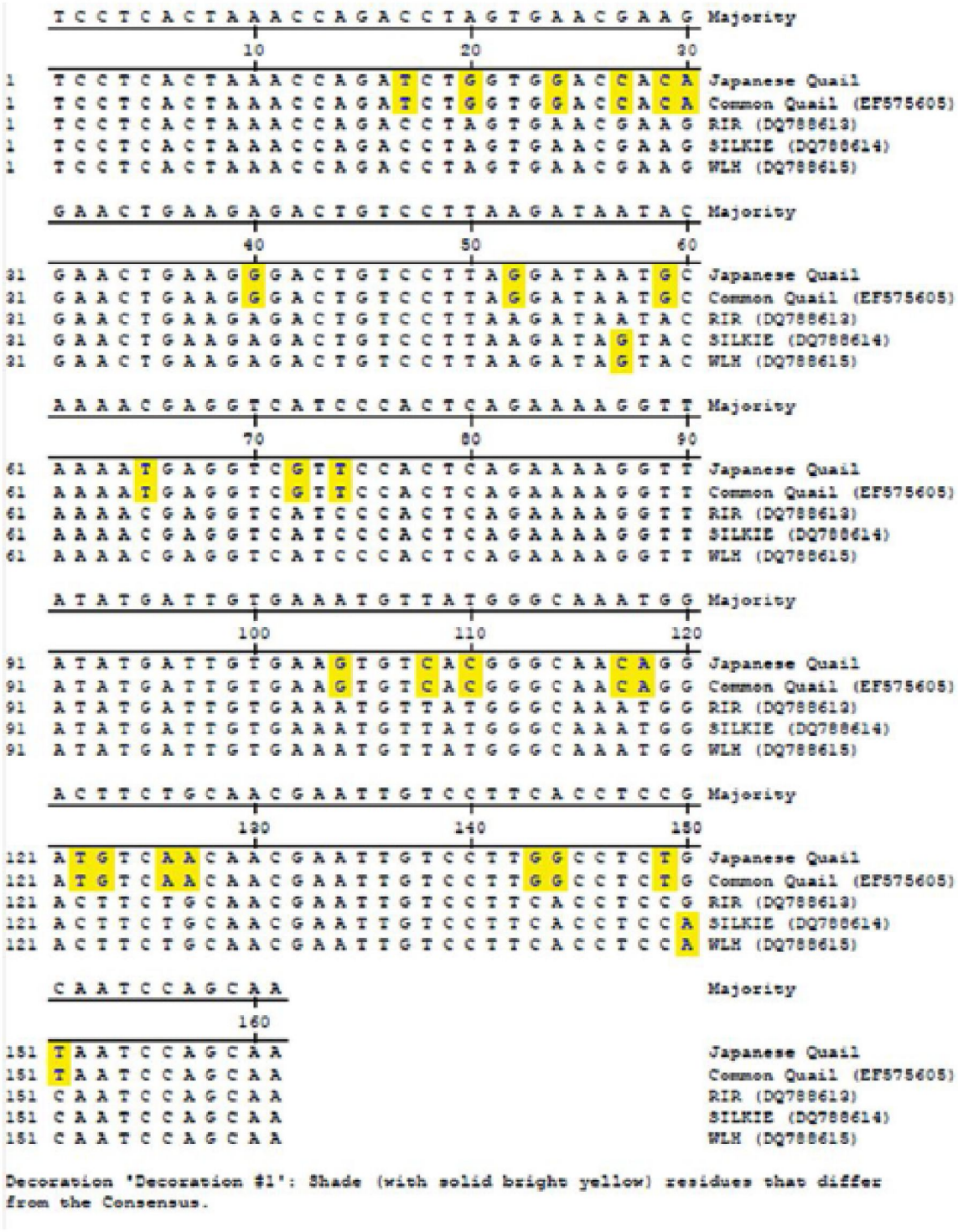


Fig.4.27 : Nucleotide sequence alignment of 161 bp fragment of *Mx 1* gene of Japanese quail with available sequences of Common quail and different breeds of chicken

Percent Identity

	1	2	3	4	5		
1		100.0	84.5	83.2	83.2	1	Japanese Quail
2	0.0		84.5	83.2	83.2	2	Common Quail (EF575605)
3	17.8	17.8		98.8	98.8	3	RIR (DQ788613)
4	19.5	19.5	1.3		100.0	4	SILKIE (DQ788614)
5	19.5	19.5	1.3	0.0		5	WLH (DQ788615)
	1	2	3	4	5		

Divergence

Fig. 4.28 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of 161 bp fragment of *Mx 1* gene

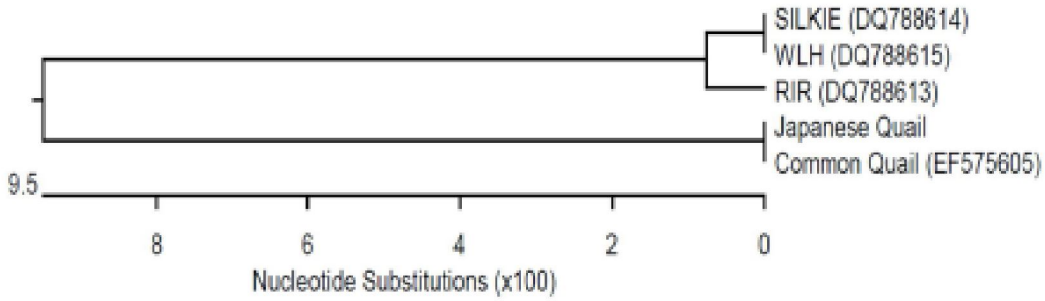


Fig. 4.29 : Phylogenetic tree based on 161 bp fragment of *Mx 1* gene in Japanese quail

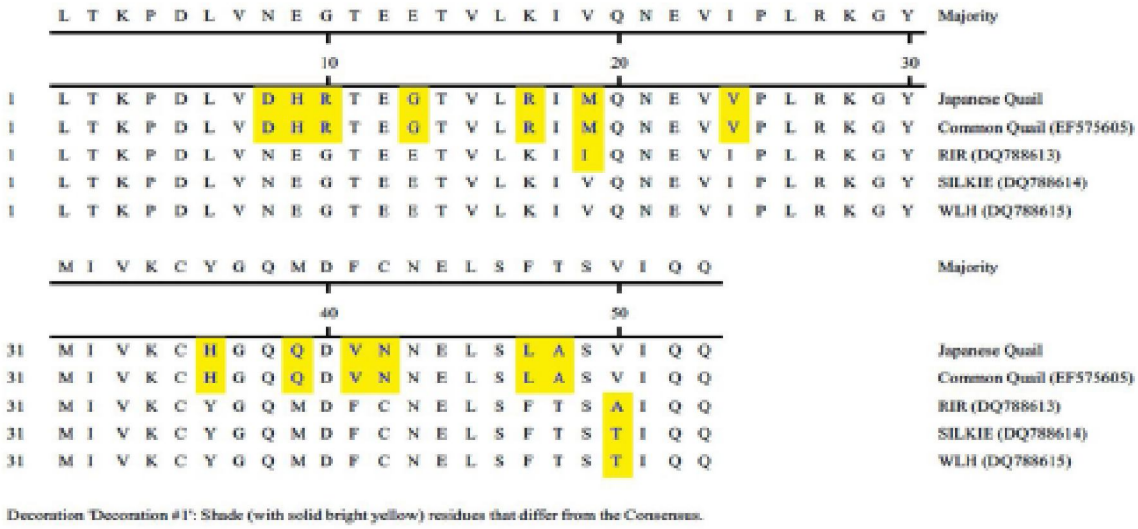


Fig. 4.30 : Amino acid sequence alignment of 161 bp fragment of Mx 1 gene in Japanese quail

