Materials and Methods
3.1. Germplasm

Healthy adult red jungle fowl, kept at Central Avian Research Institute, Izatnagar, were used.

3.2. Amplification of BF2 gene

3.2.1. Primer Designing

The two sets of primers were designed to amplify the different exons of BF2 gene taking cDNA as template (Fig 3.1). In the first set, the forward primer was derived from 5' UTR region, while the reverse primer was taken from the 5' end of exon-4 and was expected to amplify a 673 bp fragment consisting of complete exon-1, exon-2, exon-3 and partial exon-4. In the second set, the forward primer was derived from initial part of exon-4, while the reverse primer was 3' UTR region and was expected to amplify a 593 bp fragment.

**Table 3.1. Details of the different set of primers used to amplify the BF2 CDS**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primers</th>
<th>Primer sequence (5' - 3')</th>
<th>Expected fragment size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Set1-F</td>
<td>TGGGTGCGGCGGACTTGA</td>
<td>673</td>
</tr>
<tr>
<td></td>
<td>Set1-R</td>
<td>GCCTCCTTCCCCCCACACTCG</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Set2-F</td>
<td>GGCCCGAGGTGCCAGTGAGTGT</td>
<td>593</td>
</tr>
<tr>
<td></td>
<td>Set2-R</td>
<td>GCAGGCACTCATTTATTATTCACAGGA</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2. Blood collection and separation of Peripheral Blood Mononuclear Cells

Approximately 3 ml of blood from individual bird was collected from Jugular vein under sterile conditions. Three milliliter of LSM was
Set | 5'-3' Sequence | Expected size of amplicon
---|-----------------|------------------------
Set I-F | TGGGTGCGGCGGACTTGA | 673 bp
Set I-R | GCCTCCTTCCCCACACTCG | 
Set II-F | GGGCCGAGGGCGAGGTGT | 593 bp
Set II-R | GCCCATCATTTTTATTTTCACAGGA | 

Fig 3.1. Systematic presentation of BF2 gene in chicken and designing of primers for amplification of complete CDS of BF2 in red jungle fowl
added to 15 ml conical centrifuge tube at room temperature and 3 ml of whole blood was carefully layered over the column and centrifuged at 1700 rpm (900 x g) for 30 min at room temperature. After centrifugation, the opaque inter-phase containing mononuclear cells was carefully aspirated with a Pasteur pipette. The upper layer was discarded. The opaque inter-phase was carefully transferred to a clean centrifuge tube and equal volume of ice chilled DEPC treated isotonic PBS solution was added and mixed gently, then centrifuged at 3000 rpm (1400 x g) for 10 minutes. The supernatant was discarded and cell pellet was re-suspended in equal volume of ice chilled DEPC treated isotonic PBS solution and centrifuged in 3000 rpm (1400 x g) for 10 minutes. This step was repeated two times and the cell pellet suspended finally in 0.5 ml of RPMI-1640 medium containing 10% FCS. Viability of these cells was determined by Trypan blue staining method (0.4 %). After determining the viability, the cells were counted in WBC counting chambers of a haemocytometer using Trypan blue staining. Cell count was calculated employing the following equations:

Cells per ml = the average count per square x dilution factor x 104

Percent cell viability = (Number of viable cell / (Total number of viable plus non-viable cells) * 100

The final concentration of the cells were adjusted to 106 cells/ml in known volume of RPMI-1640 medium (Sigma inc., USA) containing 10 % FCS.

