Summary
In the present study, an attempt has been made to characterize the BF2 gene in red jungle fowl. An attempt was also made to study PCR-RFLP in BF2 in red jungle fowl resource population.

The different exons of BF2 gene were amplified in red jungle fowl using the chicken specific primers and cDNA as template. The monocytes were separated from Whole Blood, cultured in RPMI-1640 Medium supplemented with Fetal Bovine Serum and the cells were stimulated with CON A (mitogen) for 1 hour at 37°C in CO2 incubator (5%). The total RNA was isolated using 'RNAgent™ - Total RNA isolation system' (Promega, Madison, WI, USA) and was reverse transcribed using the 'RevertAid™ - first strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) and used as template.

The red jungle fowl BF2 gene was amplified using two sets of specific primers based on available BF2 sequence from chicken. Using set 1 primers, a 673 bp fragment was amplified, while the set 2 primers amplified a 593 bp fragment. These fragments were purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN Inc. Valencia, CA, USA) as per the manufacturer’s protocol. These purified product were directly cloned into the T/A cloning vector (e.g., pTZ57R/T vector, MBI Fermentas)) using T/A-cloning strategy, separately and sequenced. The Sequencing confirmed the size of the these amplified BLB2 fragment to be 673 bp and 593 bp.

The comparison of the nucleotide sequence of 673 bp fragment amplified from RJF with the nucleotide sequence from known chicken B haplotype showed that this fragment is comprised of 38 nucleotide from 3' end of 5' UTR region, 64 nucleotide of exon-1, 264 nucleotide of exon-2, 273 nucleotide of exon-3 and 34 nucleotide from 5' end of
exon-4. Similarly the nucleotide sequence comparisons of 593 bp fragment amplified from RJF showed that this fragment is comprised of 270 bp of exon-4, 108 bp of exon-5, 33 bp of exon-6, 182 bp of exon-8. The exon-7 found in chicken sequences was not found in RJF sequence. The complete CDS comprising of 1033 bp was obtained after editing the sequences of 673 bp and 593 bp. The 1033 bp CDS was composed 64 bp of exon-1, 264 bp exon-2, 273 bp exon-3, 273 bp exon-4, 108 bp exon-5, 33 bp exon-6, 18 bp exon-8. In RJF the signal peptide was comprised of 21 amino acids, while the mature peptide was comprised of 323 amino acids. The mature peptide of RJF same to be 11 amino acids shorter than the chicken and this difference is due to absence of 11 amino acids of cytoplasmic domain II. The 323 amino acids mature peptide was composed of 3 extra cellular domain i.e. a1 domain, a2 domain, a3 domain, transmembrane domain, cytoplasmic domain I and cytoplasmic domain III.

The nucleotide sequences of BF2 exons in different chicken B haplotypes were retrieved from database and studied for sequence homology within them selves as well as with the RJF. Within the chicken B haplotypes, exon-2 and exon-3 were found showed high polymorphism, showing 14.65-14.78 % polymorphism, where as the % polymorphism ranged from 3.03-6.48 % in other exons. Within chicken B haplotypes, the ratio of transition to transversion nucleotide substitutions was 1:1 in exon-2 and exon-3 but in exon-4 and exon-5, a bias for transition was observed as the ratio of transition to transversion was 5:1. Similarly, an upward bias for non-synonymous nucleotide substitutions was observed in exon-1, exon-2, exon-3 and exon-4 as the ratio of synonymous and non-synonymous substitutions ranged from 1:2 in exon-4 to 1:9 in exon-3. In other exons, the ratio of synonymous and non-synonymous nucleotide substitutions was close to 1:1.

Between RJF and chicken B haplotypes the percent polymorphism was maximum in exon-2 and exon-3 (15.15 and 15.38 % respectively) followed by exon-1 (9.4%). In other exons, the %
polymorphism ranged from 3.03 to 6.48 %. While the ratio of transition to transversion nucleotide substitutions was 1:1 in exon-2 and exon-3, an upward bias for transition was observed in exon-4 and exon-5 as the ratio of transition to transversion was ~ 6:1. Similarly, a highly upward bias for non-synonymous nucleotide substitutions was observed in exon-2 and exon-3 (1:5 to 1:9), while in other exons, this ratio ranged from (1.2:1 to 1:2).