Percent Identity

	1	2	3	4	5		
1		100.0	73.6	73.6	73.6	1	Japanese Quail
2	0.0		73.6	73.6	73.6	2	Common Quail (EF575605)
3	32.6	32.6		96.2	100.0	3	WLH (DQ788615)
4	32.6	32.6	3.9		96.2	4	RIR (DQ788613)
5	32.6	32.6	0.0	3.9		5	SILKIE (DQ788614)
	1	2	3	4	5		

Divergence

Fig.4.31 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of Amino acid sequence alignment of 161 bp fragment of Mx 1 gene

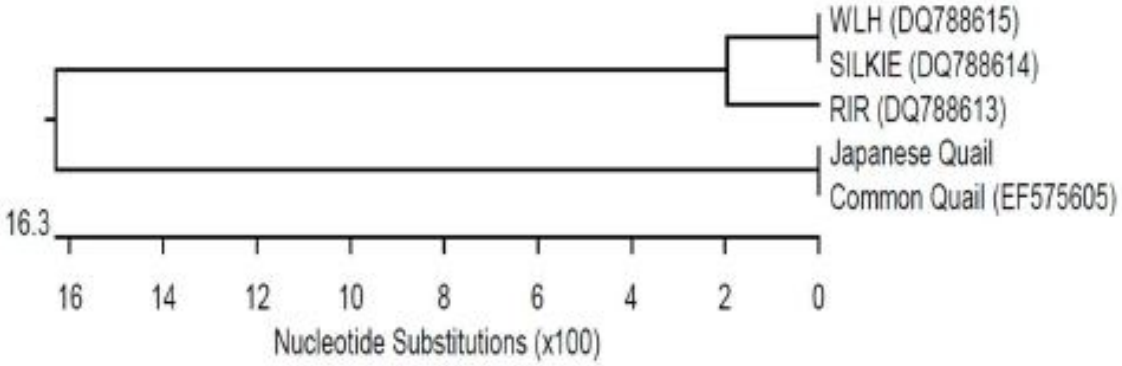


Fig. 4.32 : Phylogenetic tree on the basis of Amino acid sequence alignment of 161 bp fragment of *Mx 1* gene in Japanese quail

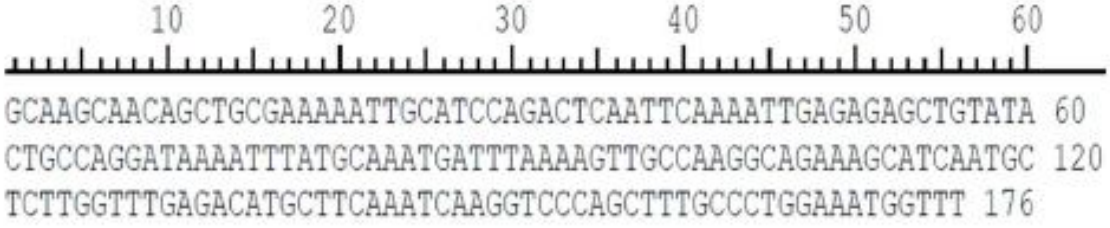


Fig. 4.33 : Nucleotide sequence of 176 bp fragment of *Mx 1* gene of Japanese quail

Table 4.9 : Comparative analysis of 161 bp Frag.III of Japanese quail with Common quail and different breeds of chicken

Sl No	Breeds	Nucleotide Position																									
		17th	20th	24th	27th	29th	30th	40th	52nd	57th	59th	65th	72nd	74th	104th	108th	117th	118th	122nd	123rd	126th	127th	143rd	144th	149th	150th	151st
1	Japanese quail	**T**	**G**	**G**	**C**	**C**	**A**	**G**	**G**	**A**	**G**	**T**	**G**	**T**	**G**	**C**	**C**	**A**	**T**	**G**	**A**	**A**	**G**	**G**	**T**	**G**	**T**
2	Common quail	**T**	**G**	**G**	**C**	**C**	**A**	**G**	**G**	**A**	**G**	**T**	**G**	**T**	**G**	**C**	**C**	**A**	**T**	**G**	**A**	**A**	**G**	**G**	**T**	**G**	**T**
3	RIR	C	A	A	G	A	G	A	A	A	A	C	A	C	A	T	A	T	C	T	T	G	C	A	C	G	C
4	SILKI	C	A	A	G	A	G	A	A	G	A	C	A	C	A	T	A	T	C	T	T	G	C	A	C	A	C
5	WLH	C	A	A	G	A	G	A	A	G	A	C	A	C	A	T	A	T	C	T	T	G	C	A	C	A	C

The major difference in amino acid alignment is between quail and chicken group at 14 places. As per the report, the Japanese quail shows 100% homology with common quail and quails are placed in separate cluster under phylogenetic tree.

4.4.4 Nucleotide sequencing of 176 bp Frag.IV of Mx 1 gene

Two different SSCP patterns of 176 bp fragment in Exon 13 region of Mx 1 gene of Japanese quail were sequenced, however the sequencing results confirmed both giving exactly similar sequences, indicating presence of a single allele only in the population (Fig.4.33). The sequencing confirmed the amplification of 176 bp fragment of this gene. The sequences obtained were subjected to NCBI BLAST and the similar sequences were downloaded from the database.

4.4.4.1 Comparative study of 176 bp Frag.IV

The sequence of 176 bp fragment of Mx 1 gene of Japanese quail was aligned along with the published sequence of Common quail and different breeds of chicken viz. RIR, SILKIE and WLH, using MEGALIGN programme of DNASTAR software (Fig.4.34, Table 4.10). After analyzing the sequences of this fragment of Mx 1 gene, it was found that the Common quail as well as different breeds of chicken are having this fragment of length 194 bp. The Common quail sequence contains 18 bp length of highly variable region from 119^{th} to 136^{th} position which otherwise is having confirmed sequence in chicken breeds. On deleting this 18 bp variable sequence from Common quail fragment, it was observed that the rest sequence comes in alignment with Japanese quail and matches properly. These results show that the Japanese quail has undergone deletion mutation for this 18 bp region.