3.2.3. Peripheral Blood Mononuclear Cells Culture

The PBMCs suspended in RPMI-1640 medium supplemented with 10% FCS, were plated in 6 well plate and induced with final concentration of 15 μg/ml of Con A (mitogen) for the expression of mRNA of BLB2/BF2 genes. The Plate was incubated under 5% CO2 tension in humidified atmosphere for one hour at 37°C in a CO2 incubator.
3.2.4. Total RNA isolation

Total RNA from stimulated PBMCs were isolated using RNAgents™ total RNA isolation system (Promega Inc., USA). Adherent cells were collected from plate by gentle friction followed by centrifugation at 13000 rpm (12000 x g). Pelleted cells were washed twice with chilled PBS and then 600 µl of denaturing solution was added to the cells. This mixture was vortexed properly and 60 µl of 2M-sodium acetate was added and mixed thoroughly by inverting the tube 4-5 times. The lower organic phase of the phenol: chloroform: Isoamyl alcohol (600µl) was added and mixed carefully by inverting the tube 3-4 times, then shaked vigorously for 10 seconds. This mixture was chilled in ice for 15 minutes, transferred to a fresh 0.1 % DEPC treated microcentrifuge tube and centrifuged at 13000 rpm (12000 x g) for 20 minutes at 4°C. The top aqueous phase containing the RNA was carefully removed and transferred to a new sterile DEPC treated eppendorf tube. RNA was precipitated by adding equal volume of Isopropanol to the aqueous phase and incubated at -20°C for overnight to precipitate the RNA. The RNA was pelleted by centrifugation at 13000 rpm (12000 x g) for 10 minutes at 4°C. The supernatant was discarded and pellet was washed with 1ml of 70% ethanol at 13000 rpm (12000 x g). The supernatant was discarded and pellet was air dried and dissolved in known 50 µl of nuclease free water. The integrity of RNA was checked on 1.0% agarose gel using 1x TBE as electrophoresis buffer. The concentrations and quality of RNA preparations were determined spectrophotometrically at OD260 and OD280. This dissolved RNA was stored in -80°C until used for cDNA synthesis.

3.2.5. cDNA synthesis

First strand of cDNA from RNA sample was prepared by Revert Aid™ First strand cDNA synthesis kit (MBI Fermentas). Following reaction mixture was prepared in a tube on ice:
Template RNA (Total RNA 1.0 µg/µl)  5 µl
Oligo (dT)18 primer (0.5 µg/µl)  1 µl
Deionized nuclease free water  6 µl

The mixture was incubated at 70°C for 5 minutes and chilled ice immediately. The tube was placed on ice and the following components were added in the indicated order:

- 5x reaction buffer  4 µl
- Ribonuclease inhibitor (20 U/µl)  1 µl
- 10 mM dNTP mix  2 µl

The above mixture was incubated at 37°C for 5 minutes. To this 1 µl of Revert Aid™ M.MuLV Reverse transcriptase (200 U/µl) was added and the mixture incubated at 42°C for 60 minutes. The reaction was then stopped by heating the mixture to 70°C for 10 minutes. This first strand cDNA was synthesized and used directly for amplification of BF2 gene by PCR. The resultant cDNA was stored frozen at -20°C until used.

**3.2.6. Amplification of BF2 gene**

Polymerase chain reaction (PCR) was carried out for amplification of BF2 gene with first strand cDNA as template using specific forward and reverse primers of individual gene in duplicate tubes. The details of primers used in present study are given in Table.

The PCR reactions were performed in thermal cycler (iCycler; Bio-rad, Hercules, CA, USA) on equal aliquots of cDNA, in separate tubes, for the amplification of BF2 genes. The amplification mixture for each sample made up to final volume of 25 µl contained 10 pmol each of a 3' and a 5' gene-specific primer, 2 µl cDNA, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH8.8, 0.1% Triton X-100, 0.01% gelatin, 200 µM of each dNTP, 1 unit of Taq DNA polymerase enzyme (Promega) and 10 Pico mole of each forward and reverse primer. Amplification conditions were: 94°C for 3 min and 35 cycles of 45s at 94°C, 45s at 56°C for BF2 and 1 min at 72°C and final extension of 10 min at 72°C.
The PCR products were tested for amplification of specific gene by agarose gel electrophoresis using 1.6% agarose gel in 1x TBE. A total volume of 20 ml of 1.6% agarose (Life Technologies Inc.) was prepared in 1x TBE and placed in microwave oven until melted. Molten agarose was allowed to cool to about 45°C and ethidium bromide was added to give a final concentration of 0.5μg/ml. The gel was poured on to electrophoresis trough fitted with comb. The gel was allowed to set on a flat surface for about 15 minutes. Electrophoresis trough was placed in an electrophoresis tank filled with 1x TBE. Samples were prepared on a parafilm by mixing 2 μl loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cynol and 40 w/v sucrose in water) and 5 μl of PCR products were loaded in parallel with 100 bp ladder (Gene ruler 100 bp ladder, MBI Fermentas). Electrophoresis was done at 90 volts for 10 minutes, then at 50 volts for 2 hour. Gel was viewed under a UV Trans-illuminator and photographed with gel documentation system (Syngene) for future analysis.