Within chicken B haplotypes, the percent amino acids polymorphism was maximum in a1 domain and a2 domain (27.27% and 26.37% respectively) followed by signal peptide (14.28 %) and trans-membrane domain (11.11 %). The a3 domain showed comparatively much lower percent amino acids variability i.e. 7.69 % as compared to a1 domain and a2 domain. While the cytoplasmic domain I was completely conserved, 16.66 % polymorphism was observed in cytoplasmic domain III.

Between RJF and chicken B haplotypes, a1 domain and a2 domain showed maximum percent amino acids polymorphism i.e. 27.27% and 26.37%, respectively, followed by 14.28 % in signal peptide and 11.11 in trans-membrane domain (11.11 %). The a3 domain showed comparatively much lower percent amino acids variability i.e. 8.79 % as compared to a1 domain and a2 domain. Cytoplasmic domain I showed complete conservation, but 16.66 % polymorphism was observed in cytoplasmic domain III.

A total of 5 nucleotide sequences of α1 and α2 domains (Table 4.6) i.e. chicken, guinea fowl, quail, goose, duck were retrieved from the data base (www.ncbi.nlm.nih.gov) and these nucleotides sequences were aligned with the respective sequences from RJF and compared for sequence homology between themselves using MEGA4.0 software. While red jungle fowl showed least sequence variability of 3.40 % in exon-2 and 5.49 % in exon-3, with chicken, much higher variability was observed between RJF and poultry species other than chicken i.e. 19.26 % to 27.65 % and 11.23 % to 25.72 % in exon-2 and exon-3, respectively. The ratio of transition to transversion
nucleotide substitution was close to 1:1 between red jungle fowl and poultry species including chicken, however an upward biasness was observed for non-synonymous between the red jungle fowl and poultry species other than chicken as the ratio of synonymous to non-synonymous nucleotide substitution 1:2 to 1:4 in exon-2 and exon-3. While red jungle fowl showed least sequence variability i.e. 6.81 % and 8.79 % in $\alpha_1$ domain and $\alpha_2$ domain, much higher variability was observed between RJF and poultry species other than chicken in these regions (30.00 % to 48.86 % and 17.39 % to 34.78 %, respectively).

Between the RJF and chicken B haplotypes, the RJF $\alpha_1$, $\alpha_2$ and $\alpha_3$ domains showed the overall conservation of structure of the PBR region. Two disulphide binding cysteines i.e. C$^{99}$ and C$^{161}$ in $\alpha_2$ domain and two disulphide binding cysteines i.e. C$^{199}$ and C$^{255}$ in $\alpha_3$ domain; a potential N-glycosylation site i.e. N$^{85}$ in $\alpha_1$ domain were conserved. All the conserved residues interacting with the amino terminus of the bonds peptide in the HLA/H2 PBS i.e. Y$^7$, Y$^{59}$, Y$^{159}$ and Y$^{171}$ were conserved in RJF also i.e. Y$^7$, Y$^{58}$, Y$^{156}$ and Y$^{168}$. Three of the four conserved residues, which interacts with the carboxyl terminus of the peptides in HLA/H2 i.e. T$^{143}$, K$^{146}$ and W$^{146}$ were also conserved in RJF i.e. T$^{140}$, K$^{143}$ and W$^{144}$. However, the residue Y$^{94}$ was not conserved and replaced by R$^{83}$ in red jungle fowl and chicken.