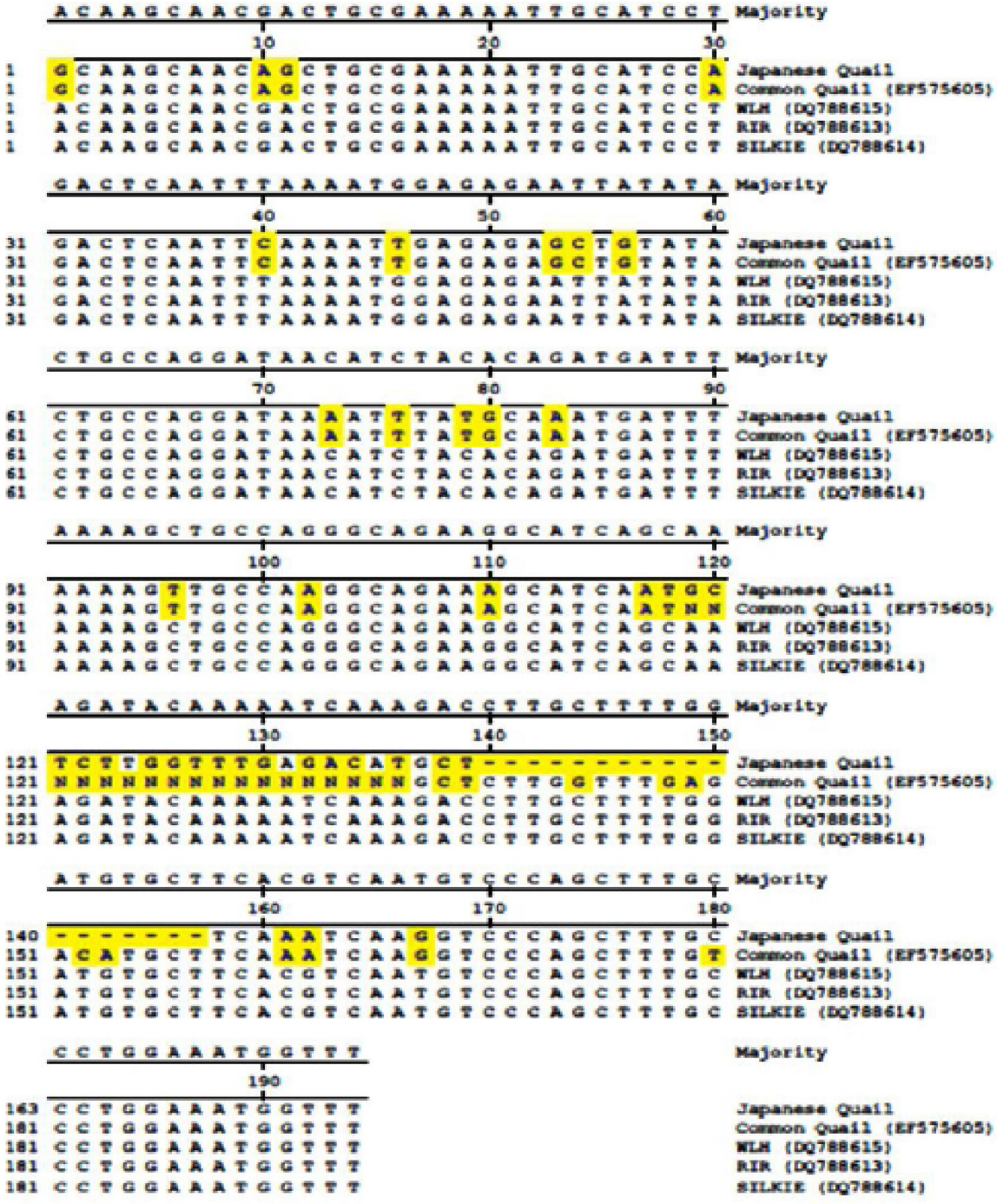


Fig. 4.34 : Nucleotide sequence alignment of 176 bp fragment of *Mx 1* gene of Japanese quail with available sequences of Common quail and different breeds of chicken

Table 4.10 : Comparative analysis of 176 bp Frag.IV of Japanese quail with Common quail and different breeds of chicken

Sl No	Breeds	Nucleotide Position																			
		1st	10th	11th	30nd	40rd	53rd	54th	56th	73rd	79th	80th	83rd	96th	102nd	110th	117th	118th	161st	162nd	167th	180th
1	Japanese quail	**G**	**A**	**G**	**A**	**C**	**G**	**C**	**G**	**A**	**T**	**G**	**A**	**T**	**A**	**A**	**A**	**T**	**A**	**A**	**G**	**C**
2	Common quail	**G**	**A**	**G**	**A**	**C**	**G**	**C**	**G**	**A**	**T**	**G**	**A**	**T**	**A**	**A**	**A**	**T**	**A**	**A**	**G**	**T**
3	RIR	A	G	A	T	T	A	T	A	C	C	A	G	C	G	G	G	C	C	G	T	C
4	SILKI	A	G	A	T	T	A	T	A	C	C	A	G	C	G	G	G	C	C	G	T	C
5	WLH	A	G	A	T	T	A	T	A	C	C	A	G	C	G	G	G	C	C	G	T	C

4.4.4.2 Percentage similarity and phylogenetic tree study between alleles

Percentage similarity study was carried out between nucleotide sequences of different breeds of Mx 1 gene (**NCBI Acc No. DQ788613, DQ788614 and DQ788615**) along with Japanese quail by using DNASTAR software are presented in Fig. 4.35 and Fig. 4.36. The Japanese quail shows 99.4% homology with Common quail for this fragment (Fig. 4.35). Japanese quail forms separate cluster with common quail under phylogenetic tree (Fig. 4.36).

4.4.4.3 Comparison of amino acid sequences of 176 bp Frag.IV

The amino acid sequence of the 176 bp fragment of Mx 1 gene of Japanese quail was deduced from its nucleotide sequence and was compared with those of the published sequences available in NCBI database. The alignment report, percentage similarity and phylogenetic tree of the deduced amino acid sequence of this region is shown in Fig.4.37, Fig.4.38 & Fig.4.39.

The amino acid alignment shows that Japanese quail amino acid sequence is missing six amino acids from 40^{th} to 45^{th} position, which is probably due to deletion mutation of the 18 nucleotide sequence (equivalent to six genetic codons i.e. six amino acids) in that region. At this region, common quail is having unclear amino acids, which is due to presence of highly variable nucleotides for this region, as shown by its nucleotide sequence. In contrast to it chicken breeds are having definite amino acids at those positions.