3.2.7. Gel purification of PCR products

- The PCR products were purified from gel using QIAquick Gel Extraction Kit (QIAGEN Inc. Valencia, CA, USA) as follows:
- A preparative gel was prepared in TAE buffer and electrophoresis was done at 30 V for 6 hours. The gel was briefly visualized in low range UV light and the desired band was cut using a sterile paragon blade. The gel slice was collected in a pre-weighed sterile 1.5 ml micro centrifuge tube.
- The gel slice was weighed and 3 volumes of buffer QG was added to 1 volume of gel slice and incubated at 50°C for 10 min. with intermittent vortexing every 2-3 min.
- After the gel slice was completely dissolved, isopropanol equivalent to gel volume was added and mixed. Then, the sample was applied to the QIAquick spin column, placed in 2 ml collection tube provided in the kit.
• QIAquick spin column was centrifuged for 1 min at 12000 rpm.
• The flow-through was discarded and the QIAquick spin column was placed back in the same collection tube.
• 500 µl of buffer QG was added to QIAquick spin column and centrifuged for another 1 min at 12000 rpm.
• For washing, 750 µl of Buffer PE was added to column and then centrifuged at 12000 rpm for 1 min.
• The flow-through was discarded and the column was placed back in the same collection tube and centrifuged at 12000 rpm for additional 2 min.
• The column was then transferred to a clean 1.5 ml microcentrifuge tube.
• For elution of the Plasmid DNA, 30 µl of buffer EB was added to the center of the column, kept as such for 5 min and then centrifuged at 12000 rpm for 1 min.
• 5 µl of the purified PCR product was run in 1.6% agarose gel in 1x TBE.

3.2.8. Cloning of the purified PCR products in pTZ57 R/T vector

3.2.8.1. Preparation of ligation reaction

The gel purified PCR products were cloned into pTZ57 R/T vector system (Fig 3.3, MBI Fermentas), as per the manufacturer's protocol. The ligation reaction was prepared in 10 µl reaction in a 0.5ml microcentrifuge tube:

- 5X Rapid ligation buffer: 2.0 µl
- pTZ57 R/T (50 ng): 1.0 µl
- Purified PCR product (100ng): 5.0 µl
- T4 DNA ligase (5 U): 1.0 µl
- Nuclease free water: 2.0 µl

The reaction mixture was incubated at 4°C overnight. The ligated DNA was diluted to 200 µl in 1.1x TCM solutions and kept at 4°C until used for transformation.
3.2.8.2 Preparation of competent cells and transformation

In order to transform DH5a cells (E. coli cells) with the ligated DNA, fresh competent cells were prepared. The following steps were followed:

- Fresh cultures of DH5a cells were grown in 25ml LB medium in shaking incubator at 37°C overnight.
- The cells were diluted 200 times in LB medium and incubated in shaking incubator at 37°C for about 2-3 hrs until culture Attained an O.D. of 0.3-0.4 at A600 nm.
- The cells were kept on ice for 20 min.
- The cells were pelleted at 6000 rpm for 10 min at 4°C and the pellet was re-suspended in 1/10 volume of chilled TSS solution and incubated on ice for 1 hour.
- Competent cells were then ready for transformation and kept at -20°C until further use.
- 200 µl of competent cells were gently added to the 200 µl ligated DNA (diluted in TCM), mixed and incubated on ice for 20 min.
- The DNA-competent cells mixture was subjected to heat shock at 42°C for 45-50 sec. and immediately snap-chilled on ice for 2 min.
- 950 µl SOC medium was added to the transformants and incubated at 37°C for 1.5 hrs with shaking.
- Transformants were then plated on LB/Amp/X-gal/IPTG plates and incubated overnight at 37°C.
- The white colonies were picked on the next day and grown in 10 ml LB/Amp broth for overnight at 37°C with shaking for plasmid extraction.

3.2.8.3 Screening of recombinant clones

In order to minimize the number of clones to be handled, clones were initially checked by performing the colony PCR and later confirmed by plasmid-PCR and RE digestion of the plasmid.
3.2.8.3.1 Preparation of master plate

To ensure in situ maintenance of the clones until the analysis is over, a master plate was prepared with LB agar with ampicillin and marked appropriately for identification of the colonies. The single white colony was picked using a sterile toothpick. For each colony, picking the colony, the respective toothpick was first touched onto the marked place of the master plate and then dipped into prenumbered PCR tube containing MilliQ water. Finally, the toothpick was immersed into the test tube containing LB broth plus ampicillin. The master plate was incubated at 37°C for overnight and the LB broth was cultured overnight at 37°C with shaking. The PCR tube was subjected to colony PCR.

3.2.8.3.2 Colony PCR

The toothpick, after touching the master plate was transferred to prenumbered PCR tube containing 10 μl of autoclaved MilliQ water. The colonies were dissolved properly by using the pipette. Colony PCR was performed using the same PCR protocol as used for the amplification of BF2 genes. PCR was performed in 25 μl reaction volume. The amplified PCR product (5 μl) was run in 1.6% agarose gel in 1x TBE along with GeneRuler 100 bp Ladder Plus, (MBI Fermentas). The amplified product was visualized, documented and its size was estimated. The clones showing the amplification were propagated for further analysis.

3.2.8.4 Isolation of plasmid DNA from PCR-positive clones

As mentioned earlier, following the master plate preparation, the tooth pick was immersed in the 50 ml test tubes containing 10 ml LB broth with the antibiotic ampicillin @ 1μl/ml of broth (100 mg/ml final concentration). The tubes were incubated at 37°C in the shaker incubator at 150 rpm for over night. A glycerol stock of the positive clones (bacterial cultures) was prepared and stored at -70°C.
From these overnight grown cultures the plasmid DNA was extracted following the small scale alkali lysis method as described by Sambrook and Russel (2001) as follows:

- Bacterial culture (1.5 ml twice in the same tube) was centrifuged at 13000 rpm (12000 g) at 4°C for 1 min and supernatant was discarded thoroughly.

- The pellet was re-suspended in 100 µl of ice-cold Solution I by vigorous vortexing. Then 200 µl of freshly prepared Solution II (RT) was added to the suspension and mixed gently by inversion. Next, 150 µl of ice-cold Solution III was added and inverted gently for mixing. The viscous lysate was kept on ice for 3-5 minute.

- The bacterial lysate was centrifuged at 13000 rpm (12000 g) at 4°C for 5 min. The supernatant was transferred to a new microcentrifuge tube.

- Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed well by vortexing. The emulsion was centrifuged at 13000 rpm at 4°C for 2 min and the upper aqueous layer was transferred to a new microfuge tube.

- Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm at 4°C for 2 min and the upper aqueous layer was transferred to a new microfuge tube.

- The nucleic acid was precipitated by adding two volumes of ethanol at RT and incubated for 2-5 min at RT after brief vortexing. Then it was centrifuged at 13000 rpm at 4°C for 5 min.

- The supernatant was discarded thoroughly by inverting the tube onto a paper towel and 1 ml of 70% ethanol was added to the pellet. The tube was inverted several times for proper mixing and then centrifuged at 13000 rpm (12000 g) at 4°C for 2 min.