Between RJF and chicken B haplotypes, majority of polymorphism residue (~ 70%) was observed in and around the region that from a helix as compared to 20 % polymorphism in $\beta$ strand of a1 domain, but in a2 domain $\beta$ strand region showed comparatively more polymorphism 60% in comparison as compared to that observed in a helix region (40%). Among the presumed sites interacting with peptide, 45 % were polymorphic in $\alpha_1$ domain, while in $\alpha_2$ domain, only 21.25 % were polymorphic. Among the presumed sites coming in contact with $\beta$ micoglobulin, only 10 % of sites were polymorphic in each domain. However for the sites interacting with T cell receptor, 40
% were polymorphic in α₂ domain, while in α₁ and α₃ domains, only 16.66 % were polymorphic.

Between the RJF and different poultry species, RJF showed the overall conservation of α₁ and α₂ domains. Two disulphide binding cysteines i.e. C⁹⁹ and C¹⁶¹ in α₂ domain and a potential N-glycosylation site i.e. N⁸⁵ in α₁ domain were conserved. Sequence alignment with mammalian MHC showed that many of the conserved features of mammalian MHC class I glycoprotein was conserved in guinea fowl. Out of the four conserved residues interacting with the amino terminus of the bonds peptide in the HLA/H2 PBS i.e. Y⁷, Y⁵⁹, Y¹⁵⁹ and Y¹⁷¹ three were conserved in red jungle fowl and different poultry species i.e. Y⁷, Y⁵⁸ and Y¹⁵⁶, while Y¹⁶⁸ was replaced by H in duck. Three of the four conserved residues, which interacts with the carboxyl terminus of the peptides in HLA/H2 i.e. T¹⁴³, K¹⁴⁶ and W¹⁴⁶ were conserved in red jungle fowl and different poultry species i.e. T¹⁴⁰, K¹⁴³ and W¹⁴⁴, except in quail, where K¹⁴³ has been replaced by A. Residue Y⁸⁴ was not conserved and replaced by R⁸⁵ in red jungle fowl and different poultry species except in quail, where it has been replaced by L.

The genetic distances between red jungle fowl and chicken B haplotypes were estimated using the cumulative nucleotide variability (Kimura 2-parameter) as well as cumulative amino acid variability (poisson correction) in α₁ domain and α₂ domain. Between the red jungle fowl and different chicken B haplotypes, genetic distances were very low and ranged from 0.046 to 0.087. Similarly, the genetic distances (Poisson correction) were estimated using the cumulative amino acid variability in α₁ domain and α₂ domain between the red jungle fowl and different chicken B haplotypes were very low and ranged from 0.081 to 0.157. Using the pair wise genetic distances based on nucleotide as well as amino acid variability in α₁ and α₂ domains between red jungle fowl and chicken B haplotypes, the phylogenetic tree was constructed. Both the phylogenetic trees revealed that RJF cluster along with the chicken B21 haplotype.
The genetic distances between red jungle fowl and different poultry species were estimated using the cumulative nucleotide variability (Kimura 2-parameter) as well as cumulative amino acid variability (poisson correction) in $\alpha_1$ domain and $\alpha_2$ domain. Red jungle fowl showed very low genetic distances with chicken based on either cumulative nucleotide sequence variability in $\alpha_1$ domain and $\alpha_2$ domain (0.047) or based on cumulative amino acid variability in $\alpha_1$ domain and $\alpha_2$ domain (0.546). However, between RJF and poultry species other than chicken, the estimates ranged from 0.206 to 0.345, when based on nucleotide variability and from 0.318 to 0.546, when based on amino acid variability. Between the poultry species, estimates ranged from 0.200 between duck and goose to 0.404 between quail and goose, when based on nucleotide variability and from 0.273 between duck and goose to 0.596 between quail and goose as well as between quail and duck, when based on amino acid variability. Phylogenetic tree constructed by using pair wise genetic distances based on nucleotide variability as well as on amino acid variability revealed two major clusters, comprising of guinea fowl, quail, chicken and RJF in one, while duck and goose in other. In first cluster, RJF grouped with chicken.