Japanese quail shows 96.6% homology with common quail as depicted by similarity diagramme and is placed in separate cluster with common quail under phylogenetic tree.

Percent Identity

	1	2	3	4	5		
1		99.4	84.1	84.1	84.1	**1**	Japanese Quail
2	0.6		75.3	75.3	75.3	**2**	Common Quail (EF575605)
3	26.6	19.9		100.0	100.0	**3**	WLH (DQ788615)
4	26.6	19.9	0.0		100.0	**4**	RIR (DQ788613)
5	26.6	19.9	0.0	0.0		**5**	SILKIE (DQ788614)
	1	**2**	**3**	**4**	**5**		

Divergence

Fig. 4.35 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of 176 bp fragment of *Mx 1* gene

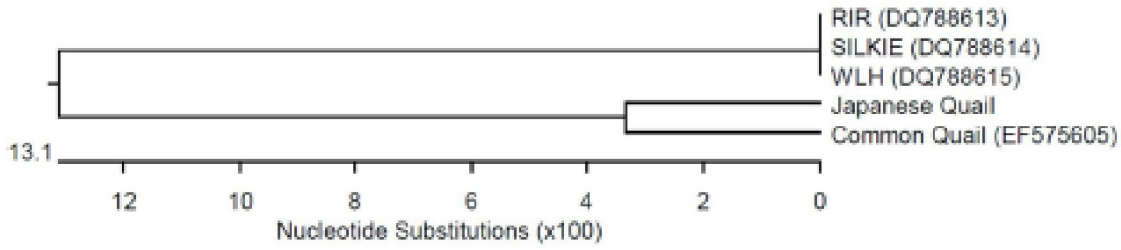


Fig. 4.36 : Phylogenetic tree based on 176 bp fragment of Mx 1 gene in Japanese quail

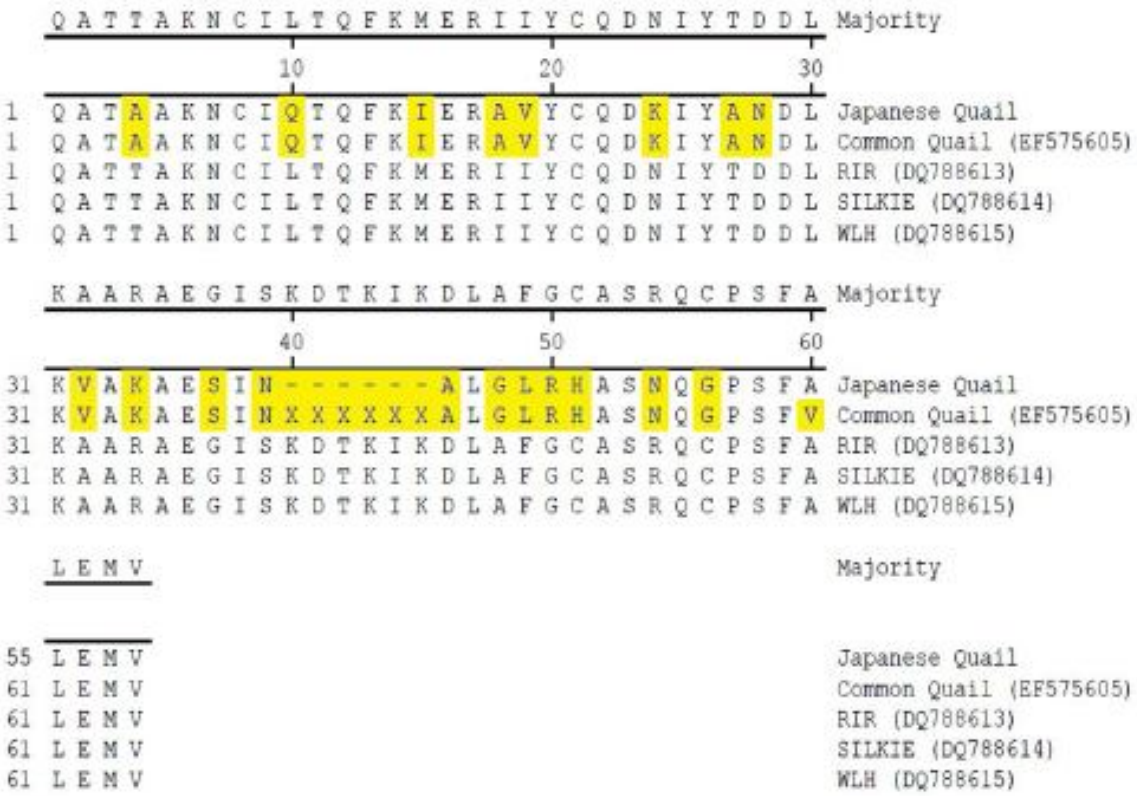


Fig. 4.37 : Amino acid sequence alignment of 176 bp fragment of *Mx 1* gene in Japanese quail

Percent Identity

	1	2	3	4	5	
1		96.6	67.2	67.2	67.2	1
2	1.7		59.4	59.4	59.4	2
3	42.9	57.8		100.0	100.0	3
4	42.9	57.8	0.0		100.0	4
5	42.9	57.8	0.0	0.0		5
	1	2	3	4	5	

Divergence

1 Japanese Quail
2 Common Quail (EF575605)
3 RIR (DQ788613)
4 SILKIE (DQ788614)
5 WLH (DQ788615)

Fig.4.38 : Similarity and divergence between Japanese quail, Common quail and different breeds of chicken on the basis of Amino acid sequence alignment of 176 bp fragment of *Mx 1* gene

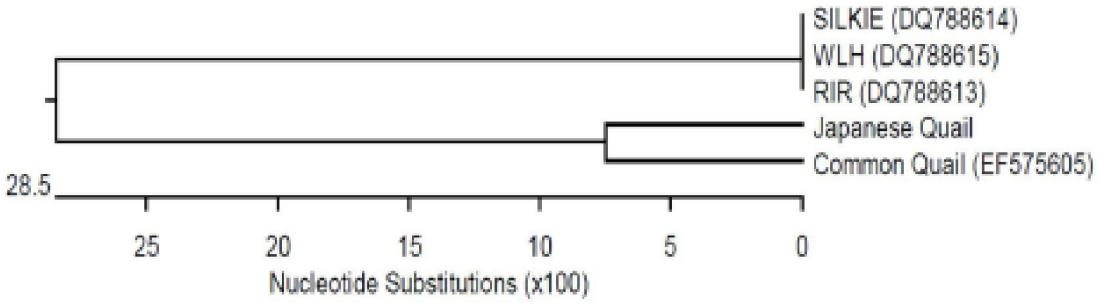
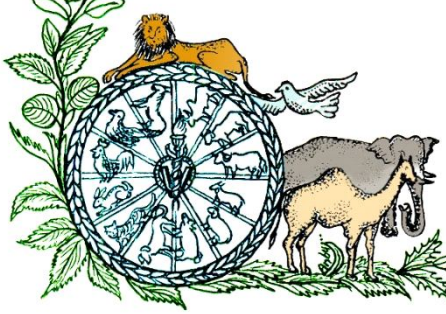