- The supernatant was removed completely and the residual ethanol was evaporated by keeping the tube open at RT for few minutes.
The plasmid was dissolved in 25 μl TE buffer (pH 8.0) containing 20 μg/ml DNase-free RNase A and briefly vortexed and incubated at RT for 30 min.

The quality of the plasmid DNA was checked in 1% agarose gel. The plasmid was then stored at -20°C until further use.

3.2.8.4.1 Restriction enzyme analysis of plasmid DNA

The recombinants were confirmed for the presence of desired insert using RE digestion of the plasmid DNA with the suitable Restriction enzymes to release the insert.

The plasmid DNA was double-digested with Restriction enzymes, EcoRI (GAATTCC) and PstI (CTGCA?G) having cutting sites on either side of the MCS in the cloning vector pTZ57R/T. Accordingly, RE digestion reaction was set up in 1.5 ml microcentrifuge tube using EcoRI and PstI (MBI Fermentas) as follows:

- Plasmid DNA: 3.0 μl
- Tango Buffer (2X): 4.0 μl
- EcoRI (10 U/μl): 0.5 μl
- PstI (10 U/μl): 0.51
- Nuclease free water: 12 μl

The contents were gently mixed by pipetting and incubated in a waterbath at 37°C for 5-6 hrs. The RE digestion was studied by running the 5 μl aliquot of the reaction on 1% agarose gel along with the DNA molecular weight marker (100 bp ladder, MBI Fermentas).

3.2.9. Sequencing of Red Jungle fowl B-F2 genes

Sequencing was done at DNA sequencing facility, Department of Biochemistry, University of Delhi South campus, New Delhi (UDSC) by automated sequencer using Sanger’s dideoxy chain termination method. The cloned PCR products from red jungle fowl were submitted in the form of the stab culture. Sequencing was done 5’-3’ as well as 3’-5’ using the M13 Forward and M13 reverse primers, respectively.
3.2.10. Analysis of red jungle fowl BF2 gene sequences

The sequences obtained were first checked manually and blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of BF2 gene. The related sequences identified from blast results were retrieved from Genbank (www.ncbi.nlm.nih.gov). The different sequences were edited and the sequences corresponding to the concerned region were cut and saved. Subsequently, the sequences were aligned using CLUSTALW (Thompson et al., 1994), website (http://www.cbi.ac.uk/clustalw/). The Molecular Evolutionary Genetic Analysis (MEGA Version 4.0) software was used to estimate nucleotide as well as amino acid variability. The genetic distances were estimated as Kimura 2-parameter distances, while the genetic distances between the amino acid sequences from different poultry species were estimated as poisson correction distances using MEGA software. Phylogenetic trees were constructed with neighbour joining (NJ) procedure using MEGA Version 4.0. Support of the clusters was evaluated by bootstrap, as percentage recurrence of clusters based on 100 bootstrapped replications with MEGA Version 4.0.

3.3. PCR-RFLP studies for BF Gene

3.2.1. Resource population

A resource population comprising of 12 red jungle fowl was used for PCR RFLP. The genomic DNA was extracted as follows.

3.2.2. Amplification of 370 bp fragment

3.2.2.1 Primers

A set of primer was designed to amplify of BF2 gene using the chicken BF2 sequence (Table 3.2). While the forward primer was taken from first quarter of the exon-2, while the reverse primer was taken from middle region of exon-3 (Fig 3.2). These primers were expected to amplify a 599 bp fragment.
5'-3' SEQUENCE

FP     GGGCAGCCGTTGGTTCGTG
RP     TGGTGGGAACTGCCTCTGGA

EXPECTED SIZE OF BAND

599 BP

Fig 3.2  Systematic presentation of BF2 gene in chicken and designing of the primers for PCR-RFLP
Table 3.2. Details of the primers used for amplifying the 599 bp size fragment