The primers were designed to amplify a 599 bp fragment comprising most of exon-2, intron between exon-2 and exon-3 and half of the exon-3 for PCR-RFLP. Using these primers, a 599 bp fragment was successfully amplified in red jungle fowl. While the PCR RFLP of 599 bp BF2 fragment with Hae II revealed the polymorphic profile in red jungle fowl, the PCR RFLP with Xho I could not detect polymorphism. The genotypic frequencies of AA, BB and AB genotypes observed in PCR RFLP with Hae II were 0.50, 0.17 and 0.33, respectively, showing low amount of heterozygocity.
Hence from the above, following conclusions could be drawn-

- A 673 bp fragment comprising of 38 nt from 3' end of 5' UTR region, 64 nt of exon-1, 264 nt of exon-2, 273 nt of exon-3 and 34 nt from 5' end of exon-4 was amplified, cloned and sequenced in RJF.

- A 593 bp fragment comprised of 270 bp of exon-4, 108 bp of exon-5, 33 bp of exon-6, 182 bp of exon-8 was amplified, cloned and sequenced in RJF.

- The 1033 bp complete CDS, comprising was composed 64 bp of exon-1, 264 bp exon-2, 273 bp exon-3, 273 bp exon-4, 108 bp exon-5, 33 bp exon-6, 18 bp exon-8 was obtained by annotating the above two sequences.

- In RJF the signal peptide was comprised of 21 aas, while the mature peptide was comprised of 323 aas, which seem to be 11 aas shorter than the chicken due to absence of 11 aas of cytoplasmic domain II.

- Between RJF and chicken B haplotypes the percent polymorphism was maximum in exon-2 and exon-3. While the ratio of transition to transversion nt substitutions was 1:1 in exon-2 and exon-3, an upward bias for transition was observed in exon-4 and exon-5. A highly upward bias for non-synonymous nt substitutions was observed in exon-2 and exon-3.

- Between RJF and chicken B haplotypes, a1 domain and a2 domain showed maximum percent aas polymorphism i.e. 27.27% and 26.37%, respectively. The a3 domain showed comparatively much lower percent aas variability.

- Red jungle fowl showed least sequence variability of 3.40 % in exon 2 and 5.49 % in exon-3 with chicken, much higher variability was observed between RJF and poultry species other than chicken.

- The ratio of transition to transversion nt substitution was close to 1:1 between red jungle fowl and poultry species including chicken, however an upward biasness was observed for non-synonymous between the red jungle fowl and poultry species.

- Between the RJF and chicken B haplotypes, the RJF α1, α2 and α3 domains showed the overall conservation of structure of the PBR region.

- Between RJF and chicken B haplotypes, majority of polymorphism residue (~ 70%) was observed in and around the region that from a helix as compared to 20 % polymorphism in β strand of a1 domain, but in a2 domain β strand region showed comparatively more polymorphism 60% in comparison as compared to that observed in a helix region (40%).
Between RJF and chicken B haplotypes, among the presumed sites interacting with peptide, 45 % were polymorphic in $\alpha_1$ domain, while in $\alpha_2$ domain, only 21.25 % were polymorphic. However, for the sites interacting with T cell receptor, 40 % were polymorphic in $\alpha_2$ domain, while in $\alpha_1$ and $\alpha_3$ domains, only 16.66 % were polymorphic.

Between the RJF and different poultry species, RJF showed the overall conservation of $\alpha_1$ and $\alpha_2$ domains.

The genetic distances between red jungle fowl and chicken B haplotypes based on cumulative nt variability as well as cumulative aa variability were very low.

Phylogenetic tree constructed using cumulative nt variability as well as cumulative aa variability revealed that RJF cluster along with the chicken B21 haplotype.

Based on cumulative nt variability as well as cumulative aa variability, Red jungle fowl showed very low genetic distances with chicken in comparison to the species other than chicken.

Phylogenetic tree constructed by using nt variability as well as aa variability revealed two major clusters, comprising of guinea fowl, quail, chicken and RJF in one, while duck and goose in other. In first cluster, RJF grouped with chicken.

PCR-RFLP of a 599 bp fragment with Hae II detected the polymorphic profile in red jungle fowl, while with Xho I, no polymorphism could be detected.