Fig. 4.39 : Phylogenetic tree on the basis of Amino acid sequence alignment of 176 bp fragment of *Mx 1* gene in Japanese quail

Summary and Conclusions



5 Summary and Conclusions

India holds 2.7% of the world's total geographical area while supports more than 11% of the world's total human population. This has lead to increased demand of nutritious food as well as unemployment. In this situation poultry sector is playing a major role as a good source of animal protein as well as providing employment to millions of people in India. Now India stands at 3rd rank in poultry egg production and 6th in poultry broiler production in the world. Despite this, the per capita availability of egg and chicken meat per annum came to be around 51 eggs and 2.26 kg chicken meat only, in 2009-10 while the recommended requirement is 180 eggs and 11 kg chicken meat, respectively. Thus there is wide gap between demand and supply of nutritious food. To decrease this gap, farming of alternative poultry species like Japanese quail can be very promising. With high growth rate, early marketing age, early sexual maturity and high economic production parameters, Japanese quail farming is in the process of commercialization.

The production performances are severely affected due to out break of various diseases which ultimately affects the country's whole economy to a large extent. Japanese quail is thought to be resistant against several diseases and therefore possesses a vast diversified reservoir of poultry genetic resources for resistant traits.

Mx 1 protein in chicken is a 79 KDa basic protein with potent antiviral activity against single stranded RNA viruses and may be one

of the causes of genetic resistance to influenza virus in chicken. In this context, Mx 1 gene has been suggested as a candidate gene for improvement of genetic resistance of birds. This would require the demonstration that a variation in DNA sequence correlates with trait improvement, and that the genetic polymorphisms under covering such a variation might be introgressed into elite stock. The characterization and the polymorphism study of Mx 1 gene has not been done yet in Japanese quail. Hence, keeping in view of Mx 1 gene as a potential candidate gene for general immune response as well as influenza resistance and the difference in specific activity, the gene needs to be characterized and the search of different variants in population is essential to exploit the variation in traditional breeding programmes.

Marker Assisted Selection is commonly used to study polymorphism. Among the several techniques used under Marker Assisted Selection (MAS), PCR-RFLP and PCR-SSCP are two simple and reliable methods for detecting mutation and thus, used in the present study for population screening.

The present investigation was undertaken on Mx 1 gene of Japanese quail birds with the objectives : (i). to identify single nucleotide polymorphic patterns by PCR-RFLP and PCR-SSCP methods, (ii). to sequence the various identified alleles. A total of 250 birds of Japanese quail maintained at CARI, Izatnagar, Bareilly, India were used under present investigation.

Venous blood was collected to isolate the genomic DNA (Sambrook and Russel, 2001). DNA was checked for its quality and quantity and good quality DNA was used for further investigation. A total of five fragments were studied in the present investigation. First fragment was RFLP fragment of 100 bp spanning over partial introns 13 and partial

exon 14 gene region of Mx 1 gene and other four fragments for SSCP study viz. Frag-I of 185 bp consisting of Exon 3 region, Frag-II of 148 bp consisting of Exon 5 region, Frag-III of 161 bp consisting of Exon 7 region and Frag-IV of 176 bp consisting of Exon 13 region of Mx 1 gene in Japanese quail were amplified by Polymerase Chain Reaction. PCR conditions and programme were optimized by using different concentrations and combination of components of reaction mixture, annealing temperature and amplification cycles. To detect polymorphism, the amplified fragments Mx 1 gene were subjected for PCR-RFLP (100 bp frag.) and PCR-SSCP analysis (four SSCP frag.) for all the birds.

In 100 bp RFLP fragment, two restriction enzymes (*Rsa* I and *Ssp* I) were used to detect the polymorphisms. Due to unclear and nonspecific amplification, the result of RFLP was ambiguous, so further analysis (PCR-RFLP and nucleotide sequencing) of this fragment could not be done.

Among the four SSCP fragments, PCR-SSCP analysis of Fragment-I of 185 bp revealed three genotypes (AA, BC and AC) and three alleles (A, B and C). The frequencies of AA, BC and AC genotypes and A, B and C alleles were estimated. The AC genotype was most frequent (0.40) followed by BC (0.38) and AA (0.22) genotype. The allelic frequencies of A, B and C alleles were 0.42, 0.19 and 0.39, respectively.

PCR-SSCP analysis of Fragment-II of 148 bp revealed four genotypes (AA, BB, CC and DD) and four alleles (A, B, C and D). The frequencies of AA, BB, CC and DD genotypes and A, B, C and D alleles were estimated. The BB genotype was most frequent (0.44) followed by AA (0.23), CC (0.18) and DD (0.15) genotype. The allelic frequencies of A, B, C and D alleles were 0.23, 0.44, 0.18 and 0.15, respectively.

PCR-SSCP analysis of Fragment-III of 161 bp revealed single genotype (monomorphic) which was named as AA having genotype frequency of 1.0 and allelic frequency of 1.0 for allele A.

Similarly, PCR-SSCP analysis of Fragment-IV of 176 bp revealed two genotypes (AA and BB) and two alleles (A and B). The frequencies of AA and BB genotypes and A and B alleles were estimated. The BB genotype was most frequent (0.64) followed by AA (0.36) genotype. The allelic frequencies of A and B alleles were 0.36 and 0.64 respectively.

After SSCP analysis, subsequent sequencing of different genotypes was done from both the ends using forward and reverse primers. Sequencing confirmed the amplification of the concerned fragments.

The sequencing results showed that SSCP Fragment-I of 185 bp length was monomorphic with exactly similar sequence for all the three SSCP patterns. But the Fragment-II of 148 bp length gave four different sequences variable to each other at few sites, confirmed presence of four different genotypes in the population. In case of Fragment-III of 161 bp, there was single SSCP pattern as well as single nucleotide sequence. The Fragment-IV of 176 bp also showed single nucleotide sequence for both two types of SSCP patterns, suggesting presence of single genotype.

Percentage similarity study for nucleotide as well as deduced amino acid sequence was carried out for nucleotide sequences of Japanese quail with common quail and also three breeds of chicken (RIR, SILKIE and WLH) by using the clustal W method of MegAlign Programme of Lasergene Software (DNASTAR).

For the Frag-I of 185 bp, the Japanese quail showed 100% homology with common quail. In case of Frag-II of 148 bp length, its allele A, B, C and D showed 95.3%, 99.3%, 98.6% and 100% homology respectively with common quail. Highest similarity among alleles of Frag- II was between allele B and allele D as 99.3% for nucleotide

sequence, whereas the similarity was 100% for amino acid sequence between them.