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Expected fragment size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP</td>
<td>GGGCAGCCGTTGGTGCGTG</td>
<td>370</td>
</tr>
<tr>
<td>RP</td>
<td>TGGTGCGAAACTGCTCTTGGA</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2.2 Extraction of genomic DNA

About 500 µl of blood from brachial vein was collected in tubes containing heparin from each bird and then stored at -20°C till further processing. The high molecular weight genomic DNA was isolated using the following simple method. To 50 µl of blood, 700 µl of lysis buffer (10 mM Tris, HCl, 100 mM NaCl, 1 mM EDTA, pH : 8.0 and 0.5% SDS) containing 60 µg of proteinase K (20 mg/ml) was added. The mixture was vigorously vortexed and incubated at 30°C for 10-12 hours with gentle shaking. The DNA was purified by extracting with equal volume of phenol, phenol-chloroform and chloroform isoamyl alcohol (24:1). The genomic DNA was precipitated by adding 0.1 volume of 3M sodium acetate and two volumes of ice chilled ethanol and centrifuged for 5 minutes at 14000 rpm. Then, DNA pellet was then washed with 70% ethanol, air-dried and subsequently resuspended in TE buffer (10 mM Tris. HCl, 1mM EDTA).

3.2.2.3 Purity, concentration and quality of genomic DNA

After the complete dissolution of DNA, its optical density at 260 and 280 nm was determined by spectrophotometry. Purity of DNA was checked by taking the ratio of optical density at 260 and 280 nm. Good DNA samples having OD ratio between 1.7 to 1.9 were used for the study.

One OD unit at 260 nm equals 50 µg/ml of double stranded DNA. The concentration of DNA samples was calculated by the following formula.

Concentration (µg/ml) = OD at 260 nm x dilution ratio x 50.
0.8 % w/v agarose was dissolved in 1X TBE buffer. The hot solution was cooled to 40-50°C followed by ethidium bromide (0.5 µg/ml of gel) addition and mixed gently. After sealing the edges of the gel casting plate and proper placement of comb, melted agarose was poured without air bubbles. The gel casting plate was submerged in electrophoresis tank containing 1X TBE solution. The comb was removed gently and steadily without disturbing the gel. Loading samples were prepared by adding bromophenol blue dye. Electrophoresis was performed at 2 V/cm (Max. 5 V/cm of gel) for 1 to 2 h. Finally gel was examined under UV light and photographed for documentation.

3.2.2.4 PCR Reaction mixture

Polymerase chain reaction (PCR) was carried out for amplification of BF2 gene with genomic DNA as template using designed specific forward and reverse primers. The amplification mixture for each sample made up to final volume of 25 µl, containing 10 pmol each primer. The further reaction was done as mentioned above.

3.2.2.5 PCR Reaction profile

The amplification was carried out in a i-cycler (Biorad) PCR machine. The protocol for PCR reaction consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of PCR, each cycle consisting of 45s at 94°C, 45s at 58°C and 45s at 72°C. After completion, the samples were stored at 4°C till further use. Molecular sizes of amplified products will be estimated by using appropriate molecular size markers.

3.2.3. RE analysis

3.2.3.1 Identification of Restriction Endonuclease Sites

The restriction enzyme map was developed for BF2 nucleotide sequence from red jungle fowl and chicken by using GENETOOL software. The map was compared and two restriction enzymes i.e. Hae II and Xho I were identified.
3.2.3.2 RE digestion

About 1 μg of amplified purified fragment was digested with different restriction enzymes as per the recommendations of the manufacturer in 40 μl digestion mixture. About 15 μl of the digested product was resolved on 6% native polyacrylamide gel and the resolved bands were visualized through silver staining.

3.2.4.3 Resolution on 1.6 % agarose gel

The PCR products were resolved on 1.6% agarose gel in 1x TBE. Electrophoresis was done at 90 volts for 10 minutes, then at 50 volts for 2 hour. Gel was viewed under a UV Trans-illuminator and photographed with gel documentation system.