Again the Frag-III of 161 bp length showed 100% homology with common quail. The Frag-IV of 176 bp had 99.4% homology with common quail. Altogether the quails were grouped in different cluster than chicken breeds under phylogenetic tree.

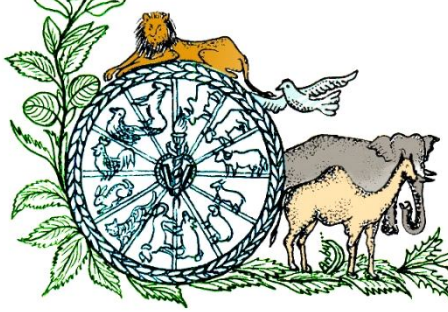
The Frag-II of 148 bp in Exon 5 region of Japanese quail is sufficiently polymorphic with respect to Mx 1 gene. The presence of different genotypes in Japanese quail can be considered as a molecular marker for breed characterization. From the present study following conclusions can be drawn:

- The Frag-II of 148 bp (Exon 5 region) was found to be polymorphic with four alleles (A, B, C & D) in Japanese quail.
- The Frag-I of 185 bp (Exon 3), Frag-III of 161 bp (Exon 7) and Frag-IV of 176 bp (Exon 13) were found to be monomorphic in Japanese quail upon sequencing.
- On the basis of the nucleotide sequencing and comparison of these four fragments, overall it was found that
  - Japanese quail is 87.77% similar with chicken (RIR, SILKIE & WLH) breeds.
  - Japanese quail showed 99.42% homology with Common quail.

FUTURE PROSPECTS

- Study may be conducted on other quail populations with larger population size.
- The whole Mx 1 gene needs to be sequenced in quail.
- The expression studies of this gene may be conducted in order to see the antiviral activity.

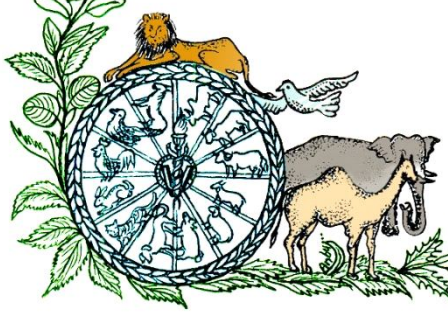
Mini Abstract



Mini Abstract

Mx 1 gene inhibits the proliferation of ssRNA viruses including influenza virus, vesicular stomatitis virus, measles virus etc. by antiviral activity of its gene product there by influencing the immune system. The present investigation was undertaken on Mx 1 gene of Japanese quail bird with the objectives (i). to identify and characterize various allelic variants of the Mx 1 gene in quail (ii). to sequence the allelic variants and compare with the published data. A total of 250 birds of Japanese quail maintained at CARI, Izatnagar, Bareilly, India were used under present investigation. The DNA of all the birds was isolated using standard protocol, followed by checking of its purity and quality. Five regions i.e. one RFLP fragment of 100 bp (partial intron 13 and partial exon 14) and other four fragments for SSCP study viz. Frag-I of 185 bp (Exon 3 region), Frag-II of 148 bp (Exon 5 region), Frag-III of 161 bp (Exon 7 region) and Frag-IV of 176 bp (Exon 13 region) of Mx 1 gene in Japanese quail were amplified and screened for polymorphism. In 100 bp RFLP fragment, two restriction enzymes (*Rsa* I and *Ssp* I) were used to detect the polymorphisms. Among four SSCP fragments, Frag-I of 185 bp (Exon 3) showed three genotypes/patterns on SSCP, with genotypes AA, BC and AC. Frequency of AC genotype was highest as 0.40. The frag-II of 148 bp (Exon 5) gave four different SSCP patterns and was found to be polymorphic with four genotypes AA, BB, CC and DD. The BB genotype was at highest frequency with 0.44. The Frag-III of 161 bp (Exon 7) gave monomorphic pattern on SSCP. The Frag-IV of 176 bp (Exon 13) showed two SSCP patterns with genotypes AA and BB. The BB genotype was most frequent with 0.64 frequency. Various genotypes observed were sequenced by the Sanger's dideoxy chain termination sequencing method in Automatic ABI Prism DNA sequencer. The alignment of the nucleotide sequences of various allelic variants was done using Lasergene Software (DNASTAR). Upon sequencing, polymorphism of Frag- II (148 bp) was confirmed by alignment and analysis of sequence, which revealed presence of four alleles A, B, C and D for this fragment in the Japanese quail population. Allele B was most frequent with frequency 0.44. The allele D of Frag-II (148 bp) was in 100% homology with common quail sequence for this fragment. In contrast to SSCP results, the sequence alignment and analysis results revealed that the Frag- I of 185 bp and Frag-IV of 176 bp were monomorphic with single type of sequences. The Frag-III (161 bp) gave single type of sequence result as like its single SSCP pattern. It can be concluded that the Mx 1 gene is polymorphic in Japanese quail, as studied by SSCP technique. The overall percentage similarity results for all the fragments/alleles studied showed that Japanese quail is 99.42% similar with common quail while similarity is 87.77% with chicken breeds (RIR, SILKIE & WLH). It is suggested that further study with sequencing of whole Mx 1 gene and expression studies of this gene may be conducted in order to see the antiviral activity of this gene in Japanese quail.

लघु सारांश



लघु सारांश

प्रस्तुत अध्ययन में बटेर पक्षी के एम.एक्स.1 जीन की पी.सी.आर.–आर.एफ.एल. पी. एवं एस.एस.सी.पी. द्वारा बहुरूपता पद्धति की पहचान की गई एवं विभिन्न चयनित एलीलों का अनुक्रमण ज्ञात किया गया। इसमें कुल 250 जापानी बटेर पक्षियों में प्रयोग किया गया। एम.एक्स. जीन का बहुगुणन कर बहुरूपता ज्ञात करने हेतु जापानी बटेर पक्षियों के एम.एक्स.–1 जीन के पाँच स्थानों जोकि 100 बी.पी. आर.एफ.एल.पी. खण्ड (आंशिक इन्ट्रान 13 एवं आंशिक एग्जोन 14) तथा अन्य चार एस.एस.सी.पी. खण्डों यथा 185 बी.पी. खण्ड–1 (एग्जोन–3), 148 बी.पी. खण्ड–2 (एग्जोन–5), 161 बी.पी. खण्ड–3 (एग्जोन–7) एवं 176 बी.पी. खण्ड–4 (एग्जोन–13) का चयन किया गया। बटेर में 148 बी.पी. (एग्जोन–5) में चार प्रकार की बहुरूपता पाई गई। स्वपीलतु ए.बी. आई. प्रिज्म डी.एन.ए. अनुक्रमण द्वारा विभिन्न जीनोद्वार का संस्गर्स डाइडिआक्सी श्रृंखला अंत अनुक्रमण पद्धति से किया गया। न्यूक्लियोटाईड अनुक्रमों की विभिन्न इलाकों की समानता लेजर जीन सॉफ्टवेयर (डी.एन.ए. स्टार) द्वारा की गई। एस.एस.सी. पी. का प्रयोग चार खण्डों के लिये किया गया। खण्ड–1 (185 बी.पी.) में तीन प्रकार के जीनोटाईप प्राप्त हुये जिनकी बारम्बारता क्रमशः 0.22 (ए.ए.), 0.38 (बी.सी.) एवं 0.40 (ए. सी.) थी। खण्ड–2 (148 बी.पी.) में चार प्रकार के जीनोटाईप प्राप्त हुये जिनकी बारम्बारता क्रमशः 0.24 (ए.ए.), 0.44 (बी.बी.), 0.18 (सी.सी.) एवं 0.15 (डी.डी.) थी। खण्ड–3 161 (बी.पी.) में एक ही प्रकार के जीनोटाईप मिले। खण्ड–4 (176 बी.पी.) में दो प्रकार के जीनोटाईप प्राप्त हुये जिनकी बारम्बारता क्रमशः 0.36 (ए.ए.) एवं 0.64 (बी. बी.) थी। तत्पश्चात संसर्ग पद्धति द्वारा ज्ञात हुआ कि खण्ड–1 (185 बी.पी.) एवं खण्ड–4 (176 बी.पी.) में एक ही प्रकार के न्यूक्लियोटाईड अनुक्रम हैं। जबकि खण्ड–2 148 (बी.पी.) में एस.एस.सी.पी. की भांति चार अलग प्रकार के अलील्स मिले। इसके अलील डी की साधारण बटेर के न्यूक्लियोटाईड क्रमों से, शत प्रतिशत समानता पाई गई। न्यूक्लियोटाईड विश्लेषण के तुलनात्मक अध्ययन से यह पाया गया कि जापानी बटेर एवं साधारण बटेर पक्षी, दोनों की आपस में काफी समानता थी और दोनों की कुक्कुट चिड़ियों (आर.आई.आर, सिल्की एवं डब्लू.एल.एच.) से कुछ स्थानों पर भिन्नता पाई गई। कुल मिलाकर यह पता चला कि अध्ययनित खण्डों के संदर्भ में जापानी बटेर पक्षी की साधारण बटेर पक्षी से 99.42 प्रतिशत सहधर्मिता मौजूद है। जबकि कुक्कुट पक्षियों से 87.77 प्रतिशत की सहधर्मिता पाई गई। इस सम्पूर्ण अध्ययन से यह निष्कर्ष निकलता है कि एम.एक्स–1 जीन, जापानी बटेर पक्षियों में एक बहुरूपीय जीन है।

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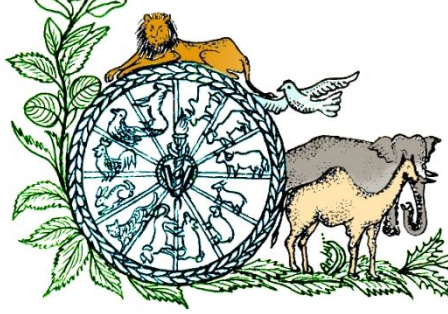
Annexure



Annexure

ANNEXURE I

CHEMICALS/EQUIPMENTS/LAB WARES/ MISCELLANEOUS ITEMS

1. Chemicals	Source
Agarose (low EEO)	SRL,GIBCO, BRL
Ammonium chloride	SRL
Boric acid	SRL
Bromophenol blue	Hi-media
Chloroform	Qualigens
EDTA di sodium salt	SRL
Ethanol	Bengal chemicals
Ethidium bromide	SRL
Formaldeyde	Qualigens
Formamide	SRL
Glacial acetic acid	Qualigens
Hydrochloric acid	Qualigens
8-Hydroxy-quinoline	Qualigens
Isoamyl alcohol (extra pure AR)	SRL
Isopropanol	SRL
Magnesium chloride	Qualigens
Phenol (extra pure AR)	SRL
Potassium bicarbonate	SRL
Potassium dichromate	Glaxo Laboratories
Sodium acetate	Qualigens
Sodium chloride	SRL
Sodium dodecyl sulfate	SRL
Sucrose	Qualigens

Sulphuric acid	Qualigens
Tris base	Qualigens
Tris HCL	SRL
Xylene cyanol	Hi-media

(2) Equipments **Sources**

Autoclave	Scientronic instruments
Centrifuge R8C	REMI
Distillation plant	Scientronic instruments
Gel documentation system	Syngen
Horizontal gel electrophoresis apparatus	Bangalore genei
Hot air oven	S. P. Scientronic Instruments
Ice box	Torson
Ice flaking machine	Scotsman
Incubator	S. P. Scientronic instruments
Laminar air flow	Tunco
Magnetic stirrer	Scientronic instruments
Micro-centrifuge RM 12C	REMI
Micropiptte (all ranges)	Qualipette
PH meter	Tunco
Power pack	Bangalore genei
Refrigerator	Godrej
Spectrophotometer	Beckman
Thermal cycler (PTC-200DNA engine)	M.J. Research Inc.
UV transiluminator	Gloworn
Vortexer	Scientronic instruments
Water bath	S.P. scientific instruments
Weighting balance (physical)	Laboratory balance Indus.
Weighing balance (Digital)	Sartorious

(3). Lab wares

3.1 Slasswares

Beakers, conical flasks measuring cylinders, Pasteur pipettes 10 ml pipettes, reagent bottles	Borosil

3.2 Plastic wares

Polypropylene centrifuge tube (15 ml and 50 ml)	Tarson
Eppendorf tube (1.5 ml and 0.5 ml)	Axygen USA
PCR tube (0.2 ml)	Axyxen USA
Microtips (All ranges)	Axygen US

(4) Miscellaneous items

Adhesive tapes	Needles
Aluminium foil	Para film
Autoclave label	Porcelain basin
Black and white films	pH paper
Blotting paper	Racks
Burette	Scissors
Butyrometer	Syringes
Cello-tape	Thermometers
Cotton	Threads
Disposable gloves	Tissue paper filters
Papers	Forceps, Marker pens

ANNEXURE II

BUFFERS AND SOLUTIONS

1. 2.7% EDTA solutions (pH 8.0)

EDTA disodium salt	2.7 gm
Double distilled water (up to)	100 ml

Adjust pH 8.0 using NaOH pellets. Sterilized by autoclaving and stored at room temperature

2. 0.5 M EDTA solution (pH 8.0)

EDTA disodium salt	186.1gm
Double distilled water (up to)	1000 ml

Adjust pH 8.0 using NaOH pellets. Autoclaved and stored at room temperature.

3. RBC lysis buffer (pH 8.0)

2M Tris HCl	2.5 ml
0.5M EDTA	5.00 ml
2M NaCl	5.0 ml

Autoclaved DW upto 100 ml and sore 4°C temperature.

4. 5 M NaCl solution

Sodium chloride	29.22 gm
Double distilled water (up to)	100 ml

Autoclaved and stored at room temperature

5. 10% SDS

SDS (Sodium Dodecyl sulfate)	100 gm
Autoclaved double distilled water (up to)	1000 ml

Adjust pH 7.2 using Conc. HCl. Heat in water bath at 60°C to dissolve and then store at room temperature

6. 3 M sodium acetate

Sodium acetate (anhydrous)	24.6 gm
Double distilled water (up to)	100 ml

Adjust pH 5.5 using glacial acetic acid. Autoclaved in batches of 20 ml.

7. 1 M Tris (pH 8.0)

Using Tris HCl

TrisHCl I	157.6g
Distilled water upto	1000 ml

Adjust pH 8.0 with NaOH pellets. Autoclave in 100 ml batches.

Using Tris base

Dissolve 121.1 g of Tris base in 800 ml of td H2O. Adujst the pH to desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H2O. Dispense into aliquots and sterilize by autoclaving. Autoclave and store at room temperature.

8. Tris saturated phenol preparation

Measure the required volume of phenol, add 8-hydroxyquinoline to a final concentration of 0.1% (it is an anti-oxidant gives yellow colour to phenol). Extract phenol once/twice with equal volume of 0.5 M Tris base (pH 10.5). Then with equal volume of Tris base pH 8.0 until the pH of the phenol phase is >7.8. Add 0.2% b-mercaptoethanol and mix (antioxidant and cleaves disulfide bond) Finally, 0.1 M Tris base (pH 8.0) is added to about 1/3rd volume of phenol and store in amber coloured bottle at 4°C.

9. Chloroform: isoamyl alcohol preparation (24:1)

Cholorform	24 ml
Isoamyl alcohol	1 ml

Mix thoroughly and store in amber coloured bottle at 4°C.

10. Phenol:chloroform: isoamyl alcohol preparation (25:24:1)

Chloroform: Isoamyl alcohol (24:1)	25 ml
Tris saturated phenol	25 ml

Mix thoroughly and store in amber colored bottle at 4°C.

11. 70% ethanol

Ethanol	70 ml
Autoclaved double distilled water	30 ml

Mix thoroughly and store in amber coloured bottle at 4°C.

12. 1 M Tris HCI (pH 8.0)

Tris HCI	157.6gm
Doubled distilled water (up to)	1000 ml

Adjust pH 8 using NaOH pellets. Autoclave and store at 4°C

13. 1 X TE

1 m Tris buffer	250ml
0.5 M EDTA (pH 8.0)	50 ml
Double distilled water (up to)	25 ml

Autoclave and store at 4°C

14. 5 X TBE

Tris base	54 gm
Boric acid	27.5 gm
0.5 M EDTA (pH 8.0)	20 ml
Autoclaved distilled water (up to)	1000 ml

Autoclave and store at room temperature.

15. 6 X Gel loading Dye (Sambrook *et al.,* 1989)

a) Type I

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Sucrose in water	40%(w/v)

Mix and store at 4°C.

b) Type II

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Mix and store at 4°C

16. 20,000 X Ethidium bromide (10mg/ml)

Ethidium bromide	10 mg
Autoclaved distilled water (up to)	1 ml

Wrap in aluminium foil and store in dark place at room temperature.

17. Phosphate buffered saline

NaCl	8gm
KCl	0.2gm
Na_2HPO_4	1.44 gm
KH_2PO_4	0.24gm
Distilled water (up to)	800 ml

Adjust pH to 7.4 with HCI. Add water to 1 liter. Autoclaved and stored at room temperature.

18. Formamide dye (100 ml)

Formamide	95 ml (95%)
Xylene cyanol	0.025gm (0.025%)
Bromophenol blue	0.025gm (0.025%)
EDTA 4 ml (0.5M)	

Autoclaved DDW make volume 100 ml. Store at 4^0 C.

19. 50:1 Acrylamide: Bisacrylamide

Acrylamide	50 gm
Bisacrylamide	1 gm
DDW (up to)	100 ml

Stirred the solution and filtered through whatmann filter paper (0.45m).

ANNEXURE III

ENZYMES AND BIOLOGICALS

1. *Taq* DNA polymerase enzyme1 units/ml

Store at –20°C.

2. 10 X *Taq* DNA polymerase buffer

Tris HCI (pH 8.8)	100 mM
KCI	500mM
$MgCl_2$	15 mM
Gelatin	0.01%

Stored at –20°C.

3. 20% Proteinase K

Proteinase-K	20 mg
Autoclaved double distilled water (up to)	1 ml

Store at –20°C

4. Primer

Working solution concentration	10 ng/ml

Store at –20°C.

5. dNTPs solution (pH 7.0)

dATP	10mM
dCTP	10mM
dGTP	10mM
dTTP	10mM

Store at –20°C

6. 100 bp Ladder DNA

It contains 10 bands of double stranded DNA fragments ranging from 100 to 1000 bp.

7. *Rsa* I restriction enzyme

*Rsa*I	10 unit/µl
10x NE buffer	10 mM Tris - HCl
	0.1 mM EDTA, 200mM NaCl
	100 mM KCl
1 mM EDTA	1 mM dithiothreitol
0.2mg/ml BSA	
50% glycerol	

Store at –20°C, pH 7.4.

8. *Ssp* I restriction enzyme

Ssp I	10 unit/µl
	10 mM Tris - HCl
	0.1 mM EDTA, 200mM NaCl
	100 mM KCl
	1 mM dithiothreitol
	1 mM EDTA
	0.2 mg/ml BSA and
	50% glycerol

Store at –20°C., pH 7.4.

Curriculum Vitae

Name	:	**Dr. Diwesh Kumar Niraj**
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Mother's Name	:	Smt. Lalita Devi
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Email address	:	diwesh12@gmail.com vetgen2k8@gmail.com

Educational Qualifications :

Degree	University	College	Marks (%)
M.V.Sc.	IVRI, Izatnagar	IVRI, Izatnagar	82.94
B.V.Sc. & A.H.	BAU, Ranchi	RVC, Ranchi	78.78
Higher Secondary	BIEC, Patna	V.N.S. College, Narma	70.22

Awards :
- ICAR Junior Research Fellowship (AIR-06)
- Best Commentator award for Live Cricket Commentary at college level and many other prizes in quize and contests at School and College level

Memberships :
- Bihar Veterinary Council, Patna
- Veterinary Council of India, New Delhi

Trainings : Participated in training on 'Bird Flu' (conducted by AHD, Govt. of Bihar) and many other programmes

Work Experiences : Two years Work Experience as 'Block Animal Husbandry Officer' under Animal Husbandry Deptt., Govt. of Bihar.